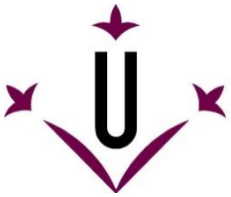
FLRT proteins act as guidance cues for migrating cortical interneurons

Catherine Fleitas Pérez



Universitat de Lleida

Ph.D. Dissertation

FLRT PROTEINS ACT AS GUIDANCE CUES FOR MIGRATING CORTICAL INTERNEURONS

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Lleida, 2015

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Mèmoria de tesi doctoral presentada per Catherine Fleitas Pérez per optar al grau de Doctor per la Universitat de Lleida (Programa de Doctorat en Salut).

Treball realizat 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 Ciènces Mèdiques Bàsiques de la Universitat de Lleida, sota la direcció del Doctor Joaquim Egea Navarro.

El treball realizat en aquesta tesi doctoral ha estat finançat pels projects de reserca del Ministerio 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 Catherine Fleitas Pérez ha gaudit durant aquest temps d'una beca predoctoral Jade Plus.

Lleida, 23 de Maig de 2015,

Doctorand | Directores de tesi

Catherine Fleitas Pérez | Joaquim Egea Navarro | Carme Espinet Mestre

A mis padres,

A abuela Mirtha

"¿Para qué, sino para poner paz entre los hombres, han de ser adelantos de la ciencia?".

José Martí

Acknowledgments

A Carme y a Joaquim, que con orgullo digo, MIS TUTORES, les estoy muy agradecida desde antes de venir. No sólo por haberme aceptado como su estudiante, sino porque sin conocerme, confiaron en mí, y me facilitaron llegar hasta aquí. Desde entonces, no han parado de ayudarme y brindarme todo lo que se necesita para sentirse realizado y afortunado haciendo una Tesis lejos de casa, me siento muy feliz por eso.

Carme desde el primer día me recibió con mucho cariño. Me enseñó con paciencia y sin ningún tipo de jerarquía las primeras cosas del laboratorio. Su alegría de siempre, sentido del humor, tolerancia, comprensión, apoyo, disposición a brindarme su conocimiento, experiencia y tiempo. Todo eso hizo que me sintiera en confianza, y más que tutora, a la cual respeto mucho, ha sido, mi compañera, con la que compartía el trabajo. Amiga, y a veces la mamá que me ha aconsejado y en la que he podido confiar. Preocupada además por mi vida fuera del laboratrio, por integrarme, nunca olvidaré que las primeras montañas de los Pirineos y la primera peli en 3D las vi con ella, ;).

Joaquim es también un magnífico tutor. A pesar de su juventud, es para mí un ejemplo de excelente científico, con una verdadera vocación, al cual respeto y admiro mucho. Fue la primera persona a la que vi tan emocionada con un resultado y siempre recordaré que me animaba a "disfrutar" con los experimentos y aunque al principio no lo entiendía, con el tiempo comprendí que es la mejor manera de hacer ciencia. Me ha sembrado la inquietud y provisto de herramientas para resolverlas. Me ha brindado muchísimos conocimientos y ha dedicado todo el tiempo que no tenía para guiarme. He aprendido de su carácter inspirador, optimista, persistente y muy rigoroso, pero sin perder la ternura, desde lo constructivo, siempre con modestia y humildad.

Con Carme y Joaquim he tenido el privilegio de contar, con lo que para mí son los verdaderos educadores, y para resumir mi agradecimiento, como una vez les dije: "Si echara el tiempo atrás y tuviera que hacer otra vez la Tesis, los elegiría, sin duda".

Si este trabajo es también un poco de alguien más, es de Bea. Al menos así lo siento yo, porque ha estado en cada minuto de esta aventura. Unicamente ella sabrá mejor que nadie, leer entre líneas, más allá, de estas páginas. GRACIAS, por haber estado incondicionalmente a mi lado, ayudándome y soportándome, Amiga. Te agradezco infinitamente por TODO, y cuando digo TODO, es TODO, lo que me has brindado. Por siempre te estaré muy agradecida. Contigo es verdad que … "con dos que tengan el alma como de cien, con dos que se quieran bien, con dos que se quieran…basta"…

No existiría esta Tesis, si no hubiera sido por ti, Omar. Me abriste la puerta y me impulsaste a volar. Me presentaste al mundo y en parte también eres responsable de este buen futuro. Siempre te estaré muy agradecida por eso.Gracias por tu mano en mis primeros pasos. Por tu gran cariño y amistad de siempre, que ha significado un gran apoyo en todo este tiempo. Gracias porque contigo me he traído una familia y un trocito de nuestra Habana.

Con mucha satisfacción puedo decir, que he podido contar con amigos, que más que amigos han sido como mi familia. Albita, Nico, Hugo y Gus, a los que siempre les estaré muy agradecida, por tanto cariño, por acogerme y apoyarme cuando lo he necesitado, por tantos momentos juntos, MUCHÍSIMAS GRACIAS, sinceramente desde mi corazón.

A Idalmys, amiga coterránea, gracias por tu afecto, tu apoyo, por tus mimos y cuidados.

Muchas Gracias a los compañeros del laboratorio por compartir sus conocimientos. Disha, M. José, Inma, Serafí, Blanca y Pau.

Agradecer a queridos amigos de otras tierras con los cuales he compartido lindos momentos: Ambika y Saravanan (por tu compañía en tantas horas extras compartidas juntos).

Gracias a las fantásticas amigas Lucía y Marta.

Gracias a las chicas de ElsOus Aquí: Elia, Alba y M. José

La Tesis para mí no ha sido solamente este trabajo, por lo que mis agradecimientos, necesariamente deben ir más allá de estos resultados. Hay personas que aunque nunca han estado en un laboratorio me han ayudado muchísimo a vencer esta etapa. Muchas Gracias a mis amigos y primeros compañeros de piso: Arcadi, Jaume y mi queridísima Jess.

Gracias a los amigos de las Remolachas Rojas, muy en especial a Martin, por su amistad, por su cariño y por complacerme con tantas canciones que me alegraban y me calmaban las nostalgias.

Y por supuesto, las GRACIAS, a mi familia, en especial a mis padres, a mi tía Cusita, a mis hermanos, mis sobrinitas, mi gran amiga Idy a Frank, que han estado como siempre muy presentes, pendientes de mis pasos, apoyándome, animándome, con tantísimo cariño acercando lejanías.

Gracias a la beca Jade Plus, que financia el Banco Santander, y que me otorgó la Oficina de Relaciones Internacionales de la UdL. En especial quiero agradecer a Paula Obregón, por su ayuda siempre.

Espero no haber olvidado a nadie, pero de cualquier forma, quiero extender mis agradecimientos a TODOS los que de una forma u otra, directa o indirectamente me han ayudado en todo este tiempo de la Tesis.

MUCHISIMAS GRACIAS !!!!

INDEX

INDEX

ABBREVIATIONS

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AP	apical progenitors
ATP	adenosine triphosphate
BDNF	brain derived neurotrophic factor
BP	basal progenitors
bRG	basal radial glial
CAM	cell adhesion molecule
CB	calbindin
CC	corpus callosum
Cdk	cyclin-dependent kinase
CGE	caudal ganglionic eminence
CNS	central nervous system
CP	cortical plate
C-R	Cajal-Retzius
CR	calretinin
CSMN	corticospinal motor neurons
Ctip	COUP-TF-interacting protein
Dab1	adaptor protein disabled-1
DAPI	4',6-diamino-2-phenylindole
DCC	deleted in colorectal cancer
DIV	days *in vitro*

Dlx	Drosophila distal less
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E	embryonic day
ECD	extracellular domain
EDTA	ethylenediamine-tetra acetic acid
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FGF	fibroblast growth factor
FLRT	fibronectin-and-leucine-rich-transmembrane protein
FNIII	fibronectin type III
GABA	gamma-aminobutyric acid
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitors
GDNF	glial cell-derived neurotrophic factor
GE	ganglionic eminences
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
HEK293T	human embryonic kidney cells

HGF	hepatocytegrowth factor
hr	hour
HRP	horseradish peroxidase
ICD	intracellular domain
Ig	immunoglobulin
IP	intermediate progenitor cells
ISH	*in situ* hybridization
ISVZ	inner subventricular zone
IZ	intermediate zone
JNK	Jun N-terminal kinase
kDa	kilo Dalton
LGE	lateral ganglionic eminence
LPD	lectin pull-down
LRR	leucine-rich-repeat
MetOH	methanol
MGE	medial ganglionic eminence
min	minute
mRNA	messenger RNA
MZ	marginal zone
NE	neuroepithelial cells
Ngn2	neurogenin 2

NPY	neuropeptide Y
NRG1	neuregulin 1
NT4	neurotrophin-4
OB	olfactory bulb
ORG	outer radial glial cells
OSVZ	outer subventricular zone
P	postnatal day
PAGE	polyacrylamide-gel-electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
POA	preoptic area
POU3F	POU domain, class 3, transcription factor
PP	preplate
PV	paralbumin
RG	radial glial cell
RMS	rostral migratory stream
RNA	ribonucleic acid
Rnd	round
Robo	roundabout
RT	room temperature

SAPs	subapical progenitors
Satb2	Special AT-rich sequence-binding protein
SDF	stromal derived factor
SEM	standard error of the mean
Sema	semaphoring
SF	scatter factor
SNP	short neural precursors
SP	subplate
SPF	specific pathogen free
SST	somatostanin
SVZ	subventricular zone
Tbr	T-brain gene
TCA	thalamocortical axons
TCL	total cell lysate
VIP	vaso-active intestinal peptide
VZ	ventricular zone
WT	wild type

ABSTRACT

ABSTRACT

The cerebral cortex is the part of our brains that has suffered the major changes during evolution and is responsible for our cognitive and memory capabilities.The complex circuitries of the cerebral cortex comprise networks produced by two major neuronal cell types: the excitatory projection neurons and the inhibitory interneurons. Although interneurons represent a minority (~20%) of the entire neocortical neuronal population (Hensch, 2005; Wang et al., 2004; Whittington and Traub, 2003), it is thought that they play a vital role in the function of the cerebral cortex. In fact, altered balance between excitation and inhibition might result in death or lead to a large variety of neurological disorders (Belforte et al., 2010; Kalanithi et al., 2005; Kitamura et al., 2009; Powell et al., 2003). The establishment of functional neuronal connectivity starts during development and depends on neuronal migration and the correct positioning of newborn neurons which integrate into specific layers of the cortex. Unlike the projection neurons, that originate from the pallial progenitors and migrate radially relatively short distances, interneurons are generated remotely in several progenitor pools of the subpallium. Therefore, they perform a complex choreography to reach their final position in the nascent cortex (Marín, 2013).

Once in the cortex, GABAergic interneurons disperse tangentially via highly stereotyped routes throughout the cerebral cortex (Lavdas et al., 1999). Interneurons then switch from tangential to radial migration to adopt their final laminar position in the cerebral cortex (Ang et al., 2003; Polleux et al., 2002; Tanaka et al., 2003). Although significant progress has been made in identifying the molecules and mechanisms that regulate the precise tangential and radial dispersion of interneurons along the cortical streams, many questions about these complex processes are just beginning to be elucidated (Abe et al., 2014; López-

Bendito et al., 2008; Myers et al., 2014; Sánchez-Alcañiz et al., 2011; Wang et al., 2011).

During the last past years, the fibronectin and leucine-rich family of transmembrane proteins (FLRT) have evolved as new regulators of several aspects during nervous system development, including neuronal migration. Interestingly, FLRTs display different mechanisms of action in several processes which demonstrate the versatility of these proteins. For instance, it was described that Unc5D-expressing neurons display a delayed migration to the FLRT2-enriched cortical plate, consistent with FLRT2 acting as a repulsive cue for $Unc5D^{+}$ cells (Yamagishi et al., 2011). Moreover, it has been recently reported that FLRT3-FLRT3 homophilic adhesion interaction regulates the tangential distribution of migrating pyramidal neurons (Seiradake et al., 2014). Thus, FLRTs have distinct functions that need to be integrated during radial and tangential patterns of pyramidal neuron migration. Taken together, all these data suggest that FLRTs are important molecules for the regulation of different processes during nervous system development. In this respect, our group has recently shown that FLRT3 plays a key role in axon guidance for the developing of the thalamocortical projections (Leyva-Díaz et al., 2014). However, we think that many of the FLRT-dependent functions in the nervous system remain to be elucidated. Therefore, this work is focused on the study of *in vivo* FLRTs involvement in the tangential migration of interneurons. For this, we have analyzed nervous system specific knockout (KO) animals for *FLRT2* and *FLRT3*, single mutants as well as the double KOs (DKO). The results revealed that deletion of only one of the two genes does not affect the normal intracortical migration pattern of Calbindin (CB) positive interneurons during development. Surprisingly, the simultaneous suppression of *FLRT2* and *FLRT3*, resulted in the appearance of several defects related to interneuron migration: a disruption of the distribution pattern of intracortical interneurons and a perturbed tangential

migration. In particular, a decrease in the number of CB^+ cells transiting through the subplate (SP) route and a consequent increase of these cells within the intermediate zone was observed. As a consequence, the interneuron tangential progression transiting through the SP stream is also affected. In order to know if FLRTs were intrinsically involved in this effect, we analyzed the DKOs using the *Emx1-Cre* line which only recombines in the pallial pyramidal neurons. The results obtained were essentially the same as we obtained with the whole nervous system deletion of *FLRT2* and *FLRT3,* indicating that FLRTs may function during interneuron migration by a non-cell autonomous mechanism, probably through Unc5 receptors. Finally, we addressed the intracellular mechanisms involved in FLRT function and considering the importance of the regulation of cytoskeleton dynamics to drive neuronal migration, we assessed the relationship between FLRT3 and the Rho GTPase Rnd3. *In vitro*, both proteins physically interact in heterologous cells. In addition, we started the analysis of *Rnd3* mutant mice, and obtained preliminary results pointing that the interneuron tangential migration is also disrupted in these animals suggesting a possible functional interaction between FLRTs and Rnds in this system.

RESUM

L'escorça cerebral és la part del cervell que presenta un nombre més gran de canvis al llarg de l'evolució i és la responsable de la nostra capacitat cognitiva i memòria. La complexitat dels circuits de l'escorça cerebral és majoritàriament conseqüència de les relacions entre dos tipus cel·lulars: les neurones excitatòries de projecció i les interneurones inhibitòries. Malgrat que les interneurones representen una minoria (~20%) del conjunt de neurones neocorticals (Hensch, 2005; Wang et al., 2004; Whittington and Traub, 2003), es creu que juguen un paper rellevant en la funcionalitat de l'escorça cerebral. De fet, l'alteració del balanç entre aquestes dues poblacions pot produir la mort o comportar una gran varietat de símptomes neurològics (Belforte et al., 2010; Kalanithi et al., 2005; Kitamura et al., 2009; Powell et al., 2003). L'establiment de les connectivitats neuronals a l'escorça en desenvolupament depèn de la migració i del correcte posicionament de les noves neurones que integren específicament les diferents capes corticals. A diferència de les neurones de projecció, que s'originen a partir dels progenitors del pal·li i migren radialment distàncies relativament curtes, les interneurones es generen en llocs allunyats, en diversos grups de cèl·lules progenitores, al subpal·li. En conjunt, estableixen una complexa coreografia per aconseguir la posició correcta final a l'escorça en desenvolupament (Marín, 2013).

Un cop a l'escorça, les interneurones GABAèrgiques es dispersen tangencialment a través de vies altament predefinides (Lavdas et al., 1999). A continuació, les interneurones canvien d'una migració tangencial a una de radial per adoptar finalment una disposició laminar a aquesta estructura (Ang et al., 2003; Polleux et al., 2002; Tanaka et al., 2003). Malgrat els progressos significatius en la identificació de les molècules i mecanismes que regulen la precisa dispersió tangencial i radial de les interneurones al llarg dels corrents corticals, moltes de

les qüestions relacionades amb aquests processos complexos, tot just comencen a ser esbrinades (Abe et al., 2014; López-Bendito et al., 2008; Myers et al., 2014; Sánchez-Alcañiz et al., 2011; Wang et al., 2011).

En els darrers anys, les proteïnes transmembrana riques en fibronectina i leucina (FLRT) han estat identificades com a nous reguladors de diversos aspectes en el desenvolupament del sistema nerviós, incloent la migració neuronal. Especialment interessant és el fet que els FLRTs presenten mecanismes d'acció diferents en diversos processos, el quals demostren la versatilitat d'aquestes proteïnes. Per exemple, s'ha descrit que les neurones que expressen Unc5D presenten un endarreriment en la migració cap a la placa cortical rica en FLRT2, basat en la repulsió que estableix FLRT2 sobre les cèl·lules que expressen Unc5D (Yamagishi et al., 2011). A més, s'ha descrit recentment que la interacció homofílica FLRT3-FLRT3 regula la distribució tangencial de les neurones piramidals migratòries (Seiradake et al., 2014). En definitiva, els FLRTs presenten diverses funcions integrades als patrons de migració radial i tangencial de les neurones piramidals. En conjunt, totes aquestes dades suggereixen que FLRTs són molècules importants per la regulació de diferents processos durant el desenvolupament del sistema nerviós. En aquest sentit, el nostre grup ha demostrat recentment que FLRT3 juga un paper clau en la guia d'axons pel desenvolupament de les projeccions talamocorticals (Leyva-Díaz et al., 2014). El present treball de tesi està centrat en l'estudi *in vivo* de la implicació dels FLRTs en la migració tangencial de les interneurones. Amb aquest propòsit, hem analitzat animals knockout (KO) de *FLRT2* i *FLRT3,* específics de sistema nerviós,com a mutants simples i també, com a dobles mutants (DKO). Els resultats indiquen que l'eliminació de l'expressió de només una de les dues proteïnes no afecta la migració normal d'interneurones positives per Calbindina (CB) durant el desenvolupament. Sorprenentment, la supressió simultània de *FLRT2* i *FLRT3,* produeix diversos defectes relacionats amb la migració de les interneurones: una

disrupció del patró de distribució de les interneurones intracorticals i una migració tangencial. En particular, s'observa una disminució en el nombre de cèl·lules CB-positives desplaçant-se per la sub-placa (SP) i un conseqüent increment d'aquestes cèl·lules a la zona intermèdia. Com a conseqüència, també es veu afectada la progressió tangencial de les interneurones a través de la SP. Per tal d'estudiar si els FLRTs es troben intrínsicament implicats en aquest efecte, hem analitzat els DKOs utilitzant la línia *Emx1-Cre*, que només recombina a les neurones piramidals del pal·li. Els resultats obtinguts són essencialment els mateixos que quan eliminem *FLRT2 i* FLRT3 al sistema nerviós complet, indicant que els FLRTs poden mediar la seva funció en la migració de les interneurones per un mecanisme extrínsec a la cèl·lula, probablement a través dels receptors Unc5. Finalment, considerant la rellevància de la regulació de la dinàmica del citoesquelet en la migració neuronal, abordem els mecanismes intracel·lulars implicats en la funció de FLRT, descrivint la relació entre FLRT3 i RhoGTPase Rnd3. *In vitro*, amb dues proteïnes interaccionen físicament en cèl·lules heteròlogues. A més, hem iniciat l'estudi del model murí mutant de *Rnd3* obtenint resultats preliminars que indiquen que la migració tangencial de les interneurones també s'hi troba alterada. Això suggereix una interacció funcional entre els FLRTs i els Rnds en aquest sistema.

RESUMEN

La corteza cerebral es la parte de nuestro cerebro que ha sufrido los mayores cambios durante la evolución y que es responsable de nuestras capacidades cognitivas y de memoria. La complejidad de la corteza cerebral comprende redes que se forman a partir de dos grandes tipos celulares de neuronas: las neuronas excitatorias de proyección y las interneuronas inhibitorias. Aunque las interneuronas representan la minoría (~20%) del total de la población neocortical neuronal (Hensch, 2005; Wang et al., 2004; Whittington and Traub, 2003), se considera que juegan un papel vital en el funcionamiento de la corteza cerebral. De hecho, alteraciones en el equilibrio entre la excitación y la inhibición podría causar la muerte o dar lugar a una gran variedad de alteraciones neurológicas (Belforte et al., 2010; Kalanithi et al., 2005; Kitamura et al., 2009; Powell et al., 2003). El establecimiento de las conectividad neuronal comienza durante el desarrollo y depende de la migración neuronal y del correcto posicionamiento de las nuevas neuronas, las cuales se integran dentro de capas específicas de la corteza. A diferencia de las neuronas de proyección, que se originan a partir de los progenitores del palio y migran radialmente distancias relativamente cortas, las interneuronas se generan en varios grupos progenitores del subpalio. Por lo tanto las interneuronas tienen que atravesar grandes distancias para alcanzar su posición final en la corteza en formación (Marín, 2013).

Una vez en la corteza, las interneuronas GABAérgicas se dispersan tangencialmente por rutas bien definidas a través de la corteza cerebral (Lavdas et al., 1999). A continuación, las interneuronas cambian de una migración tangencial a una radial para adoptar su posición laminar final en la corteza cerebral (Ang et al., 2003; Polleux et al., 2002; Tanaka et al., 2003). A pesar de los progresos significativos en la identificación de moléculas y mecanismos que regulan la dispersión tangencial y radial de las interneuronas a lo largo de las rutas

corticales, muchas de las cuestiones relacionadas con estos procesos apenas empiezan a ser dilucidadas (Abe et al., 2014; López-Bendito et al., 2008; Myers et al., 2014; Sánchez-Alcañiz et al., 2011; Wang et al., 2011).

Durante los últimos años, las proteínas transmembrana ricas en fibronectina y leucina (FLRT) han evolucionado como nuevos reguladores de varios aspectos durante el desarrollo del sistema nervioso, incluyendo la migración neuronal. Curiosamente, FLRTs muestran distintos mecanismos de acción en diferentes procesos que demuestran la versatilidad de estas proteínas. Por ejemplo, se ha descrito que las neuronas que expresan Unc5D muestran una migración retardada a la placa cortical, acorde con las señales repulsivas de FLRT2 hacia las células Unc5D^{+} (Yamagishi et al., 2011). Por otra parte, se ha publicado recientemente que la interacción de adhesión homofílica FLRT3-FLRT3 regula la distribución tangencial de las neuronas piramidales que migran (Seiradake et al., 2014). Todos estos datos en conjunto sugieren que FLRTs son moléculas relevantes para la regulación de diferentes procesos durante el desarrollo del sistema nervioso. En este sentido, nuestro grupo ha mostrado recientemente que FLRT3 juega un papel clave en la guía axonal para el desarrollo de las proyecciones talamocorticales (Leyva-Díaz et al., 2014). Sin embargo, pensamos que muchas de las funciones dependientes de FLRT en el sistema nervioso aún no se han dilucidado. Por tanto, este trabajo se centra en el estudio de la implicación *in vivo* de FLRTs en la migración tangencial de las interneuronas. Para ello, hemos analizado animales knockout (KO) específicos del sistema nervioso para *FLRT2* y *FLRT3*, simples mutantes y dobles KOs (DKO). Los resultados revelaron que la deleción de sólo uno de estos dos genes no afecta el patrón normal de migración intracortical de las interneuronas positivas para Calbindina (CB) durante el desarrollo. Sorprendentemente, la supresión simultánea de *FLRT2* y *FLRT3*, resultó en la aparición de varios defectos relacionados con la migración de las interneuronas: una alteración en el patrón de distribución de las interneuronas intracorticales y

una migración tangencial afectada. En particular, se observó una disminución en el número de células CB^+ que transitan via subplaca cortical (SP) y un consecuente incremento de estas células dentro de la zona intermedia. Como consecuencia, la progresión tangencial de las interneuronas que transitan a través de la ruta SP también se vio afectada. Para saber si FLRTs estaban implicadas intrínsecamente en este efecto, analizamos los DKOs usando la línea *Emx1-Cre,* que sólo recombina en las neuronas piramidales paliales. Los resultados obtenidos fueron básicamente los mismos que obtuvimos con la deleción completa de *FLRT2* y *FLRT3* en el sistema nervioso, indicando que FLRTs pudieran actuar durante la migración de las interneuronas mediante un mecanismo extrínseco a estas células, probablemente a través de los receptores Unc5. Por último, abordamos los mecanismos intracelulares implicados en la función de FLRT y, considerando la importancia de la regulación de la dinámica del citoesqueleto en la migración neuronal, evaluamos la relación entre FLRT3 y Rho GTPase Rnd3. *In vitro,* ambas proteínas interaccionan físicamente en células heterólogas. Además, comenzamos el análisis de los ratones mutantes *Rnd3* y obtuvimos resultados preliminares que apuntan a que la migración tangencial de las interneuronas está también alterada en estos animales, sugiriendo una posible interacción funcional entre FLRTs y Rnds en este sistema.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

The Brain, the most complex and magnificent organ in humans, gives us awareness of ourselves and of our environment. It directs our existence, controlling our voluntary movements and involuntary activities. As the mainstay of the consciousness, the brain is responsible of everything related with memories, intelligence, emotions and personalities, interpreter of the senses, initiator of body movement, and controller of behavior. The processing a constant stream of sensory data, and the unique abilities of communicating through a native language, are some of the qualities man that separates humans from the animal world. This was the result of an evolution towards increasing of brain complexity. However, it has been recognized that the physical differences of the human brain are not sufficient to explain the power that defines human ingenuity. Despite of the advances in the field of brain research most of the processes remain a mystery and that's why the numerous efforts began in the 90s with the decade of the brain and human brain project creation, it continues today in order to have a more complete understanding of this fascinating organ. In addition, nowadays increases the risk of diseases affecting the brain and new ones are emerging, as the most serious neurodegenerative diseases are Parkinson's disease or Alzheimer's, mental illness or the process that is so genuinely human aging. The research continues to bring new insights into the brain behavior, in turn, the neuroscience improved understanding makes the development of new treatment options possible and the research challenge is to discover the molecular mechanisms that govern these diseases and whether damage to the brain can be reversed. Besides this not only aims, moreover it is intended to go further in search of our privacy as human beings whose goals reach beyond the simple molecular and cellular understanding of the brain and trying to understand the complex mechanism that governing human mind and behavior to know the codes of how our brain works.

1.1 Development and organization of the mammalian cerebral cortex

The forebrain is undoubtedly one of the most intricate regions of the mammalian brain, the principal organ of the central nervous system (CNS) (Marín and Rubenstein, 2003) and comprises a complex set of structures that derive from the most anterior region of the neural tube, the prosencepahlon (Marín and Rubenstein, 2002). The prosencephalon consists of the diencephalon and telencephalic vesicles, which evaginate from the dorsal aspect of the rostral diencephalon. The telencephalon is an extremely complex biological entity and is responsible for the higher functions of the CNS (Sultan et al., 2013). It has two major regions: the pallium (roof) and the subpallium (base). The pallium gives rise to the cerebral cortex and hippocampus, whereas the subpallium consists of three primary subdivisions: the striatal, pallidal, and telencephalic stalk domains, all of which extend medially into the septum. Finally, the olfactory bulbs (OB) develop as bilateral evaginations from a region of the prosencephalic neural plate intercalated between the septal and the cortical anlage (Cobos et al., 2001; Rubenstein et al., 1998) (Figure 1).

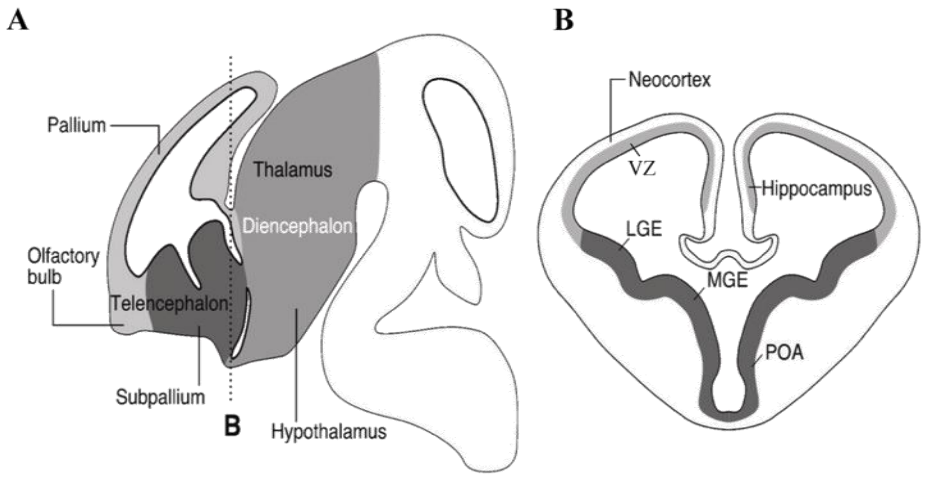

Figure 1. Anatomical organization of the developing forebrain. (A) Schema of a sagittal section through the brain of an E12.5 mouse showing the main subdivisions of the forebrain, the diencephalon and the telencephalon. In the telencephalon, the pallium is depicted in lighter gray

than the subpallium. (B) Schema of a transversal section through the telencephalon of an E12.5 mouse, indicating some of its main subdivisions. Abbreviations: VZ, ventricular zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; POA, preoptic area. Figure adapted from (Marín and Rubenstein, 2003)**.**

Over the course of evolution, the mammalian brain massively expanded its size and complexity, which is thought to underlie the growth of intellectual capacity believed to be responsible for an increase in cognitive functions and intellectual skills. Most of this expansion is due to a massive increase in surface area of the multilayered sheet of neurons forming cerebral cortex (Finlay and Darlington, 1995). Such expansion in cortical surface area, which is about 1000 times larger in humans than in mice, is not accompanied by a proportionate difference in thickness (only~2 times thicker in humans than mice) but rather comes together with the appearance of convolutions of the cortical sheet (gyrencephaly), with folds and fissures known as gyri and sulci (Welker, 1990; Rakic, 1995; Reillo et al., 2011). Largely based on the anatomical differences between the developing cortex of lissencephalic and gyrencephalic brains, several hypotheses have been formulated aiming to explain the massive increase in size and induction of brain folding during mammalian evolution (Ghosh and Jessberger, 2013) (Figure 2).

The formation of the cerebral cortex during development occurs via highly complicated processes and fundamental principles. How to organize something as complex as the cerebral cortex?. It is precisely the target research of many neuroscientists, who through animals models are studying these processes in order to extrapolate to humans. Although for over a century the mouse became the preferred animal model for the research, several studies demonstrate that are decisive histological differences precisely distinguished the gyrencephalic from the lissencephalic cerebral cortex in species like mouse and guinea pig (Reillo et al., 2011). Thus, lately are used a variety of non primate gyrencephalic species,

including ferret (Fietz et al., 2010; Reillo et al., 2011; Reillo and Borrell, 2012), as more appropriate models to better understand the process in the human brain.

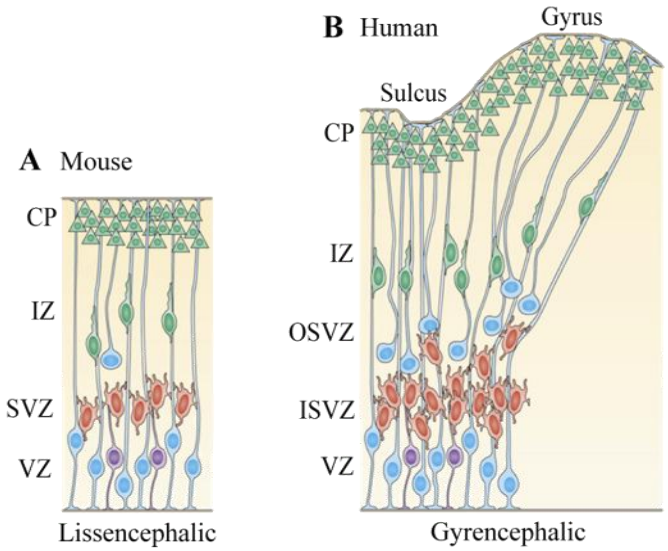

Figure 2. Cerebral cortex expansion during evolution. Based on cortical folding, mammals can be divided into lissencephalic species (such as mice), which have smooth-surfaced cortices (A), and gyrencephalic species (such as ferrets and most primates), which exhibit a cortical surface expansion results in folded structures called sulci and gyri (B). Abbreviations: CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; OSVZ, outer SVZ; ISVZ, inner SVZ; VZ, ventricular zone. Figure adapted from (Sun and Hevner, 2014).

Although the laminar organization of the cortex is relatively similar in all mammals, the expansion in cortical surface area underlies the transformation from smooth cortex to the highly folded primate neocortex (Rakic, 1988). The expansion of the neocortex may be explained by some evolutionary changes in corticogenesis (Charvet and Striedter, 2011). Growth of the neocortex results from an increase in neuron numbers that populate larger cortical surface areas (lateral expansion) in thicker cortical walls (radial growth) (Lui et al., 2011; Rakic, 2007). The greater increase in neuronal production obviously underlies the remarkable expansion of the cortex (Betizeau et al., 2013; Fish et al., 2008; Gertz et al., 2014;

Lewitus et al., 2013; Lui et al., 2011; Martínez-Cerdeño et al., 2006; Noctor et al., 1997; Rakic, 1995; Borrell and Götz, 2014).

Most notably in primates, there is an expanded subventricular zone (SVZ), which can be subdivided into inner (ISVZ) and outer (OSVZ) regions (Fietz et al., 2010; Lui et al., 2011; Smart et al., 2002) (see Figure 2). In addition to increased neurogenesis, have been studied cellular mechanisms involved in the tangential expansion of the cerebral cortex in higher mammals. This process relies on the proliferation of OSVZ progenitors driving the tangential dispersion of radially migrating neurons and, finally, the expansion in surface area of the cerebral cortex (Reillo et al., 2011). The molecular mechanisms of cortical growth have been recently summarized (Sun and Hevner, 2014).

The hallmark in mammals is the emergence of the newest part of the cerebral cortex, the neocortex (isocortex), defined by its six-layer organization. The cortex also includes the hippocampus and rhinal cortex (allocortex) (Guérout et al., 2014). The development of the cortex requires a continuous rearrangement of a primordial structure that progresses through successive steps including proliferation, specification, migration, and neuronal differentiation. Disrupting the completion of one or several of these events can lead to brain malformations such as microcephaly or lissencephaly (see below) (Laguesse et al., 2015).

Each layer is cytoarchitectonically and functionally distinct and contains neurons with similar morphology, functional properties, connections; as well as time and place of origin (Kwan et al., 2012). There are two major types of neurons: excitatory projection neurons (pyramidal neurons) and inhibitory interneurons distributed along the layers. Although inhibitory interneurons comprise only about 20% of cortical neurons and thus constitute a clear minority compared to the vast number of excitatory projection neurons, they play a vital role in modulating

neuronal excitability. This role is depicted in an eloquent analogy by Di Cristo "to compare interneuron function to the music director of a symphony orchestra, who structures and coordinates the overall musical performance. Without proper direction, the ensemble cannot produce the right melody" (Di Cristo, 2007).

Interneurons, of all the cells within the forebrain, are the most diverse in terms of morphology, molecular and functional properties. The best effort to date by physiologists, anatomists and developmental neurobiologists to come to a common nomenclature for gamma-aminobutyric acid (GABA)ergic interneurons is a unifying nomenclature of GABAergic interneurons in the cortex, the Petilla terminology (Petilla Interneuron Nomenclature et al., 2008). The expression of molecular markers is probably the simplest and most commonly classification used. These include: the calcium-binding proteins: parvalbumin (PV), calbindin (CB) and calretinin (CR); certain neuropeptides, such as somatostatin (SST), vaso-active intestinal peptide (VIP), neuropeptide Y (NPY), cholecystokinin and corticotropin-releasing factor (Kawaguchi and Kondo, 2002; Kubota et al., 2011; Xu and Callaway, 2009). Also, combining molecular and physiological properties, the large variety of interneurons subtypes will fall in one of four major groups: (a) fast spiking, PV-expressing interneurons; (b) burst spiking or adapting non-fast spiking SST-expressing interneurons; (c) non-fast spiking and fast adapting CR- and/or VIP-expressing interneurons; (d) rapidly adapting neuropeptide Y (NPY)- and/or Reelin-expressing interneurons (Gelman and Marín, 2010) (Figure 3).

The projection neurons and interneurons originate from two different germinal areas. All cortical pyramidal neurons are born locally along the neuroepithelium in the dorsal pallium ventricular zone (VZ), whereas cortical interneurons most are born in subcortical domains (subpallium), the ganglionic eminence (GE), that includes the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE) and the preoptic area (POA) (Marín

and Rubenstein, 2003) (see Figure 1). These two classes of neurons follow distinct migration modes to get their intended destinations in the neocortex (see below).

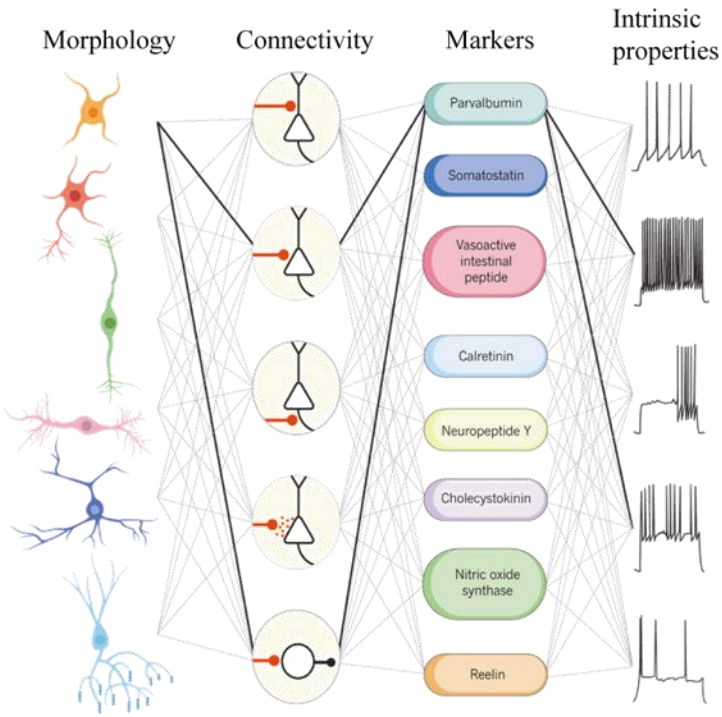

Figure 3. Multiple dimensions of interneuron diversity. Interneuron cell types are usually defined using a combination of criteria based on morphology, connectivity pattern, synaptic properties, marker expression and intrinsic firing properties. Figure adapted from (Kepecs and Fishell, 2014).

1.2 Cortical progenitors and corticogenesis

Corticogenesis is a highly dynamic process that requires the generation of different classes of neurons that are later distributed within layers regionally organized into sensory, motor and association areas (Rash and Grove, 2006). Cortical lamination in the mouse begins around embryonic day (E)11 and is completed by approximately postnatal day (P)14. During early corticogenesis (up to E14.5) (Faux et al., 2012), cortical layers originate from the proliferation of

progenitor cells in the neurogenic compartment of the developing neocortex lining the VZ. Cortical progenitors of projections neurons are classified into two groups according to the location of their mitosis in cortical wall in the developing cortex: the apical progenitors (AP) they comprise neuroepithelial cells (NE), radial glial cells (RG) and short neural precursors (SNP) and the basal progenitors (BP) include intermediate progenitor cells (IP) and outer radial glial cells (ORG) (Götz and Huttner, 2005; Laguesse et al., 2015) (Figure 4). During embryogenesis, NE in the dorsal telencephalon symmetrically divide until E9-E10. Initially, there is a single sheet of pseudostratified NE undergoing symmetric cell divisions to expand the pool of multipotent progenitors as well as a smaller percentage of asymmetric cell divisions to generate the earliest born neurons (Götz and Huttner, 2005). These NE then become RG with glial cell features and radial processes extending from the pial surface to the lateral ventricle. The RG are connected to each other by adherence junctions forming the VZ (Haubensak et al., 2004). RG can divide both symmetrically to expand their population and asymmetrically to generate one RG daughter cell and one non-RG daughter cell, with 10-20% of RG directly generating neurons. Most of the non-RGC daughter cells are known as IP. IP are the other major type of neuron-producing progenitor and are located in the SVZ (the adyacent part of VZ that starts to form at E13.5 in the mouse and expands significantly during late corticogenesis), and in the basal VZ early in neurogenesis before the formation of the SVZ (Molyneaux et al., 2007). Over the course of cortical neurogenesis, IP cells migrate away from the VZ and form the new germinal layer, the SVZ (Noctor et al., 2004). Progenitors cells residing in the VZ and SVZ produce the projection neurons of the different neocortical layers in a tightly controlled temporal order from E11.5 to E17.5 in the mouse (Caviness and Takahashi, 1995), and postmitotic neurons position themselves in the developing neocortex through defined modes of migration (Britanova et al., 2006). Of note, the SVZ has increased in size with evolution leading to thicker upper-layer

neurons involved in higher cognitive functions, which underlines the importance of RG regulation (Guérout et al., 2014). In addition, the basal processes of RG contact meninges which secrete regulatory factors for progenitor maintenance. β1-integrins anchor RG basal processes to the extracellular matrix, allowing exposure to meningeal-derived trophic signals that maintain progenitor survival and proliferation (Radakovits et al., 2009) (Figure 4).

The earliest born neurons appear around E10.5 in the mouse and form a layered structure termed the preplate (PP), which is later split into the more superficial marginal zone (MZ) and the deeply located subplate (SP). The next wave (E13.5) of post-mitotic neural progenitors forms CP by entering the PP and splitting it into a superficial layer, the MZ or layer I and the deep SP (Gupta et al., 2002; Marín and Rubenstein, 2003). Intercalation within the PP also marks the nascent CP, which further develops into layers II to VI. In mice, this occurs approximately at E11.5 (Angevine and Sidman, 1961). The CP, which will give rise to the multilayered neocortex, begins to develop in between these two layers (Bayer and Altman, 1991), such that later born neurons arriving at the CP migrate past earlier born neurons through a mechanisms that is known as "inside-out" layering (Caviness and Takahashi, 1995).

Because the layers are conventionally number from the top, layers II/III contain the youngest neurons and the layer VI the oldest. Inside-out layering means that each neuronal precursor has to migrate outward from the ventricle, pass beyond its predecessors and then stop, undergo terminal differentiation and establish its synaptic connections (Cooper, 2008) (Figure 5A). Because the Cajal-Retzius (C-R) neurons, which localize in the most superficial layer (MZ/layer I) (Soriano and Del Río, 2005), are continually pushed outward as the cortex grows, they are an exception to the inside-out birth order of the rest of the CP (Cooper, 2008).

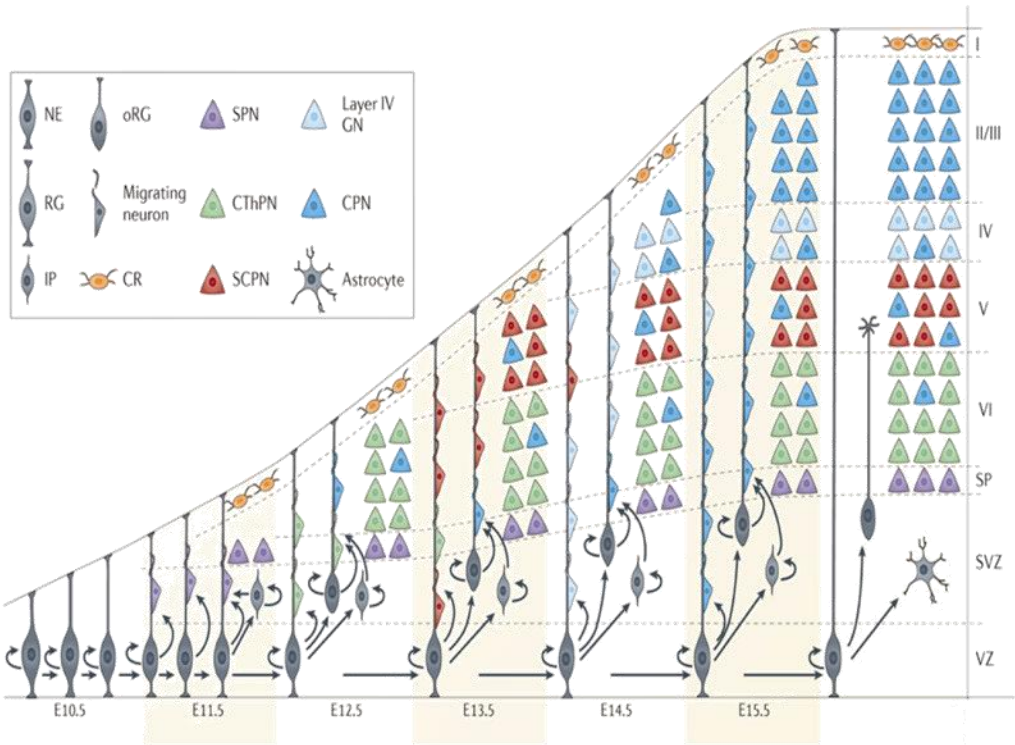

Figure 4. Neurogenesis in the mouse cortex. Neocortical projection neurons are generated by diverse progenitor types in the VZ and SVZ. This schematic depicts the sequential generation of neocortical projection neuron subtypes and their migration to appropriate layers over the course of mouse embryonic development. NE initially divide symmetrically to expand their pool and progressively transform into RG progenitor cells. They divide symmetrically, and asymmetrically to produce neurons that migrate radially to form the CP. RG in the VZ begin to produce projection neurons around E11.5. At the same time, RG generate IP and ORG, which establish the SVZ and act as transit-amplifying cells to increase neuronal production. Abbreviations: RG, radial glial cells; VZ, ventricular zone; IP, intermediate progenitor cells; ORG, outer RG; SVZ, subventricular zone; NE, neuroepithelial cells; E, embryonic day. Figure adapted from (Greig et al., 2013).

Unlike pyramidal neurons, inhibitory interneurons derive from RG cells in the MGE and CGE of the ventral telencephalon (Guérout et al., 2014), do not always follow an inside-out pattern. Birth-date analysis of specific interneuron subtypes suggests that interneurons follow heterogeneous developmental rules for laminar positioning (Ang et al., 2003; McConnell and Kaznowski, 1991; Métin et al., 2006; Pla et al., 2006; Valcanis and Tan, 2003; Yozu et al., 2004).The laminar

distribution and subtypes of interneurons dependon their gene expression, origin within the GE and birthdate (Miyoshi and Fishell, 2011).

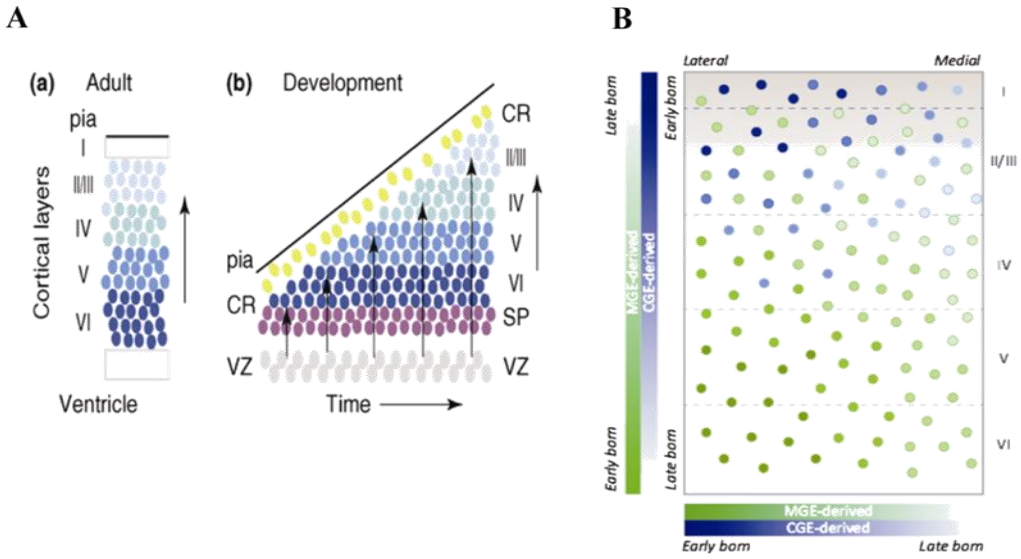

Figure 5. Inside-out layering of cortical projection neurons and developmental distribution of interneuron subtypes. (A) The normal adult CP is divided into layers I through VI, numbered from outside (pial surface) to inside (ventricular surface). In the adult, layer I and the region below the CP are crowded with neurites (gray). Projection neurons in the CP are layered with oldest neurons below and youngest ones above (color coded in different shades of blue). The arrow on the right shows the order of layer formation. Layer thickness is not to scale. (B) Schematic of a coronal section through the mouse neonatal cerebral cortex showing the areal and laminar positioning of MGE- and CGE-derived interneurons. Both MGE- and CGE-derived interneurons reach their final areal positions in a lateral-to-medial gradient (i.e., arriving first in lateral regions of the cortex). MGE-derived interneurons show an inside-out pattern of distribution, whereas CGE-derived interneurons exhibit an outside-in pattern of distribution. MGE-derived interneurons distribute relatively evenly in the neocortex, whereas CGE-derived interneurons preferentially distribute in superficial layers. Abbreviations: CP, cortical plate; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; SP, subplate (purple); VZ, ventricular zone; CR, Cajal-Retzius cells (yellow). Figure adapted from (Cooper, 2008; Guo and Anton, 2014).

MGE- and POA-derived interneurons subtypes show a time-dependent, inside-out pattern of positioning that is similar to projection neurons. By contrast, CGE-derived interneurons show an outside-in placement pattern (Ang et al., 2003; Rymar and Sadikot, 2007). Further, VIP- and NPY-expressing interneurons do not

show a strict inside-out layering pattern, but preferentially localize to superficial layers or scatter widely within the cortex, respectively (Métin et al., 2006; Rymar and Sadikot, 2007). The final cortical distribution of interneurons therefore depends on the temporal and spatial origin of interneurons and subtype specification, as well as on interactions with the RG scaffold and projection neurons (Guo and Anton, 2014). Moreover, it was recently shown that subtypes of pyramidal neurons extrinsically influence the fate of interneurons by regulating their laminar fate and circuitry (Lodato et al., 2011a) (Figure 5B).

Cerebral cortical functions depend on the accurate construction of neural circuits, which require balanced and coordinated activities between the glutamatergic excitatory projection neurons and GABAergic inhibitory interneurons (Guo and Anton, 2014). The achievement of such a highly complex architecture relies on an exquisite orchestration between the spatio-temporal generation of distinct cell types in progenitors, the control of their migration and final settling position (often via long migration journeys), and finally cell death (Pierani and Wassef, 2009). The neuronal migration plays essential roles in the establishment of this expanding laminar structure, and the molecular mechanisms that regulate these processes are being unraveled and remain a highly active area of research.

1.3 Neuronal migration

The proper development of cortical circuits requires highly orchestrated cell migratory events to establish specific laminar position, orientation, and connectivity of neurons (Kriegstein and Noctor, 2004; Rakic, 2007). Neocortical circuits are assembled from subtypes of glutamatergic excitatory and GABAergic inhibitory neurons with divergent anatomical and molecular signatures and unique physiological properties (Marín and Müller, 2014). Although multiple genes and

signaling networks have been implicated in cortical neuron migration, the molecular mechanisms underlying this process are still poorly understood.

The importance of neuronal migration for cerebral cortical formation and function is evident in neurodevelopmental disorders resulting from disrupted neuronal migration. Major neuronal migration defects lead to severe brain malformations including lissencephaly, heterotopias, polymicrogrya, schizencephaly, and focal cortical dysgenesis (Spalice et al., 2009). The mutations in cytoskeletal regulators, extracellular matrix molecules, or posttranslational modifiers tend to affect both radial and tangential migration (Valiente and Marín, 2010). However, minor changes in temporal or spatial patterns of neuronal migration may contribute to the formation of aberrant neuronal circuitry underlying these disorders, which have been recently summarized (Evsyukova et al., 2013). Especially disruption of cortical GABAergic interneurons function has been linked to various neurodevelopmental disorders, including epilepsy, mental retardation, autism, and schizophrenia (Akbarian and Huang, 2006; Cossart et al., 2005; Levitt et al., 2004; Lewis et al., 2005; Rossignol, 2011).

One of the structures that better illustrates how the types of migrations are integrated during brain development is the cerebral cortex (Marín et al., 2010). Two major types of neuronal migration have been implicated in corticogenesis: radial migration of excitatory neuron precursors and tangential migration of interneurons as well as C-R cells. In the past several years, significant progress has been made in understanding how these parallel events are regulated and coordinated during corticogenesis (Huang, 2009).

During radial migration, neurons follow a trajectory that is perpendicular to the ventricular surface, moving alongside RG fibers expanding the thickness of the neural tube. In contrast, tangentially migrating neurons move in trajectories that

are parallel to the ventricular surface and orthogonal to the RG palisade (Figure 6). Besides their relative orientation, some of the basic mechanisms underlying the movement of cells using each of these two modes of migration are also different. For example, radially migrating neurons often use RG fibers as substrate, whereas tangentially migrating neurons do not seem to require their support to migrate. Even so, neurons may alternate from radial to tangential movement and vice versa during the course of their migration. In fact, tangentially migrating interneurons switch to radial migration as they move toward specific laminar locations within the CP (Figure 6) (Faux et al., 2012; Nadarajah and Parnavelas, 2002; Yokota et al., 2007) (see below).Thus, the different types of neurons (projection neurons and interneurons) coordinate their oriented migratory activities within the developing cerebral cortex to ultimately produce functional laminar organization (Evsyukova et al., 2013). This suggests that both types of migrations share common principles, in particular those directly related to the cell biology of movement (Marín et al., 2010).

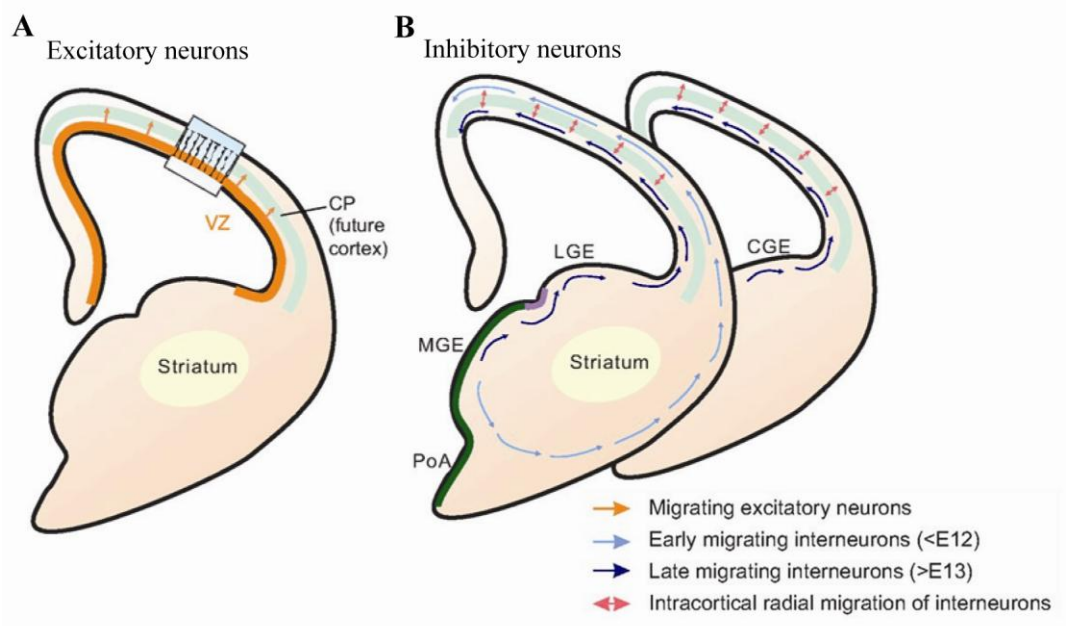

Figure 6. Generation and migration of neocortical excitatory and inhibitory neurons. (A,B) Excitatory and inhibitory neurons originate from different germinal zones of the embryonic

telencephalon. (A) Cortical excitatory neurons are generated from progenitor cells in the VZ of the dorsal telencephalon. Newborn excitatory neurons undergo RG fiber-guided radial migration (orange arrows) and settle into the developing CP (light green). (B) Cortical inhibitory interneurons are predominantly generated from progenitor cells located in the proliferative zone of the ventral telencephalon, mainly within the MGE (dark green) and the CGE. A small population of cortical inhibitory interneurons is produced from the POA. Newborn inhibitory interneurons follow two tangentially oriented migratory streams to enter the cortex: a superficially migrating early cohort (pale-blue arrows) migrates through the MZ, and a deeply migrating second and more prominent cohort (dark-blue arrows) migrates through the lower IZ and SVZ. Upon reaching the cortex, they switch to radial migration (pink double-headed arrows) and settle into their final laminar position in the CP. Abbreviations: VZ, ventricular zone; SVZ, subventricular zone; RG, radial glial cells; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; CP, cortical plate; MZ, marginal zone; IZ, intermediate zone; POA, preoptic area. Figure adapted from (Gao et al., 2013).

1.4 Cellular mechanisms in neuronal migration

Neuronal migration is a dynamic and directional process. Neurons must coordinate the extension and branching of their leading processes, cell movement with axon specification and extension, switching between actin and microtubule motors, and attachment and recycling of diverse adhesion proteins (Cooper, 2013). Migration of neurons is a tightly regulated by varios extracellular cues that ultimately trigger rearrangement of the cytoskeletal components. Migrating neurons are highly polarized in the direction of their movement. The standard movement of neurons is commonly known as locomotion (Nadarajah and Parnavelas, 2002). Neurons undergoing locomotion follow three cellular events synchronized steps to move (Ayala et al., 2007; Marín and López-Bendito, 2006) (Figure 7).

First, the cell extends a leading process, which is tipped by structures that are similar to the growth cones of migrating axons, and they are thought to play an important role in sensing the surrounding microenvironment and thereby contributing to the guidance of neurons (Rakic, 1990; Yee et al., 1999). The generation, maintenance, and remodeling of a leading process marks the direction

followed by the cell. The leading process acts as the compass of migrating neurons, selecting the direction of migration in response to chemotactic cues (Marín et al., 2010). The morphology of the leading process varies in different neuronal types, probably reflecting an adaptation to different migratory requirements. In cortical interneurons, for instance, the leading process branches as part of the migratory cycle (Bellion, 2005; Kappeler et al., 2006; Martini et al., 2009). This seems to be common to many tangentially migrating neurons (Okada et al., 2007). In contrast, radially migrating neurons seem to have a single leading process (Rakic, 1972).

Second, the nucleus translocates into the leading process, a step referred to as nucleokinesis that occurs in two steps. First, a cytoplasmic swelling forms in the leading process, immediately proximal to the nucleus. The centrosome, which is normally positioned in front of the nucleus, moves into this swelling (Bellion, 2005; Schaar and McConnell, 2005; Tsai and Gleeson, 2005). The centrosome is accompanied by additional organelles, including the Golgi apparatus, mitochondria, and the rough endoplasmic reticulum. Second, the nucleus follows the centrosome. These two steps are repeated producing the typical saltatory movement of migrating neurons. Third, in the final step, the migrating neuron eliminates its trailing process, which leads to the net movement of the cell (Marín et al., 2010).

1.5 Projection neurons: radial migration

Newly born projection neurons, which constitute the majority of cortical neurons, reach their target locations within the developing cortex via radial migration (Ayala et al., 2007; Marín and Rubenstein, 2003; Marín et al., 2010) (Figure 6A). Proper cortical lamination thus requires precise guidance of these neurons from

the VZ to CP in order for them to reach appropriate cortical layers (Ayala et al., 2007; Rakic, 2007).

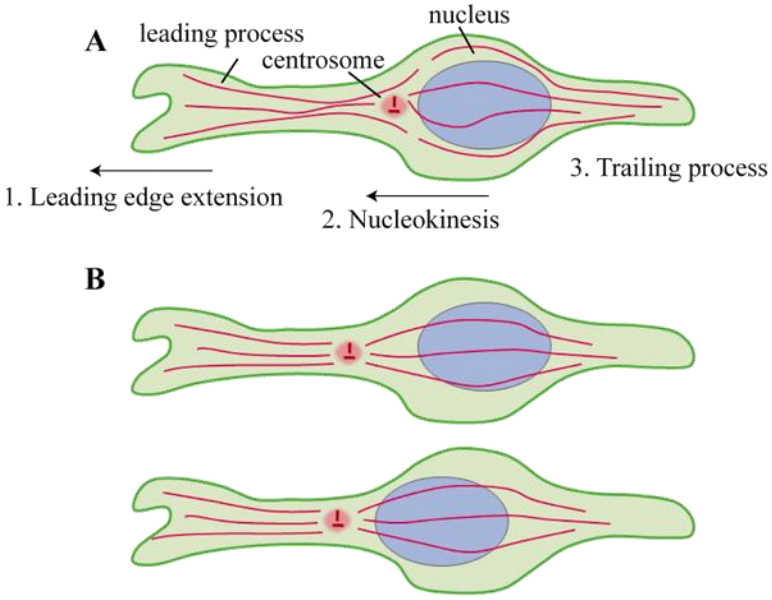

Figure 7. Cellular events in neurons locomotion. (A) Neuronal migration occurs in three stages. First, the leading process advances in the direction of migration (1). This is followed by advance of the centrosome into the leading process. Subsequently, the nucleus translocates forward in a saltatory fashion (nucleokinesis) (2) and (B), and the trailing process of the neuron undergoes remodeling (3). Neuronal migration results from repeating of this basic sequence of events. Figure adapted from (Tsai and Gleeson, 2005).

Radial migration occurs in two different modes: somal translocation and locomotion (Kriegstein and Noctor, 2004; Marín and Rubenstein, 2003; Nadarajah and Parnavelas, 2002; Nadarajah et al., 2001) (Figure 8). The earliest neurons that form the PP use somal translocation, whereas most cortical neurons forming the CP employ locomotion (Evsyukova et al., 2013).

1.6 Cellular mechanism of radial neuron migration

During early corticogenesis, somal translocation is the predominant mode and some neurons appear to migrate solely by this mode. Somally translocating neurons move continuously, without significant pausing (Nadarajah et al., 2001). Early-born cortical neurons possess a long, branched leading process attached to the pial surface (Miyata et al., 2001; Nadarajah et al., 2001). The leading process gets progressively shorter as the cell soma translocates upward (Nadarajah et al., 2001). Importantly, this mode of migration does not depend on RG guides, but attachment of the leading process to the intact pial basement membrane is likely required (Hawthorne et al., 2010; Marín and Rubenstein, 2003) (Figure 8).

The fundamental feature of migration via locomotion is the involvement of RG, highly polarized cortical progenitor cells that not only serve as precursors to the majority of cortical neurons but also provide an instructive scaffold for neuronal migration (Marín and Rubenstein, 2003; Marín et al., 2010; Noctor et al., 2001; Rakic, 1972). As the thickness of the developing cortex grows, neurons undergoing locomotion use these fibers as a guide to reach the CP (Campbell and Götz, 2002; Rakic, 1972). Polarized RG have a characteristic pear-shaped soma positioned in the VZ and posses long processes that extend spanning the entire thickness of the developing cortex from the ventricular wall to the pial surface (Ayala et al., 2007). After the final division, immature neurons transiently become multipolar, with multiple neurites within SVZ and lower intermediate zone (IZ), which is also known as the premigratory zone (Ohshima et al., 2007). These multipolar neurons do not seem to require RG and move at a slower speed in the direction of the pial surface, occasionally reverting back to the ventricular surface and then reassuming pia-oriented migration (Tabata and Nakajima, 2003; Tabata et al., 2009) (Figure 8).

Importantly, the multipolar stage is transient and is followed by a switch back to the bipolar morphology and locomotion-based mechanism of migration (Noctor et al., 2004; Tabata et al., 2009), with a long leading process oriented toward the pial surface and a shorter trailing process in the direction of the VZ.

Neurons migrating by locomotion switch to somal translocation during the final stages of their migration, right after their leading process makes contact with the MZ (Nadarajah et al., 2001), which implies that these two modes of radial migration are not entirely neuronal-type specific. However, considering differences in the morphologies of locomoting and translocating cells, as well as differences in how they move (continuous translocation versus saltatory movement of locomoting neurons), the two modes of radial migration are likely mediated by different mechanisms (Franco et al., 2011; Marín and Rubenstein, 2003; Nadarajah et al., 2001).

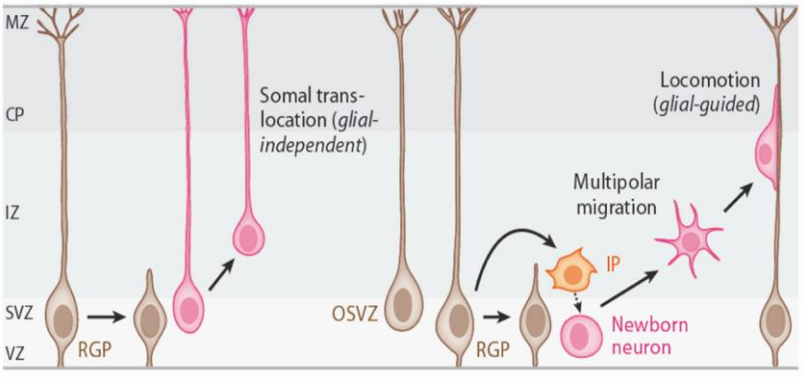

Figure 8. Principals cellular mechanism in radial migration. Projection neurons, generated from RG progenitors (brown) or IP (orange), migrate using either RG-independent somal translocation or glial-guided locomotion. Newborn neurons undergo a multipolar transition phase prior to glial-guided radial migration. Abbreviations: RG, radial glial cells; IP, intermediate progenitors. Figure adapted from (Evsyukova et al., 2013).

1.7 Regulation of projection neuron migration

Multiple cellular events are precisely regulated and coordinated to ensure proper neuronal migration (Ayala et al., 2007; Bielas et al., 2004; Creppe et al., 2009; Guerrier et al., 2009). The mechanisms and cues guiding radial neuronal migrations are highly diverse. Here will be addressed those related with: RG-neuron interactions, transcriptional and cues regulating radial migration.

RG cells play a vital role in neuronal migration, since neurons undergoing locomotion use these fibers as a guide to reach the CP. Various membrane-bound cell adhesion molecules (CAM), including astrotactin, neuregulin, and several integrins, mediate the interaction of migrating neurons and RG fibers (Adams et al., 2002; Anton et al., 1997; Edmondson et al., 1988; Fishell and Hatten, 1991; Stitt and Hatten, 1990). In the case of integrins, several studies indicate a wide spectrum of cell adhesion functions mediated by the integrin family, which cooperatively modulate the radial migration of cortical neurons (Anton et al., 1999; Dulabon et al., 2000; Graus-Porta et al., 2001; Marín and Rubenstein, 2003; Schmid et al., 2004). Integrin activation is translated by intracellular mediators to modifications of the microtubule and actin networks as well as cell-cell adhesion, both of which are precisely modulated during neuronal migration (Marín et al., 2010). Recent studies have shown that the interaction between RG fibers and migrating neurons also relies on the adhesive properties of Gap junctions (Cina et al., 2009; Elias et al., 2007). Several connexins, the component of Gap junctions, are expressed in both RG and migrating neurons, and their association in *trans* is required for glial-guided migration. The mechanisms regulating the dynamic assembly and disassembly of these transient contacts between RG fibers and migrating neurons are currently unknown (Marín et al., 2010).

Recent data suggest that transcriptional programs regulate neuronal migration, positioning, and acquisition of correct neuronal identity in the developing neocortex (Kwan et al., 2012). For example, transcription factors that are expressed by both early- and late-born projection neurons (Sox5, POU3F2 and -3) regulate neuronal migration in a neuronal type-specific manner (Kwan et al., 2012, 2008; Lai et al., 2008). However, early neuron transcription factor Tbr1 and late neuron transcription factor Satb2 regulate neuronal migration in a layer-specific manner (Alcamo et al., 2008; Bedogni et al., 2010; Britanova et al., 2008; Kwan et al., 2012). The downstream mechanisms of this transcriptional regulation have not yet been explored fully, but it is intriguing that POU3 transcription factors exhibit their regulatory effect via Cdk5 and Dab1, suggesting that migration-dependent transcriptional programs modulate intracellular signaling pathways known to be essential for oriented neuronal migration (Evsyukova et al., 2013). KLF4, Scratch2 and FoxG1 transcription factors are required for the multipolar-to-bipolar transition, migration during the multipolar phase and exit from the multipolar stage, respectively (Miyoshi and Fishell, 2012; Paul et al., 2014; Qin and Zhang, 2012).

Since leading process extension of migrating neurons is highly similar to growth cone extension by navigating axons, guidance cues may operate similarly in migrating neurons as in growth cones and mainly affect leading process extension (Huang, 2009). A large number of secreted extracellular molecules have been shown to regulate migration, including: Slits, Netrins, Semaphorins, and Reelin. The functions of Slits, Netrins, and Semaphorins have been characterized in tangential migration, but their role in radial migration is not as well studied as Reelin (Marín et al., 2010).

Reelin, a gene encoding for a large extracellular glycoprotein, is mutated in *reeler* mice (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995;

Sheppard and Pearlman, 1997). During cortical development, Reelin is expressed by C-R cells in MZ (Soriano and Del Río, 2005), and from E18 onward it is expressed by interneurons (Alcántara et al., 1998; Borrell et al., 1999; Drakew et al., 1998). The spontaneous mutant *reeler* mouse display layering defects in multiple brain regions, but most prominently in cortical areas (Lambert de Rouvroit and Goffinet, 2001; Rice and Curran, 1999). In the neocortex, the PP forms normally, but the next cohort of cortical neurons fails to divide it into the MZ and the SP. Loss of Reelin signalling reverses the lamination of the cortex to an outside-in pattern, whereby late-born pyramidal neurons are unable to migrate past the early-born cells (Caviness, 1982; Hevner et al., 2004). Reelin plays multiple roles in regulating the proper laminar position of neurons in the cortex by acting as a stop signal to terminate the radial migration of cortical neurons at the top of the CP (Frotscher, 2010; Magdaleno et al., 2002).

Reelin binds to two members of the lipoprotein family receptors, VLDLR and ApoER2 (D'Arcangelo et al., 1999; Hiesberger et al., 1999) and induces tyrosine phosphorylation of Dab1 (Howell et al., 2000, 1999), which triggers a signaling cascade that instructs neurons to adopt their proper destination in the cortex. Dab1 phosphorylation is finally translated into the regulation of microtubule dynamics, as supported by several lines of evidence (Marín et al., 2010). Also, Dab1 interacts with Lis1, aprotein implicated in the development of a human brain developmental disorder called lissencephaly. Reelin/Lis1 heterozygous mice show a greater degree of cortical malformation than the individual heterozygotes, suggesting an epistatic relationship of the two genes (Assadi et al., 2003).

Significant insights into cortical neuron migration have also come from studies of mouse mutants in cyclin-dependent kinase 5 (cdk5) and related genes (Huang, 2009). In cdk5 mutants the cortical neurons appear to fail a multipolar to bipolar transition before initiation of radial migration (Ohshima et al., 2007). Thus, Cdk5

appears to regulate cytoskeletal dynamics in migrating neurons. Indeed, several molecular targets of Cdk5 have been identified that modulate either the microtubule or actin cytoskeleton, or both (Huang, 2009). Recently was described the role of Mst3, a serine/threonine kinase family member, in neuronal positioning. The activity of Mst3 is regulated via Cdk5 signaling and regulates neuronal migration via RhoA-dependent actin dynamics (J. Tang et al., 2014). As RG are an intrinsically asymmetric cell type, it is conceivable that this asymmetry may contribute to the directionality of radial neuron migration. Alternatively, additional pathways may act to regulate this process. Indeed, recent data suggest that Semaphorin 3A and 3F (Sema3A and 3F) in the developing CP may regulate the directionality of cortical neuron migration (Chen et al., 2008).

1.8 Interneurons

1.8.1 Generation and specification of cortical interneurons

The discovery, approximately 15 years ago, that cortical GABAergic interneurons originate outside the pallium has revolutionized our understanding of the development of the cerebral cortex (Marín, 2013). While the MGE, CGE and POA contribute to the generation of cortical interneurons (Flames et al., 2007; Rubin et al., 2011), the LGE is mostly involved in striatal and OB histogenesis (Yun et al., 2003).

Despite these observations, what is well known is that cortical interneurons, especially from the MGE, migrate through a corridor in the LGE on their way to the cortex and that LGE plays an important instructive role in their migration (Flames et al., 2004). These distinct subpallial regions differ in progenitor domain composition and also in the ability to generate interneurons subtypes characterized

by specific networks of transcription factors. In addition to genetic programs, diffusing molecules also participate in shaping the timing, space and specificity of cortical interneurons subtype production (Peyre et al., 2015).

During mouse embryogenesis MGE histogenesis starts at around E9, followed by the generation of LGE at E10 and CGE at E11 (Smart, 1976). More recently, genetic lineage tracing experiments have confirmed that most cortical interneurons originate from the MGE and CGE (Fogarty et al., 2007; Miyoshi et al., 2010; Rubin et al., 2010; Xu et al., 2008), and adding proved as well that the POA acts as the source of a small fraction of cortical interneurons (Gelman et al., 2011, 2009). MGE, CGE and POA were estimated to contribute to cortical interneurons in a ratio of 6:3:1, respectively (Marín, 2013).

The molecular rules governing cell proliferation in the ventral telencephalon as well as the characterization of the distinct interneurons progenitor behavior has just started to be unveiled. For some time it was anticipated that GE progenitors would display a proliferative behavior similar to progenitors in the cerebral cortex (Ross, 2011). Brown and collaborators identified the presence of RG in the VZ of MGE and POA that undergo interkinetic nuclear migration and divide asymmetrically in the VZ to self-renew and produce IP or differentiating interneurons (Brown et al., 2011). Later was proposed a more complex hierarchical classification for ventral progenitors. RG sit at the base of this classification and divide asymmetrically to generate both an amplifying and a self-renewal branch. These cells give rise to SNP. Both RG and SNP generate subapical progenitors (SAP) which in turn divide to produce basal radial glia (bRG) or BP and they contribute to the great SVZ expansion (Pilz et al., 2013).

The interneurons are specified at the time of birth and therefore subtype specification is largely defined within the GE by transcription factors in a

temporal regulation (Faux et al., 2012). GE histogenesis requires a complex interplay between morphogens and transcription factors to ventralize the structure and promote interneurons subtype production (Peyre et al., 2015). The analysis of the expression of several transcription factors within the VZ of the MGE have led to the proposal that this region can be compartmentalized into five different progenitor domains (Flames et al., 2007). The dorsal region of the MGE (dMGE) preferentially gives rise to SST-expressing interneurons. In contrast, the ventral part of the MGE (vMGE) was shown to mostly generate PV-expressing interneurons (Xu et al., 2010). On the other part, MGE transcription factor expression [Nkx2.1, Lhx6, Lhx8(7), Sox6] promotes the specification of interneuron subtypes (Azim et al., 2009; Batista-Brito et al., 2009; Butt et al., 2008; Flandin et al., 2011; Fragkouli et al., 2005; Liodis et al., 2007; Zhao et al., 2008, 2003). *Dlx* genes also contribute to interneuron specification and maturation. *Dlx* genes expression is temporally regulated, following the sequence: *Dlx2*, *Dlx1*, *Dlx5*, and *Dlx6* (Eisenstat et al., 1999; Liu et al., 1997). *Dlx1/2* gene seems to be particularly important for the acquisition of SST, CR, NPY and Reelin fates (Cobos et al., 2005) as its absence leads to an abnormal expression of cortical markers in the ventral telencephalon (Long et al., 2009a, 2009b). Several studies have demonstrated that CGE derived interneurons acquire either a CR and/or VIP (Butt et al., 2005; Pleasure et al., 2000) or Reelin identity (Miyoshi et al., 2010). Gsh or Gsx homeobox transcription factors act at the top of the genetic network involved in CGE cell specification (Pei et al., 2011; Xu et al., 2010). Interestingly, the control of the choice between proliferation and differentiation by *Gsh* genes seems to involve the downstream target Mash1 (Fode et al., 2000). In Mash1 loss of function there is premature differentiation of progenitors located in the SVZ and precocious expression of *Dlx* genes (Casarosa et al., 1999; Yun et al., 2002), downstream effectors. Other CGE transcription factors include *Nrf2f1* and *Nrf2f2* or *Couptf1* and *Couptf2*, respectively, as well as *SP8*. These genes are

however not exclusive from CGE, as they have been identified in the dMGE and POA (Lodato et al., 2011b). Finally, the POA primarily produces NPY-containing interneurons and a smaller proportion of PV-containing and SST-containing interneurons. In terms of molecular markers expression, the cells generated by the POA resemble the ones originating from the CGE (Gelman et al., 2009). *Shh* and *Nkx2.1* but not *Lhx6* are also expressed in the POA (Flames et al., 2007). *Dbx1* and *Nkx6.2* are respectively markers of the dorsal and ventral POA. The function of these genes remains, however, elusive.

It also became clear that there is a strong correspondence between interneuron and its temporal production. For example: interneuron genesis in mice takes place between E9 and E16, and the peak production from the MGE occurs around E12-E13 (Faux et al., 2012). In contrast, the initiation and peak production of interneurons from the CGE is around E12 and E15-E16, respectively (Miyoshi et al., 2010, 2007). This temporal pattern is reflected by the subtypes that are generated, for example, most SST positive Martinotti cells are predominantly born at E9, SST-and CR-double-positive cells at E12 and most VIP-positive cells at E15 (Miyoshi et al., 2007; Sousa et al., 2009).

1.8.2 Tangential migratory streams of interneurons in the developing brain

Whereas pyramidal cells migrate radially to adopt their corresponding laminar position in the nascent cortex, interneurons originating from the ventral telencephalon choreograph a complex pattern of migration to reach their final position (Guo and Anton, 2014). Interneurons do not only migrate dorsally to the cortex. They also tangentially migrate toward other destinations within the developing brain; ventrolaterally to the striatum, caudally to the hippocampus, and rostrally to the OB (Ang et al., 2003; Gelman and Marín, 2010; Lois and Alvarez-

Buylla, 1994; Nery et al., 2002; Stenman et al., 2003; Sussel et al., 1999; Yozu et al., 2005).

There are three general consecutive phases through which neocortical interneurons adopt their final position in the cortex during corticogenesis. First, they reach the pallium along well-defined long tangential migration routes from the subpallium; towards the corticostriatal junction and into the cortical wall, second they spread out tangentially within specific migratory paths to occupy the entire cerebral cortex, and the third involves a shift towards a radial trajectory so as to enter the CP and they integrate into specific layers of the cortex (Faux et al., 2012; Marín, 2013). These phases in turn can be broadly divided into six decision-making steps involved in interneuron migration (Guo and Anton, 2014) (Figure 9).

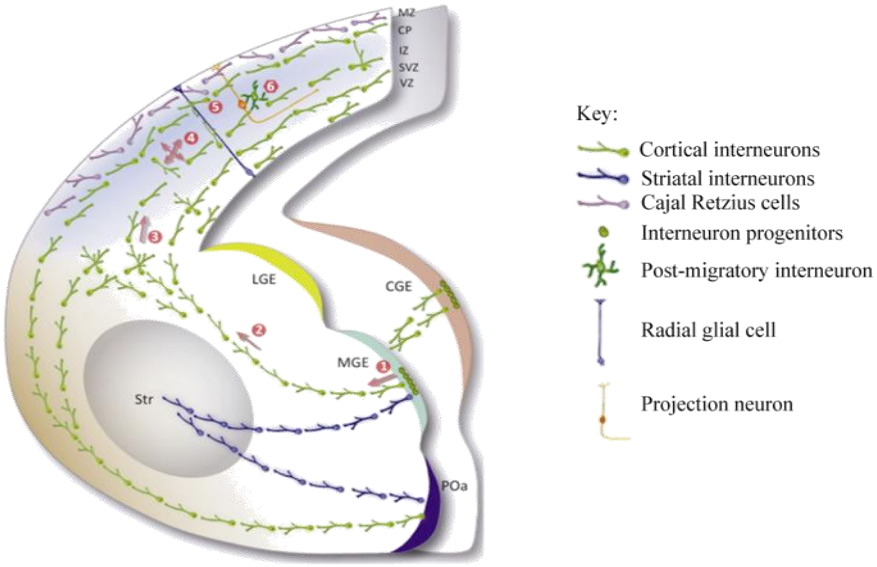

Figure 9. Patterns of interneuron migration in the developing telencephalon. This schema shows a rostral and caudal hemisection through the mouse telencephalon at the mid-embryonic stage (E15). The major decision-making steps (1-6) involved in the migration of cortical interneurons derived from the subpallium are illustrated. Interneurons derived from the MGE (green), the POA (purple), or the CGE (orange) exit the proliferative zones and initiate their migration toward the developing neocortex and striatum. Cortical interneurons traverse around the developing striatum, transit across the corticosubpallial boundary, and course tangentially into the cortex, whereas striatal interneurons migrate ventrolaterally into the developing striatum. Cortical

interneurons transit the neocortex mainly through the MZ, SP, and IZ/SVZ migratory streams. Once in the neocortex, tangentially migrating interneurons undergo multimodal local migration as they reach and settle in specific areal and laminar locations within the emerging CP before forming functional synaptic contacts with appropriate projection neuron partners. Multiple decision-making steps are involved in this process. These include: (1) exit from the proliferative zone and initiation of migration in the subpallium; (2) selection of migratory route toward the dorsal cortex; (3) choice of migratory stream within the neocortex; (4) local orientation of migration within the cerebral wall; (5) identification of the final areal and laminar location; and (6) termination of migration at the appropriate cortical layer. Arrows indicate net directionality of movement. Abbreviations: MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; CGE, caudal ganglionic eminence; POA, preoptic area; Str, striatum; MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Figure adapted from (Guo and Anton, 2014).

Newborn interneurons have an enormous ability to migrate throughout the developing telencephalon, a feature that is common to all interneurons, independent of their origin (Marín, 2013). They cluster around RG fibers or coalesce as a migratory stream as they exit from the subpallial proliferative zone (Brown et al., 2011; Hansen et al., 2013). Interneurons with different temporal and spatial origin in the subpallium follow specific migratory routes, suggesting that the distinct origins of interneurons help to prespecify their migratory routes (Guo and Anton, 2014).

Once generated in the MGE, postmitotic interneurons journey towards the cortex by first traversing the developing LGE (striatum primordium) en route to the corticostriatal junction. During their transit through the subpallium, cortical interneurons actively avoid entering the POA and the striatum, two structures that develop in close proximity to the MGE. As compared with MGE-derived interneurons, relatively little is known about the tangential migration of CGE-derived and POA derived interneurons. However, the routes followed by CGE-derived and POA-derived interneurons to reach the cortex are largely distinct from those used by MGE-derived interneurons, suggesting that interneurons born in the CGE and POA respond, at least in part, to a different set of guidance cues (Faux et al., 2012; Marín, 2013). In particular, most CGE-derived interneurons colonize the

cortex through its caudal pole (Yozu et al., 2005); whereas POA interneurons reach the cortex via a route that courses superficially to the striatum (Gelman et al., 2009; Marín and Rubenstein, 2001; Zimmer et al., 2010). Interneurons that reach the cortex through routes that course superficially (putatively POA-derived) or deep (putatively MGE-derived) to the striatum seem to express different guidance receptors (Zimmer et al., 2011).

1.8.3 Intracortical dispersion of interneurons

Travel across the pallial-subpallial boundary and course tangentially into the cortex marks the termination of a phase in the interneurons migratory program. The second phase in the migration of cortical interneurons primarily involves the homogeneous dispersion of interneurons throughout the entire cerebral cortex, which do not disperse in an indiscriminate way, but rather use a very specific set of routes or migratory streams. Most interneurons migrate through one of two large migratory streams, a superficial route that courses through the MZ, and a deep route that largely overlaps with the SVZ. A smaller fraction of interneurons migrate through the SP (Lavdas et al., 1999; Wichterle et al., 2001; Nadarajah et al., 2002; Yokota et al., 2007) (Figure 10). A first cohort of interneurons (E11.5 in mouse) from the MGE migrates dorsolaterally and forms a stream onto the top of the PP. Later during corticogenesis (E13-E15 in mouse), a second and more prominent stream of interneurons, mainly from the MGE, rapidly migrate into the neocortex, through the IZ (Marín and Rubenstein, 2001). At later stages of corticogenesis, and after de formation of the CP, interneurons enter the cortex, where they disperse tangentially via highly stereotyped streams: largely through the lower IZ and SVZ, as well as through migratory streams in the SP and MZ (Anderson et al., 2001; Lavdas et al., 1999; Métin et al., 2006). Especifically the SP was defined as a transient zone (Bystron et al., 2008; Rakic, 1977) that contains residential SP cells, and numerous other migrating cells and extending

fibers through the region (Montiel et al., 2011). SP has received renewed attention because of its functional relevance in cerebral cortex development (Ayoub and Kostovic, 2009; Hoerder-Suabedissen and Molnár, 2015). The 3D profile of cortical interneuron migration indicates that, simultaneously with the MGE-derived streams, a wave of interneurons originating from the CGE migrate in a lateral and medial direction to enter the caudal-most end of the cerebral cortex (Ang et al., 2003; Nery et al., 2002; Yozu et al., 2005).

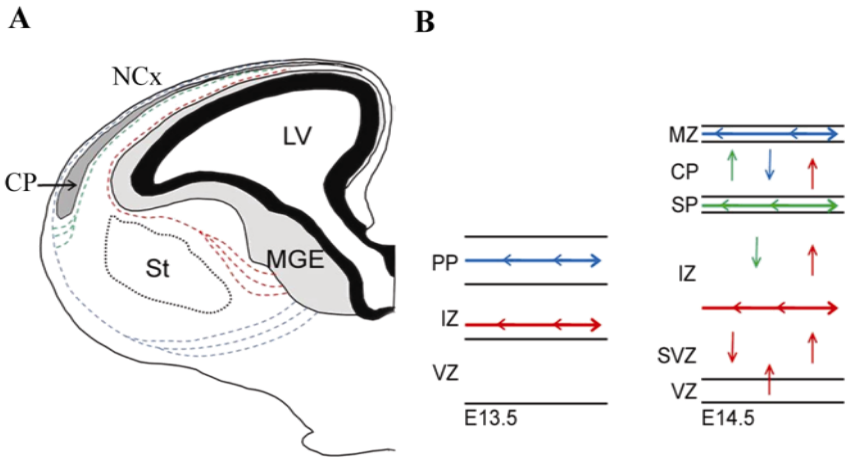

Figure 10. Tangential migration of cortical interneuron. (A) Drawing of a coronal section of E15.5 mouse brain showing the tangential paths of early- (blue broken lines), and late (red broken lines)-born interneurons. Upon the emergence of the CP, an additional migratory path is formed within the SP (green broken lines). (B) Schema showing the tangential and radial movements of interneurons within the cortical wall at E13.5 and E14.5. Abbreviations: LV, lateral ventricle; PP, preplate; NCx, neocortex; SP, subplate; Sp, septum; St, striatum. Figure adapted from (Hernández-Miranda et al., 2010).

Several lines of evidence suggest that the interneurons are not distributed randomly across these streams. Cell-intrinsic determinants, acquired either before or after their entry into the cortex (Avila et al., 2013; Crandall et al., 2007; Ferguson et al., 2005; López-Bendito et al., 2003), in combination with extracellular cues released within the cerebral wall (Alcántara et al., 2006; Antypa

et al., 2011; Shinozaki et al., 2002; Stanco et al., 2009; Zarbalis et al., 2012); dictate the choice of distinct interneuron migratory route within the cerebral wall. Evidence comes from the observation that populations of cortical interneurons migrating along the different cortical streams do not show the same gene expression profile (Antypa et al., 2011). Also, GABA signaling, in addition to its migration promoting role, seems to be implicated in the choice of the migratory route during cortical migration (López-Bendito et al., 2003).

Remarkably, interneurons stay away from the CP during this phase, whereas pyramidal cells begin forming cortical layers in this location. This observation suggests that the dispersion of cortical interneurons throughout the cortex requires the active avoidance of the CP, their final place of residence. Interestingly, the region of origin (MGE vs. CGE) does not seem to influence the choice of migratory stream by cortical interneurons (Miyoshi and Fishell, 2011). The confinement of interneurons to specific migratory streams in the cortex seems to generally rely on chemokine signaling, but it is unclear whether specific interneurons show a preference for a particular route of migration.

Within the developing cortex, that migration of cortical interneurons appears to occur primarily in two streams, in the cortical MZ and the IZ/SVZ (Kriegstein and Noctor, 2004); the most prominent cortical migratory streams compared to the SP stream. In fact, most of the studies to elucidate the mechanisms that regulate the preferential interneurons migration through the streams refer to the cues regulating the interneurons confining to the MZ and IZ/SVZ; but have been less reported about the SP stream (Abe et al., 2014; Li et al., 2008; López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011; Wang et al., 2011). The SP is a transient cortical structure that forms during mammalian brain development (Allendoerfer and Shatz, 1994) and has numerous developmental functions (Hoerder-Suabedissen and Molnár, 2015). At embryonic ages, SP cells are involved in

thalamocortical and corticofugal axon guidance, including establishment of topographical projections (Ghosh et al., 1990; Grant et al., 2012; McConnell et al., 1989). SP neurons are a heterogeneous population of neurons that are among the earliest generated in the cerebral cortex (Allendoerfer and Shatz, 1994; Hoerder-Suabedissen and Molnár, 2013; Price et al., 1997; Rakic, 1974; Robertson et al., 2000) and are recognized to be important players in cortical development and maturation, with distinct roles at different developmental ages (Kanold and Luhmann, 2010). A recent study identified a further source of tangentially migrating SP neurons (rostro-medial telencephalic wall), although these are non-GABAergic (Pedraza et al., 2014). Exactly what interneuronal subtypes participate in this migratory pathway is not yet well understood.

Once interneurons arrive into the cortex, different modes of migration are employed as intracortical migration. These migratory behaviours include: (1) the multidirectional migration within tangential routes (Ang et al., 2003; Tanaka et al., 2003, 2009, 2006), (2) the radial migration for cells moving away from the tangential routes into the CP (Martini et al., 2009) and (3) the ventricle-directed migration from the IZ/SVZ towards the VZ (Nadarajah and Parnavelas, 2002). The second migratory mode encompasses both inward radial migration towards the CP from the MZ (Ang et al., 2003; Polleux et al., 2002; Tanaka et al., 2003) and outward radial migration towards the CP from the IZ/SVZ (Hevner et al., 2004; Polleux et al., 2002; Tanaka et al., 2003, 2006) (Figure 11).

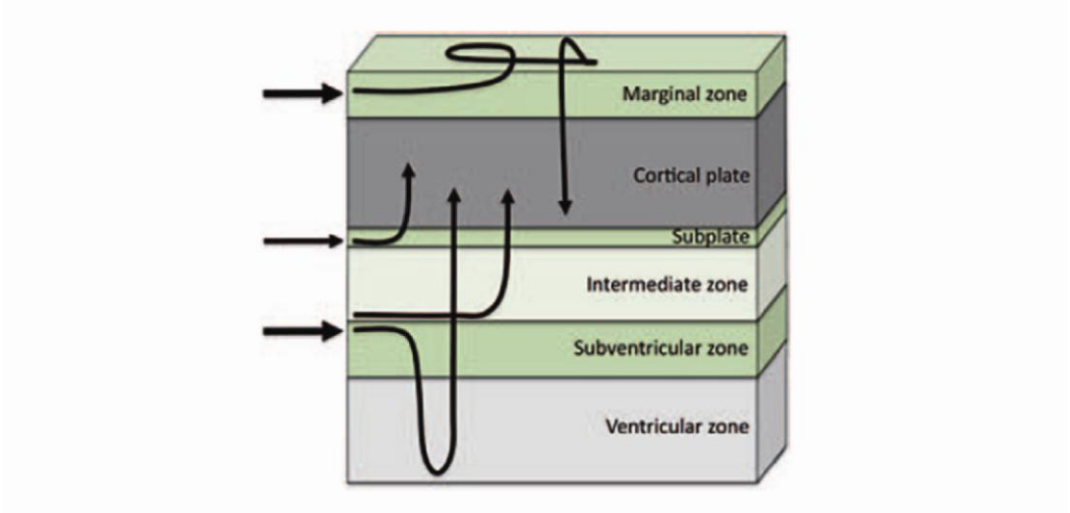

Figure 11. Intracortical migration of interneurons. Intracortical migration represented as multidirectional migration within the MZ, the inward and outward radial migration from the tangential paths into the CP and the ventricle-directed migration towards the VZ. Abbreviations: MZ, marginal zone; CP, cortical plate; VZ, ventricular zone. Figure adapted from (Faux et al., 2012).

Alternatively, changing streams routes might only reflect transient, exploratory movements that migrating interneurons make as they progress through the cortex. In this context, the spreading of interneurons in the MZ stream exhibit a particular migratory behavior called "random walk", leading to constant multidirectional changes (Tanaka et al., 2009). This behavior of interneurons is believed to contribute to the tangential dispersion of interneurons to appropriate cortical areal positions, but it is tempting to speculate that interneurons in the MZ may actually undergo contact repulsion to disperse through the surface of the cortex, as recently shown for C-R cells (Villar-Cerviño and Marín, 2012). It is tempting to speculate that similar contact-repulsive interactions may exist between individual interneurons within the MZ stream, between C-R cells and interneurons, or between interneurons and RG endfeet and may thus contribute to the appropriate dispersion of interneurons within the cerebral cortex (Guo and Anton, 2014).

1.8.3.1 Switch tangential to radial migration

The final stages of intracortical dispersion of interneurons depend on a tangential-to-radial switch of the interneuronal migratory mode. The mechanisms that trigger the switching from tangential to radial of cortical interneurons remain unclear. It has been demostrated that interneurons of different birthdates remain within the tangential migration streams for similar amounts of time (~48 h) (López-Bendito et al., 2008). However, it has been shown that the timing of exit from the migratory streams is related with the different chemoattractive activities present in the embryonic cortex, which must be hierarchically organized (Marín, 2013). In fact it correlates with the loss of responsiveness to Cxcl12 as an attractant (Li et al., 2008). One possibility is that interneurons have an internal clock that determines their maturation, independently of the environment and this would explain why MGE-derived interneurons invade the CP progressively, with early-born interneurons entering the CP earlier than late-born interneurons (López-Bendito et al., 2008; Pla et al., 2006).

During normal embryonic development interneurons normally tend to avoid the CP. The CP undergoes invasion by MGE-derived neurons from both the IZ (moving outward) and from the MZ (moving inward). Also it has been proposed that MGE-derived interneurons migrate first to the cortical SVZ, then from the SVZ to the CP, accumulating transiently in the MZ. The existence of this outward migration was confirmed and identified as being characteristic of late-born interneurons (after E15.5) (Hevner et al., 2004; Polleux et al., 2002; Tanaka et al., 2009). Finally, there is evidence that a subpopulation of cortical interneurons within the IZ may migrate radially inwardly towards the VZ, in what has been termed “ventricle-directed migration”, and pause at the bottom of the VZ, extending multiple processes to scan the ventricular surface, possibly to obtain positional information or modulate progenitor proliferation, that may ultimately

assist them in correctly integrating into the cortex, before migrating up radially toward the CP (Nadarajah et al., 2002; Yokota et al., 2007).

From a cellular perspective interneurons seem to rely on RG to enter the CP during their tangential-to-radial switch in migration (Ang et al., 2003; Tanaka et al., 2003, 2009, 2006; Yokota et al., 2007). The interactions with the basal processes of RG might be similarly to the glial-dependent migration of pyramidal cells (Elias et al., 2010, 2007; Valiente et al., 2011). Although the molecular cues governing this switch in trajectory are still largely unknown, the RG scaffold is instructive in interneurons tangential-to-radial migration transition (Polleux et al., 2002; Yokota et al., 2007). On the other hand it has been suggested that the switch from tangential to radial migration is dependent on neurite branching dynamics. During migration through the tangential streams, interneurons maintain the orientation of the leading neurite parallel to the ventricular surface/pia. Once received the signal to move into the CP, the angle of the leading branch changes from small to nearly orthogonal, and the switch from tangential to a radial migratory mode is achieved (Martini et al., 2009).

1.8.3.2 Laminar and areal allocation

After arriving at the appropriate cortical area, and within the CP, interneurons settle into specific laminar positions before forming functional synaptic contacts with appropriate projection neuronal partners (Ang et al., 2003; Hevner et al., 2004; Pla et al., 2006; Polleux et al., 2002; Tanaka et al., 2003, 2006). The final phase in the migration of cortical interneurons corresponds to their allocation (i.e. their soma) to specific layers of the cortex which occurs during the first postnatal days (Hevner et al., 2004; Miyoshi and Fishell, 2011; Pla et al., 2006), and is likely to be regulated by mechanisms different from those that recruit interneurons within the CP (Marín, 2013).

Birth-date analysis of specific interneuron subtypes suggests that interneurons follow heterogeneous developmental rules for laminar positioning (Ang et al., 2003; McConnell and Kaznowski, 1991; Métin et al., 2006; Pla et al., 2006; Valcanis and Tan, 2003; Yozu et al., 2004). The final cortical distribution of interneurons therefore depends on the temporal and spatial origin of interneurons and subtype specification, as well as on interactions with the RG scaffold and projection neurons (Guo and Anton, 2014). In addition to signals from projection neurons, postnatal neuronal activity can also affect interneuron positioning (De Marco García et al., 2011). These observations thus suggest that the laminar allocation of interneurons is strictly regulated, probably in a cell class-specific way (Marín, 2013). Once at their final laminar localization, interneurons cease migration by altering their intracellular calcium transients in response to ambient GABA and glutamate signals (Bortone and Polleux, 2009).

1.8.4 Cellular dynamics of migrating interneurons

Distinct types of neurons possess morphologically distinct leading processes (i.e., the single unbranched leading process of the radially migrating projection neurons, the multiple thin leading processes of multipolar neurons, and the constantly branching leading process of the tangentially migrating interneurons), but in all cases the leading process serves as a compass that directs oriented migration by responding to various chemotactic, chemoattractant, or chemorepellent gradients (Faux et al., 2012; Marín et al., 2010; Trivedi and Solecki, 2011).

Unlike the stereotypical migratory behavior of many neurons that extend a single leading process in the direction of migration, the leading process (neurite) of interneurons branches continuously during the migratory cycle (Martini et al., 2009; Yanagida et al., 2012). A single branch is selected and oriented toward the

direction of movement, and the rest of the branches retract. Similar to growing axons, interneurons use their leading process as a compass, having a growth-cone-like structure at the distal end. Stabilization of the selected leading process is followed by translocation of the centrosome and the Golgi complex toward the selected branch, followed by nuclear translocation and trailing-process retraction (Faux et al., 2012; Marín et al., 2010; Trivedi and Solecki, 2011). The leading-process extension, nucleokinesis, and trailing-process retraction of the migrating neurons depend on integration of extrinsic cues and the resultant dynamic rearrangements of the cytoskeletal functions (Evsyukova et al., 2013) (Figure 12).

This active branching is hypothesized to facilitate the finding of the appropriate route of migration by constantly measuring the concentrations of chemoattractants or repellents across a broad area (Valiente and Marín, 2010). Whereas a single growth cone can only compare the concentrations of attractant or repellant cues across its width (Zheng, 1996), a cell may be able to compare attractant and repellant concentrations at widely different locations using multiple growth cones.

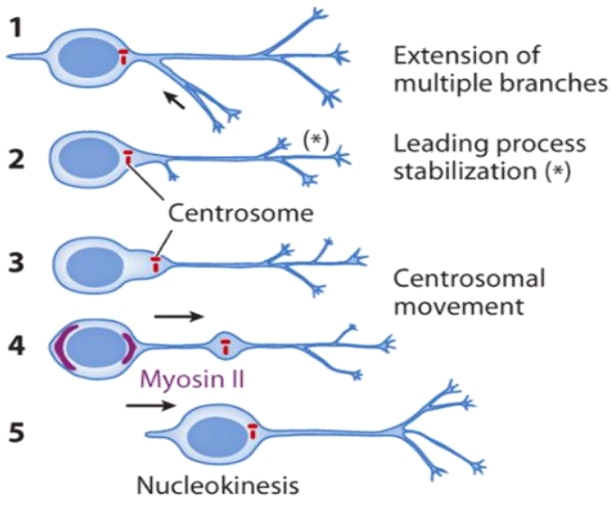

Figure 12. Morphological remodeling during interneuron migration. They extend multiple leading branches (1), followed by branch stabilization (2), centrosomal movement (3), (4), and forward nucleokinesis (5). Figure adapted from (Evsyukova et al., 2013).

Indeed, the leading process does not turn when the source of attractant changes (Martini et al., 2009; Ward et al., 2005); rather, branches are selectively stabilized based on proximity to the source of attractant: the branch whose growth cone is nearer the attractant is stabilized while the others retract. Once the migratory direction is decided, interneurons advance forward by performing a repeated cycle of two-phase nucleokinesis (Bellion, 2005). Competition between different branches also steers pontine neurons from tangential to radial paths (Watanabe and Murakami, 2009).

1.8.5 Molecular mechanisms controlling the tangential migration of interneurons

The migration of newly born neurons is a precisely regulated process that is critical for the development of brain architecture. Appropriate interneuron migration and distribution is essential for the assembly of functional neuronal circuitry and the maintenance of excitatory/inhibitory balance in the brain. Interneuron migration is regulated by a combination of intrinsic programming signals and extracellular matrix substrates. Intricate molecular mechanisms including an array of motogenic factors, chemotactic guidance cues, transcription factors and neurotransmitters are employed by interneurons throughout tangential migration.

1.8.5.1 Transcription factors

The intrinsic migratory fate of interneurons is specified by the combinatorial expression of several key transcription factors (Briscoe et al., 1999; Cobos et al., 2005; Flames et al., 2007; Flandin et al., 2011; Long et al., 2009b; Nadarajah et al., 2002). For example, Lhx6 LIM-homeobox transcription factor and Arx homeodomain transcription factor in mouse brain slices have shown impeded tangential migration of interneurons into the cortex (Alifragis et al., 2004;

Colasante et al., 2008; Friocourt et al., 2008; Liodis et al., 2007). The homeobox genes *Dlx1* and *Dlx2* are essential not only for the migration of interneuron precursors but also for their maturation in the cortex. Recent evidence suggests that Dlx1 and Dlx2 regulation of interneuron migration depends on its ability to restrain neurite outgrowth (Anderson, 1997; Cobos et al., 2007; Le et al., 2007).

The transcription factors not only define subpallial patterning and interneuron differentiation, but also provide migratory route instructions for the newborn interneurons. The homeodomain factor Nkx2.1 is specifically expressed in MGE interneuron progenitors and required for the specification of cortical interneuron subtypes (Butt et al., 2008; Du et al., 2008). The expression of Nkx2.1, however, is downregulated in interneurons entering the migratory route in the cortex, and this downregulation is in fact an active step taken by cortical interneurons to coordinate their programmes of differentiation and migration (Nóbrega-Pereira et al., 2008). On the other hand, COUP transcription factor II (COUPTFII) is preferentially expressed in the CGE and is required for CGE-derived interneuron migration in the caudal direction. Notably, overexpression of COUP-TFII in MGE interneurons is sufficient to change their migratory orientation to the caudal direction when they are transplanted into the CGE environment, thus providing an example of how a single, locally expressed transcription factor activity is capable of determining the migratory fate of interneurons in its local environment (Kanatani et al., 2008).

1.8.5.2 Motogenic cues

Newborn interneurons seem to respond to several soluble factors that have been proposed to play a role in cortical interneuron migration by acting as motogenic factors. Several growth factors increase the migratory rate of MGE derived interneurons *in vitro* and are thought to promote the movement of these cells *in*

vivo. For instance, hepatocyte growth factor/scatter factor (HGF/SF) enhances the migration of the cells away from the ventral telencephalon and demarcate the migratory routes of migrating interneurons in the developing forebrain (Powell et al., 2001). Members of the neurotrophin family of soluble trophic factors, including glial cell line-derived neutrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and neurotrophin 4 (NT4), are widely expressed in the developing cortex and have also been shown to act as motogenic factors for migrating interneurons (Polleux et al., 2002; Pozas and Ibáñez, 2005). However, the direct involvement of these molecules in the regulation of the migration of MGE-derived interneurons *in vivo* is less clear. It is also known that BDNF signalling, modulates Reelin expression, the distribution of both C-R cells and interneurons in the MZ, and participates in the final phase of interneuron migration within the cerebral cortex (Alcántara et al., 2006).

1.8.5.3 Chemotactic molecules

Tangentially migrating neurons most frequently achieve directional migration by interpreting chemotactic gradients. An exquisite coordination of chemoattractive and chemorepulsive cues, expressed within the developing brain, allow cortical interneurons to reach the cerebral cortex and avoid subcortical areas (Andrews et al., 2007; Marín and Rubenstein, 2003; Métin et al., 2006; Parnavelas, 2000). Interestingly, almost every molecule known to influence axon guidance has also been implicated in tangential migration of interneurons, demonstrating the multi-functionality of these molecules.

1.8.5.3.1 Neuropilins/Sema

Postmitotic interneurons generated in the MGE traversing the developing LGE (striatum primordium) en route to the corticostriatal junction and cortex, actively avoid entering the POA and the striatum suggesting that both areas are hostile to

the migration of cortical interneurons. Some of the molecules involved in this avoidance have been characterized. To avoid entering the striatum, migrating interneurons express neuropilins (Nrp1 and Nrp2) and plexin co-receptors that respond to the chemorepulsive ligands semaphorin (Sema)3A and Sema3F emanating from the striatal mantle (Marín et al., 2001). In addition, the proteoglycan chondroitin-4-sulphate expressed in the striatal mantle (Zimmer et al., 2010); which, in conjunction with the Semaphorins, creates an exclusion zone for migrating interneuronsto channel them into adjacent paths and thus define the formation of migratory routes into the cortex. The Semaphorins proteins are also active in the neocortex and act to direct interneuron migration in the tangential streams, preventing them from entering the CP (Tamamaki et al., 2003).

1.8.5.3.2 Slit/Robo

Slit proteins are secreted from the VZ and SVZ of the GE (Andrews et al., 2007; Marillat et al., 2002; Yuan et al., 1999). Binding of Slit proteins to their corresponding Robo (Roundabout) receptor family, expressed by interneurons, repels interneurons from the GE; thus initiating their migration toward the neocortex (W. Andrews et al., 2008; Andrews et al., 2007). Although these proteins appear to regulate neuronal migration within the basal telencephalon, no defects in the tangential migration of interneurons were detected in the cortices of *Slit1/Slit2* double mutant, which suggests that different ligands may mediate Robo signaling (Marín et al., 2003).

Robo1-null effects could be Slit independent, and this has been confirmed with the recent discovery that Robo1 modulates semaphorin-neuropilin/plexin signalling to steer interneurons around the striatum and into the cortex (Hernández-Miranda et al., 2011). Later studies discovered that Slit/Robo signaling is indeed important for establishing correct morphology of the

interneuron population (W. Andrews et al., 2008). Recent studies also suggest that Robo1 receptors expressed in migrating interneurons may also contribute to this repulsive effect by sensing striatal Semaphorins (Hernández-Miranda et al., 2011). Also, a new member of the Robo family, Robo4, regulates radial migration, partly by suppressing the repulsive activity of Slit (Zheng et al., 2012).

1.8.5.3.3 Ephrin/Eph

The Eph-ephrin is a bidirecctional receptor system that has been related to a vast number of events in the developing and adult brain (Klein, 2004) and constitute an additional set of molecules mediating chemorepulsion for interneurons. The class-A members of the Eph/ephrin system act as a repulsive cue that restricts cortical interneurons from entering inappropriate regions and thus contributes to define the migratory route of cortical interneurons (Rudolph et al., 2010).

Further, a recent study has demonstrated that ephrin A5 acts as the repellent force to facilitate the exit of newborn interneurons from GE. Ephrin A5 and its receptor EphA4 are complementarily expressed in the VZ and SVZ of the GE, respectively, and CB-positive cells isolated from the MGE express the EphA4 receptor. *In vitro* stripe assays have demonstrated that both ephrinA5 and ephrin A3 are potent chemorepellents for MGE-derived neurons thus implicating EphA4 in mediating in part the repulsive effects of ephrin A3 (Rudolph et al., 2010; Zimmer et al., 2008). Although the molecular nature of the chemorepulsive activity present in the POA has not been identified yet, it is known that ephrin B3 expressed in the POA and its derivatives act as repulsive cues by binding to the EphA4 receptor expressed by MGE derived cortical interneurons. This repellent activity prevents MGE interneurons from migrating in a ventral direction and is possibly responsible for their dorsal orientation toward the cortex (Zimmer et al., 2011). In summary, despite previous data suggest a role of ephrins and Ephs in

some steps of interneuron migration, genetic confirmation need to be provided to clarify the function of Eph/ephrin signaling in this process *in vivo* (Marín, 2013).

1.8.5.3.4 Neuregulin-1

Addition to the inhibitory cues necessary to guide migration; interneurons are also directed towards the cortex in response to attractive cues. MGE-derived interneurons follow a gradient of increasing permissivity towards the cortex, most likely created by the diffusion of long-range chemoattractive cues from the pallium (Marín, 2013; Wichterle et al., 2003). The Neuregulin-1 (NRG1) is a protein that contains an epidermal growth factor-like domain that signals through receptor tyrosine kinases of the Erbb family (Buonanno and Fischbach, 2001; Falls, 2003). An interesting work, found that, indeed, different isoforms of NRG1 play distinct roles along the migratory path. Two different isoforms of NRG1 are expressed in the developing telencephalon: CRD-NRG1, a membrane-bound protein that is expressed in the route followedby MGE-derived interneurons towards the cortex; and Ig-NRG1, a diffusible protein that is produced by the pallium. Experimental evidence suggests that these different isoformsof NRG1 act sequentially as short-range and long-range attractants, respectively, for migrating interneurons (Flames et al., 2004).

The membrane-bound isoform of NRG1 (CRD-NRG, type III) is found highly expressed by the so-called corridor cells present in the SVZ but not the VZ of the LGE. Together with the inhibitory action of Semaphorins emanating from the striatum, a permissive corridor is created along the SVZ for interneurons to traverse the LGE. Next, in order to cross the corticostriatal notch, interneurons require the secreted isoforms of NRG1 (Ig-NRG1, types I and II), which are expressed in the neocortex and act as a long-range chemo-attractant for migrating interneurons. The immediate action of interneurons exposed to an exogenous

source of secreted NRG1 is to alter the direction of migration by the extension of a new leading neurite in the direction of the source (Flames et al., 2004; Martini et al., 2009).

Both, short and long-range NRG1 functions are mediated by Erbb4, the neuregulin receptor expressed by MGE-derived interneurons (Flames et al., 2004; Yau et al., 2003). Consistently, genetic studies have revealed that perturbation of ErbB4 function decreases the number of MGE-derived interneurons that reach the cortex (Fisahn et al., 2009; Flames et al., 2004). In the long term, when cortical NRG1 expression is reduced, there is a concomitant accumulation of ErbB4-positive interneurons at the corticostriatal junction (Ying et al., 2009) and the complete loss of NRG1 in the forebrain leaves interneurons incapable of leaving the MGE (Flames et al., 2004). Interestingly, NRG1 has reproducibly emerged as a susceptibility gene for schizophrenia (Li et al., 2006; Nicodemus et al., 2006).

1.8.5.3.5 Cxcl12/Cxcr4/Cxcr7

In the streams, tangentially migrating interneurons do not invade the CP. Avoidance of the CP do not seem to be because of the existence of chemorepulsive activity in this region (López-Bendito et al., 2008). The mechanisms that control the preferential migration of interneurons through these migratory streams are beginning to be elucidated. So far, it seems as if the streams are maintained mainly by the positive action of molecules that are secreted by cells in these regions, or immediately adjacent. To date, the only molecule that has been shown to mediate this process is the chemokine Cxcl12.

The Cxcl12 (also known as stromal derived factor or SDF1) mediates a chemoattractant effect on interneurons in the cerebral cortex. During early corticogenesis (up to E14.5), when migratory streams are well defined, Cxcl12 expression is high in the MZ and SVZ (Stumm et al., 2003; Tiveron et al., 2006)

and, to a minor extent, by cells in the SP (Stumm et al., 2007). At later stages, however, expression remains high in the MZ but is dramatically reduced in the SVZ, consistent with a more diffused stream in this region (Tiveron et al., 2006).

Cxcl12 acts through two related signaling G-protein-coupled receptor, Cxcr4 and Cxcr7, expressed by tangentially migrating interneurons (Sánchez-Alcañiz et al., 2011). Very recently has been demonstrated that loss of Cxcr7 leads to excessive Cxcl12-mediated activation and downregulation of Cxcr4 in interneurons, which sequester Cxcl12 through Cxcr7, supporting the concept that Cxcr7 acts as a Cxcl12 scavenger in these cells (Abe et al., 2014). The action of Cxcl12 through these receptors restricts the migrating cortical interneurons into confined streams by suppressing leading process branching and thereby maintaining their tangential migratory direction (Caronia-Brown and Grove, 2011; Lysko et al., 2011).

Cxcl12 is a potent long-range chemoattractant for MGE-derived interneurons (Li et al., 2008; López-Bendito et al., 2008), but its limited diffusion properties *in vivo* would explain the relative confinement of interneurons to the migratory streams found in the cortex. A gradient of Cxcl12 would be important for the regulation of cortical invasion (Wang et al., 2011). The timing of switch from the tangential to radial migration and invasion of the CP, correlates with the loss of responsiveness to Cxcl12 as an attractant (Li et al., 2008). It appears as if Cxcl12 masks an unknown chemoattractive activity present in the CP by reducing their branching frequency and thus minimizing the potential of interneurons to sense cues outside the tangential streams (Lysko et al., 2011). On the other part, disruption of Cxcr4 or Cxcr7 function results in premature exit of cortical interneurons from their migratory streams and perturbs their laminar and regional distribution within the neocortex (Li et al., 2008; López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011; Tanaka et al., 2010; Tiveron et al., 2006; Wang et al., 2011). This sophisticated fine-tuning mechanism dynamically adapts

chemokine responsiveness in migrating neurons, thereby preventing their desensitization as they migrate through these routes for a protracted period of time (Sánchez-Alcañiz et al., 2011).

Thus, Cxcl12/Cxcr signaling may play a dual role, initially attracting interneurons to the neocortex and control their tangential-to-radial switch entry into the developing CP. It is worth noting that, despite the prominent defects observed in the intracortical dispersion of interneurons in the absence of Cxcl12 signaling, interneurons reach the cortex of *Cxcr4* or *Cxcr7* mutants mice in normal numbers (Li et al., 2008; López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011; Tiveron et al., 2006; Wang et al., 2011). This observation reinforces the idea that the mechanisms driving the migration of interneurons from the subpallium to the cortex and those controlling their intracortical migration are different (Marín, 2013).

1.8.5.3.6 Neurotransmitters

Neurotransmitters are usually recognized for their central role in synaptic transmission and the functionality of cortical networks in the adult brain; however, increasing evidence suggests a role in regulating developmental processes, including interneuron migration. Several neurotransmitters and their corresponding receptors are expressed during migration, either along the migratory routes and interneurons (Crandall et al., 2007; Cuzon Carlson and Yeh, 2010; Cuzon et al., 2006; López-Bendito et al., 2003; Ohtani et al., 2003; Poluch et al., 2001; Soria and Valdeolmillos, 2002). In particular, it has been shown that they play a multiple role in guiding interneurons across the corticostriatal junction and maintaining the migratory distribution within the cortical wall (Crandall et al., 2007; Cuzon et al., 2006; López-Bendito et al., 2003).

Less is known about the cues that instruct an interneuron to stop migration in the correct laminar position and to start arborization. Probably this process is influenced by multiple mechanisms that have just begun to be identified and where neurotransmitters seem to play an important role. GABA and glutamate are thought to enhance neuronal migration in the embryo because they both depolarize the membranes of interneurons and stimulate the generation of calcium transients (Bortone and Polleux, 2009; Cuzon et al., 2006; Inada et al., 2011; Manent et al., 2006). However, GABA becomes hiperpolarizing during early postnatal development and this change in activity turns ambient GABA into a stop signal. Consequently, interneurons stop migrating in response to GABA, but only after interneurons have switched their responsiveness from a depolarizing to a hyperpolarizing state in response to GABA (Ben-Ari, 2002; Bortone and Polleux, 2009). Thus, the mechanisms controlling the transition of GABA from depolarizing to hyperpolarizing seem to be directly related to the termination of interneuron migration.

Together, these observations suggest that interneurons integrate information about their temporal and spatial origin, subtype identity, and extrinsic signals from projection neurons and the CP environment to establish their final laminar fate. As described above, a vast array of motogenic and chemotactic cues, transcription factors and neurotransmitters instruct and guide the tangential migration of the interneurons from the ventral telencephalon into the neocortex. Significant progress has been made toward defining many mechanisms underlying neuronal migration. However, we still do not fully understand how neurons integrate a multitude of signals to produce oriented and coordinated patterns of migration evident in the developing cerebral cortex. These and many more questions will drive the interneuron field into the future, as we are only beginning to understand the intricacies of generating the functional balance between pyramidal neurons and interneurons. Particularly, in the cerebral cortex, the chemoattractant cues

have been more described compared with the chemorepulsive molecules, which still remains to be explored.

1.9 Axon guidance

Neuron migration positions the cells in the correct place but for the brain to be wired correctly, axons projecting from these cells must be guided to the correct target, many times over long distances, in order to establish functional synapses. Four broad axonal tracts exist within the mammalian forebrain that have been extensively studied in order to ascertain the cellular and molecular basis of axon guidance: the corpus callosum (CC) that connect the two cortical hemispheres, the corticospinal tract (CST), that connect the cortex with the spinal cord, the corticothalamic projection (Leyva-Díaz and López-Bendito, 2013), that send axons from the cortex to the thalamus, and the thalamocortical projection that projects in the opposite direction. Other well studied systems in axon guidance include the commisural axons and the motorneuron projections in the spinal cord.

Axon guidance is highly stereotyped and includes several steps until reaching the vicinity of the appropriate target region and form a proper connection. As for neuron migration, this "axon guidance" depends on the orchestrated action of diverse, conserved families of guidance cues present in the environment and their neuronal receptors at the neuron surface (Dickson, 2002; Tessier-Lavigne and Goodman, 1996).

Neuronal connections form during embryonic development and rely on a structure, at the leading edge of the axon, known as the "growth cone". It is composed of a central domain filled with microtubules and a peripheral domain of actin-rich filopodia and lamellipodia (Figure 13A). The growth cone is a

specialized sensory and motile structure that actively extend and retract in response to extracellular cues which allows for the sensing of the environment in order to take steering decisions during axon migration (Bashaw and Klein, 2010; Carmeliet and Tessier-Lavigne, 2005; Raper and Mason, 2010; Vitriol and Zheng, 2012) (Figure 13B).

The expression of guidance cues and receptors is exquisitely tailored to allow growth cones to make appropriate pathfinding decisions at specific choice points regulated both, temporal and spatially throughout development (Figure 13C). This regulation ensures the correct presentation and receipt of guidance signals. Determining how these signaling pathways function to regulate axon growth and guidance, the integrated information from different cues and how these receptors, in turn, signal to the growth cone cytoskeleton to control steering decisions, are fundamental questions to understand nervous system wiring specificity (O'Donnell et al., 2009). No doubt that their answer will shed light on how to design possible therapeutic approaches for many neural developmental disorders.

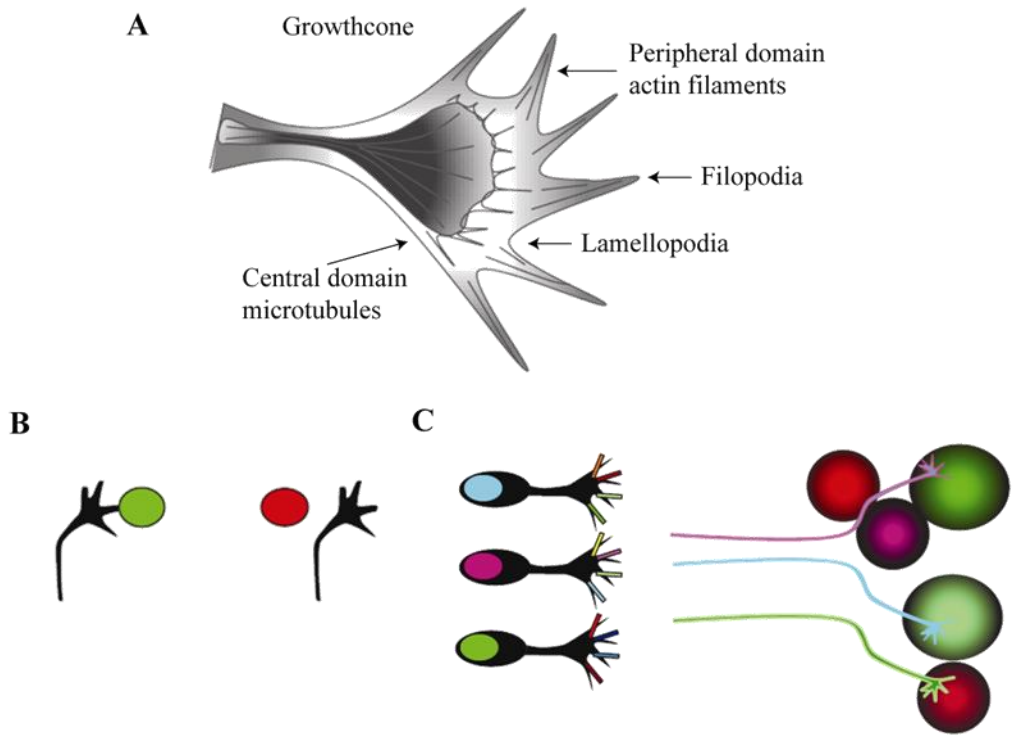

Figure 13. Structure and function of the growth cone in axon navigation. (A) The growth cone that tips the axon is a highly motile structure composed of a microtubule-rich central domain and an actin-rich peripheral domain, forming lamellipodia and filopodia. (B) During axon navigation, the trajectory is defined by environmental cues which exert attractive (in green) and repulsive (in red) effects on the growth cone. (C) Axons from different neuron types (here in blue, pink, and green) express specific combinations of receptors and thus respond differently to the same cues present in their environment and thus navigating distinct pathways. Figure adapted from (Castellani, 2013).

The guidance cues, both attractants and repellents, include contact-mediated or secreted molecules, acting over short or long distances, respectively. It is clear that many individual guidance cues can function both as repellents and attractants (Huber et al., 2003). These cues can also be divided into those that are substrate or cell membrane-bound, and so act on nearby axons (upon cell contact), and those that are secreted from distant sources and form gradients that influence the trajectories of extending axons (Huber et al., 2003; Kolodkin and Tessier-Lavigne, 2011; Tessier-Lavigne and Goodman, 1996) (Figure 14).

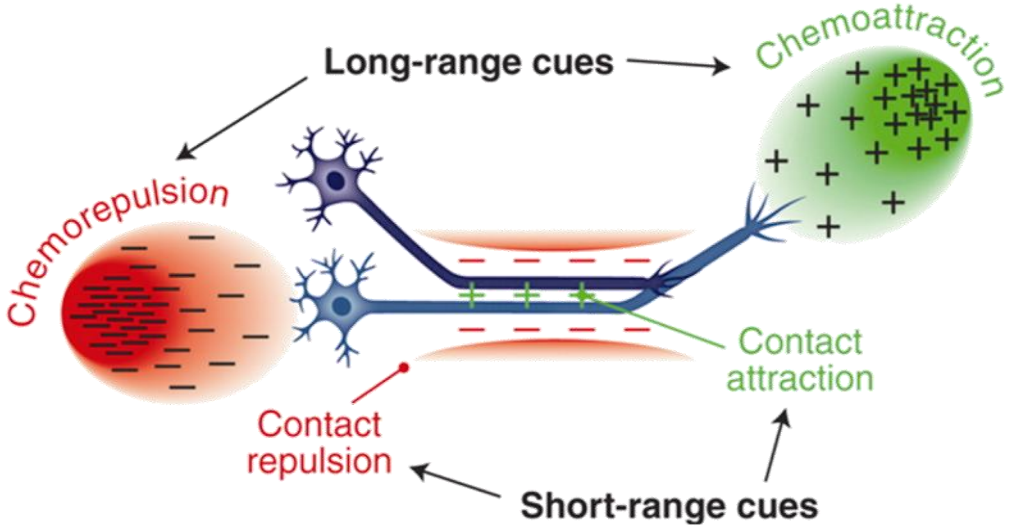

Figure 14. The diversity of neuronal guidance mechanisms. Neuronal processes are guided by cues that can function at long and short distances to mediate either attractive or repulsive guidance. Figure adapted from (Kolodkin and Tessier-Lavigne, 2011).

In the 1990s, genetic, biochemical and molecular approaches together identified four major conserved families of guidance cues (the "canonical cues") with very well-established roles in neuronal guidance and prominent developmental effects: the Netrins, Slits, Semaphorins and Ephrins (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). Netrins, Slits and some Semaphorins are secreted molecules that associate with cells or the extracellular matrix, whereas Ephrins and other Semaphorins are anchored to the cell surface. Of the classic axon guidance cue, Slits, Semaphorins, and Ephrins act primarily as repellents but can be attractive in some contexts, whereas Netrins can act as attractants or repellents. Recent evidence have shown that the activity of a given axon guidance cue depends on the cellular context and the status of the receiving neuron so, in certain contexts, molecules regarded as archetypal chemorepellents act attractively and vice versa (see below for instance in the case of Netrin) (Dickson, 2002; Dudanova and Klein, 2013).

Probably the best well studied axon guidance cue is Netrin. Netrins are bifunctional cues, capable of attracting some axons and repelling others (Colamarino and Tessier-Lavigne, 1995), and play major roles in the control of axon crossing at the midline (Wadsworth et al., 1996). For instance, Netrin-1 can act as a chemoattractant for dorsal commissural neurons in the spinal cord and as a repellent for certain classes of motor neurons (Culotti and Merzt, 1998). A mammalian Netrin, Netrin-4 (orb-Netrin), is more distantly related, having a similar overall structure but showing greater homology to the β chain of laminins (Koch et al., 2000). Netrin-5, a new member of the netrin family, was recently involved in the control of neurogenesis in the adult brain (Yamagishi et al., 2015).

The main factor that determines the type of response of an axon to Netrin-1 is the relative amount of netrin receptors on the surface of the growth cone (Round and Stein, 2007). In all species, the attractive effects of Netrins are mediated by receptors of the DCC family (Deleted in Colorectal Cancer) (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). DCC not only mediates attractive signaling through netrin-induced homodimerization of the receptor but also has been shown to participate in repulsive axon guidance (Stein et al., 2001). The Ig superfamily member DsCAM (*Drosophila* Cell-Adhesion Molecules) has been proposed to function as a co-receptor in Netrin mediated attraction in some systems (G. L. Andrews et al., 2008; Ly et al., 2008). Repulsive effects of Netrins are mediated by members of the Unc5 family (Hong et al., 1999; Keleman and Dickson, 2001). Netrins can induce the association of DCC and Unc5 in the cytoplasm, and the formation of this receptor complex result in a switch from attraction to repulsion (Hong et al., 1999). The attractive response of an axon to Netrin1 can be switched to a repulsive one, or vice versa, by modulating the levels of cytosolic cyclic AMP (cAMP) in the growth cone *in vitro* (Song et al., 1997). Thus, the nature of the response of an axon to a particular guidance signal may

depend on the receptors present in the growth cone and/or on the recent history of second-messenger activation in the growth cone (Butler and Tear, 2007).

A second well characterized guidance cues are Slits, which include a group of large secreted proteins that were implicated in axonal repulsion (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Wang et al., 1999). The repulsive actions of Slit proteins are mediated by receptors of the Robo family (Kidd et al., 1998; Zallen et al., 1998). There is evidence identifying a critical role for Slit/Robo signals to guide pioneer longitudinal axons in the embryonic brain stem. These studies indicate that Slit/Robo signals from the floor plate have dual functions: to repel longitudinal axons away from the ventral midline, and also to maintain straight longitudinal growth. These dual functions likely cooperate with other guidance cues to establish the major longitudinal tracts in the brain (Mastick et al., 2010). A very nice example that demonstrates the importance of Robo in the axon guidance, is the phenotype found in patients with horizontal gaze palsy with progressive scoliosis, in which were identified three novel homozygous ROBO3 mutations (Volk et al., 2011).

A third group of axon guidance cues include the Semaphorins, a large, phylogenetically conserved, family that is constituted by both, secreted and transmembrane guidance proteins (Yazdani and Terman, 2006). They are distributed in dynamic and complex patterns during development and function in both long-range and short-range guidance. The major receptors for Semaphorins are members of the Plexin family (Tamagnone and Comoglio, 2000). Many Semaphorins bind Plexins directly, but several secreted vertebrate Semaphorins, including Sema3A, instead bind to the obligate co-receptors Neuropilin-1 or Neuropilin-2; Neuropilins, together with a Plexin receptor, form an active holoreceptor complex. Different secreted Semaphorins require specific combinations of Neuropilin-1 or Neuropilin-2 and a specific Plexin for guidance

responses in distinct neuronal subtypes, a conclusion drawn from extensive observations both in cell culture and *in vivo* (Kolodkin and Tessier-Lavigne, 2011; Tran et al., 2007). It is believed that Sema3 proteins are among the cues that channel extending motor axons into their pathways by surround repulsion. Like in cortical neurons, the magnitude of the response to different Sema3s is determined by the set of Neuropilin receptors present on the growth cones (Dudanova and Klein, 2013). A recent study demonstrated that the balance between Neuropilin-1 and Neuropilin-2 depends on the levels of Sema3C expressed by motor neurons (Sanyas et al., 2012).

The fourth family of canonical guidance cues is the Ephrins, cell-surface signaling molecules that play important roles in a large number of developmental events including axon guidance (Klein, 2004). Ephrins have been shown to play an essential role in organizing topographic projections that connect, for example, retinal ganglion cells in the eye with their target cells in the appropriate portion of the optic tectum in lower vertebrates, or the lateral geniculate nucleus of the thalamus in higher vertebrates (Feldheim and O'Leary, 2010). These mapping functions show the versatility of Ephrins, which can function as attractants for some axons and repellents for other, as well as either positive or negative regulators of axonal branching (Kolodkin and Tessier-Lavigne, 2011).

Because ephrins are membrane attached, interactions between ephrins and Ephs require intercellular contact. It is remarkable that ephrin/Eph complexes transduce signals bidirectionally into both receptor (Eph)-expressing cells and ligand (ephrin)-expressing cells in what is known as "forward" and "reverse" signaling, respectively (Kullander and Klein, 2002). There is ample evidence to indicate that both forward and reverse modes of Ephrin-Eph signaling are critical for axon guidance during neural development (Egea and Klein, 2007; Huber et al., 2003). More recent observations indicate that Ephrins and their receptors play key roles

in the regulation of dendritic morphology and synaptogenesis in the CNS, implicating Ephrin mediated receptor tyrosine kinase signaling in the regulation of synaptic plasticity (Shen and Cowan, 2010).

When axons navigate their way *in vivo*, at every choice point they are probably confronted with several different signals acting simultaneously and in a coordinated manner, in a permissive or instructive way. Thus, the final decision is based on the integration and interpretation that the growth cone does from all the guidance cues that are presented at once in each choice point. In some cases, one cue might be dominant and suppress responses to the other. Among these hierarchical interactions, interesting insights into the regulation of the interplay between Slit/Robo and netrin/DCC signaling have been reported for the guidance of postcrossing commissural axons (Stein and Tessier-Lavigne, 2001), motor neurons of the spinal cord (Bai et al., 2011) and axons of the CC (Fothergill et al., 2014).

1.9.1 Signaling mechanisms of axon guidance

Signaling mechanisms that act downstream of axon guidance cues (Netrins, Semaphorins, Ephrins, and Slits) have been extensively studied in both invertebrate and vertebrate model systems (Bashaw and Klein, 2010). These cues detected by receptors on the axon surface, then activate a variety of downstream signaling pathways to cause axon turning and/or changes in growth rate (Zheng and Poo, 2007).

One of the most crucial components of these pathways is calcium and over the past few years many new insights have been gained into the role of calcium in axon guidance (Borodinsky and Spitzer, 2006; Gomez and Zheng, 2006; Sutherland and Goodhill, 2015; Sutherland et al., 2014). Changes in calcium concentrations appear to be instructive signals to direct growth cone (Zheng,

2000). In general, moderate amplitude increases in calcium favor attraction, whereas high or low amplitude increases favor repulsion, although differences in neuron type, growth substrate and resting calcium concentrations can affect growth cone responses. Like calcium signaling, cyclic nucleotides (cAMP or cGMP) can have profound effects on growth cone responses to guidance cues. The levels of cyclic nucleotides, specifically the ratio of cAMP to cGMP, can determine whether the response to a guidance cue will be attractive or repulsive, with high cyclic nucleotide levels (or high cAMP/cGMP ratios) favoring attraction and low levels (or low cAMP/cGMP ratios) favoring repulsion (Nishiyama et al., 2003; Song et al., 1998, 1997).

How do changes in intracellular calcium and cyclic nucleotide signaling result in directed growth cone turning?. One of the key downstream effectors target is the Rho-family small GTPases. Considerable progress has been made in establishing direct links between RhoGTPases and guidance receptors. These pathways result in growth cone collapse, or axon extension through signaling events that act locally to modulate cytoskeletal dynamics in the growth cone to achieve specific guidance outcomes (Bashaw and Klein, 2010).

1.10 Neuronal migration and axon guidance. The wiring of the cerebral cortex

Both neuronal migration and axon guidance constitute fundamental mechanisms underlying the wiring of the brain. Traditionally, these two processes have been studied independently, but it is easy to hypothesize that the normal formation of neural circuitry requires an exquisite coordination of both. In this respect, it is not surprising to observe that mechansims of migrating neurons shares many features with axon guidance, from the use of substrates to the specific cues regulating

chemotaxis. There are, however, important differences in the cell biology of these two processes. The most evident case is nucleokinesis, which is an essential component of migration that needs to be integrated within the guidance of the cell. Perhaps more surprisingly, the cellular mechanisms underlying the response of the leading process of migrating cells to guidance cues might be different to those involved ingrowth cone steering, at least for some neuronal populations (Marín et al., 2010).

Although both neuron migration and axon guidance share common mechanisms of cell biology and biochemistry, little is known about their coordinated integration during development. There are some examples that illustrate the integrationof both mechanisms. Recent studies indicate that neuronal migration regulates axonal guidance in the cerebral cortex. Guidepost neurons, cortical neurons that are positioned along axonal migration routes, have been shown to guide axonal pathways. For example, two transient populations of glutamatergic and GABAergic neurons arrive at the CC just before the arrival of callosal axons and act together with glial cells to provide attractive cues for axonal navigation (Shu et al., 2003). Glutamatergic neurons secrete semaphorin 3C, which attracts Neuropilin-1-expressing callosal axons, whereas MGE- and CGE-derived GABAergic neurons use Sema3A- and ephrin-signaling pathways, as well as cell-adhesion mechanisms, to control callosal axon navigation (Niquille et al., 2013, 2009).

Thalamocortical projections constitute one of the most prominent higher level processing connections in the mammalian brain. It was shown that proper pathfinding of thalamocortical axons (TCA) in the ventral telencephalon during development, depends on the early tangential migration, from lateral to medial, of a population of neurons derived from the SVZ/VZ of the LGE. This tangential migration contributes to the establishment of a permissive corridor that is essential

for TCA pathfinding. NRG1/ErbB4 signaling is largely responsible for creating this permissive corridor (López-Bendito et al., 2006). Importantly, the Slit/Robo signaling pathway is instrumental in repelling corridor neurons from the ventral MGE and POA, thus controlling oriented migration of these cells and their correct positioning within the corridor (Bielle et al., 2011).

Another aspect of the interplay between neuron migration and axon guidance is related to intrinsic programs of the differentiating neuron. It is generally assumed that neuronal migration is largely incompatible with differentiation because this later process drives cells into the acquisition of morphological features that are unsuited for migration. Specifically, neuronal differentiation involves the growth of dendrites and axons inspecific patterns, which may restrict their movement and break the polarity that cells require to migrate. To prevent this from happening, migrating neurons express genes that repress the differentiation program of the cell. In migrating cortical interneurons, for instance, *Dlx1* and *Dlx2* repress the expression of other genes involved in axonal growth, synaptogenesis, and axon and dendritic branching (Cobos et al., 2007). It is well known, however, that certain types of neurons, these are able to migrate as they simultaneously extend an axon in the opposite direction. This is the case of pyramidal cells, which growtheir axon as soon as they start migrating toward the CP (Marín et al., 2010; Noctor et al., 2004; Schwartz et al., 1991).

Another mechanism shared by either neuronal migration or axon guidance, is the role of cytoskeletal remodeling which plays a central role in neuronal polarization mechanisms underlying both processes. The cellular and molecular mechanisms that underlie neuronal guidance are closely related with cytoskeletal dynamics in extending neuronal growth cones and steer axons. I this context and as mentioned above, Rho GTPases are key regulators of the actin cytoskeleton in orchestrating

cytoskeletal rearrangements and neuronal polarization in different steps of development.

1.11 Rho GTPases

The establishment of precise neuronal cell morphology provides the foundation for all aspects of functional neurobiology, during development as well as during final refinement of neuronal circuits. Morphology is regulated by cytoskeleton dynamics and many factors affecting migration, axon guidance, neuron diferentiation and synaptogenesis trigger signaling pathways that ultimately modify cytoskeleton components and neuron morphology. The Rho GTPases have emerged as critical players in these pathways, regulating the cytoskeleton at the leading edge of moving neurons or at the growth cones by linking microtubule ends to actin (Hall and Lalli, 2010).

Rho GTPases constitute one of the five distinct families of the Ras superfamily (Hall, 2012; Rojas et al., 2012). The Rho GTPases subfamily consists of small, 20-30 kDa monomeric GTP-binding proteins that are highly conserved throughout evolution in a variety of organisms (Madaule and Axel, 1985). Mammalian Rho GTPases comprise a small family of 20 intracellular signalling molecules, including RhoA, Ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division control protein 42 homolog (Cdc42). These proteinsare known to play important roles in various cellular processes, as key cytoskeleton dynamics regulators (Dickson, 2001; Hall, 1998; Heasman and Ridley, 2008).

Like Ras, the majority (although not all) of Rho family members act as molecular switches to regulate signal transduction pathways, by interconverting between inactive GDP-bound and active GTP-bound conformational states (Hall, 2012).

The cycling of Rho GTPases between these two states is regulated by three sets of proteins; (*a*) guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP to activate the switch (Schmidt and Hall, 2002); (*b*) GTPase activating proteins (GAPs) that stimulate the intrinsic GTPase activity to inactivate the switch (Bernards, 2003); and (*c*) guanine nucleotide dissociation inhibitors (GDIs), whose role appears to be to block spontaneous activation (Olofsson, 1999) (Figure 15). Although most studies have focused on the so called "classically activated" Rho GTPases, the family also includes atypical members that are constitutively bound to GTP and do not detectably hydrolyse GTP. They are the Rnd ("round"), RhoH, RhoBTB1 and RhoBTB2 proteins. These proteins lack amino acids that are critical for GTPase activity, explaining why they do not hydrolyse GTP and therefore are not influenced by GEF and GAP activity and are constitutively active. Instead, regulation of the activity of these atypical Rho GTPases might come mainly from gene expression, protein stability and phosphorylation (Aspenström et al., 2007; Berthold et al., 2008; Chardin, 2006; Riou et al., 2010).

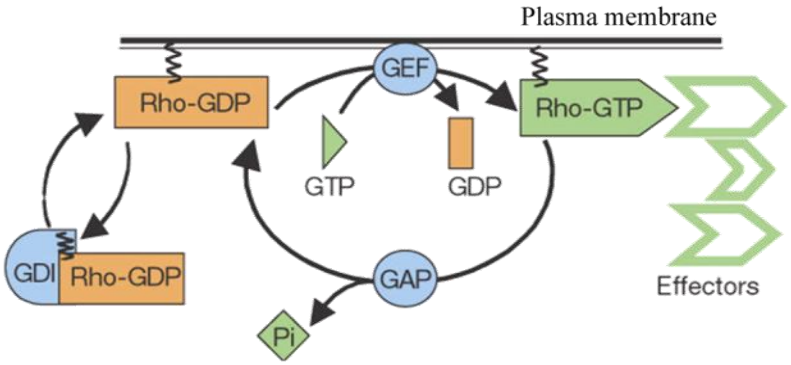

Figure 15. Rho GTPases activation/deactivation cycle. Rho GTPases are molecular switches that cycle between an inactive GDP-bound and an active GTP-bound state. Activation of Rho GTPases occurs by stimulation with a guanine exchange factor (GEF) that causes the release of GDP and the binding of GTP. In the GTP-bound form, Rho proteins undergo a conformational change becoming able to interact with effector molecules and thus initiating a downstream response. (GAPs) stimulate GTP hydrolysis, leading to inactivation; and guanine nucleotide

exchange inhibitors (GDIs) extract the inactive GTPase from membranes. The major feature of Rho GTPases, as well as of other small GTP-binding proteins, is the absolute requirement for membrane attachment in order to exert their activities. Figure adapted from (Etienne-Manneville and Hall, 2002).

Rho proteins interact with and activate downstream targets (effector proteins) when bound to GTP, thereby stimulating a variety of processes. It is in the active GTP-bound state that Rho GTPases perform their regulatory function through a conformation-specific interaction with effector proteins. Over 50 of these effectors have been identified so far for Rho, Rac, and Cdc42 that include serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases, and scaffold proteins. For the handful of targets that have been examined structurally, it appears that they exist in a closed inactive conformation that is relieved through GTPase binding (Bishop and Hall, 2000; Jaffe and Hall, 2005). The activation of Rho, Rac, or Cdc42 leads to the assembly of contractile actin:myosin filaments, protrusive actin-rich lamellipodia, and protrusive actin-rich filopodia, respectively

(Etienne-Manneville and Hall, 2002). These highly specific effects on the actin cytoskeleton point to a series of well-defined signal transduction pathways controlled by each GTPase leading to both the formation (actin polymerization) and the organization (filament bundling) of actin filaments (Jaffe and Hall, 2005).

The Rho GTPases regulate many other signal transduction pathways in addition to those linked to the actin cytoskeleton. They function in the regulation of migration, motility and invasion, adhesion, proliferation and the transduction of signals from the outside environment by these proteins have been well documented in a variety of cell types, including neurons (Bishop and Hall, 2000; Heasman and Ridley, 2008; Ito et al., 2014; Jaffe and Hall, 2005). Rho family proteins have been reported to regulate a wide range of neurnal mechanisms and functions and their alterations result in different malformations and neurological

disorders (Ballester-Lurbe et al., 2014; Cappello, 2013; Linseman and Loucks, 2008; Nadif Kasri and Van Aelst, 2008). As major regulators of the cytoskeleton, the family of small Rho GTPases has been shown to play essential functions in cerebral cortex development, especially to cortical projection neuron development (Azzarelli et al., 2015b). They are the most extensively studied group with respect to their function in the remodeling of the cytoskeleton and the establishment of polarity during cortical development. Most of the studies have focused on Rho (RhoA-C), Rac (Rac1-3), Cdc42 and to some extent Rnd1-3. Rho, Rac, Cdc42 and Rnd1-3 are highly expressed during cortical development in the VZ and SVZ, already suggesting an important role in progenitors (Pinto et al., 2008).

Particularly for cell migration, polarization in the direction of movement is the first crucial step. Experimental data indicating the importance of the Rho family of small GTPases in radial migration of excitatory neuron precursors have been accumulated and comprehensively reviewed (Govek et al., 2011). Deletion of Rac1 in the telencephalic VZ showed surprisingly that Rac1 is not essential for neuritogenesis, but that it has important functions in axonal guidance and migration. Rac1 deficiency appears to delay the onset or reduce the speed of cortical neuron migration rather than inhibit it entirely (Chen et al., 2007). This migration defect could be due, at least in part, to defects in RG organization resulting from an inability to anchor their pial endfeet to the basement membrane (Leone et al., 2010). Although the role of Cdc42 in radial migration has not been as extensively examined compared to Rac, studies suggest that Cdc42 is important for this process. In the developing neocortex, perturbation of Cdc42 activity retards radial migration (Konno et al., 2005). On the other hand, the regulation of RhoA levels and activity appears to be required for radial migration successfully (Govek et al., 2011). In the developing rodent neocortex, *RhoA* mRNA expression is high in the premigratory cortical VZ and SVZ, and low in cells migrating in the IZ, while RhoB mRNA expression is high only in the CP (Ge et al., 2006; Olenik

et al., 1999). The members of Rnds proteins also have been implicated in the radial migration as we will discuss later on (Heng et al., 2008; Pacary et al., 2013, 2011).

In contrast to the radial migration of pyramidal cells, involvement of Rho proteins in the migration of interneuron precursors is still enigmatic. Nevertheless, very recently, Ito and collaborators, summarize the roles of Rho small GTPases and their related molecules in the tangential migration of interneurons (Ito et al., 2014). To mention some examples: Rac protein have been related with migratory defects of LGE- and MGE-derived neural cells (Chen et al., 2007), perturbation of cell cycle exit and aggregation of MGE-derived neural cells in ventral telencephalon (Vidaki et al., 2012), reduction of relative number of cells that migrate to OB (Khodosevich et al., 2009), selective reduction of PV-positive cells in cortex and hippocampus and migratory defects in cortical interneuron (Vaghi et al., 2014); RhoA is implicated in as well increase and decrease of interneurons migration (Wong et al., 2001); ROCK (Rho-associated protein kinase) and Cdc42 control the number of migrating cells (Shinohara et al., 2012; Wong et al., 2001).

Development of the nervous system requires efficient extension and guidance of axons and dendrites culminating in synapse formation. As we mentioned above, axonal growth and navigation during embryogenesis are controlled by extracellular cues that affect growth cone morphology in inducing attraction or repulsion. Again, in this context, Rho-family GTPases also play an important role in regulating intracellular cytoskeletal components that facilitate axonal morphological changes and in integrate and propagate signals from various guidance cues (Govek et al., 2011; Spillane and Gallo, 2014).

1.11.1 Rnd proteins

The Rnd proteins Rnd1, Rnd2, and Rnd3 (also called RhoE) constitute a unique branch of Rho family GTPases that have a low affinity for GDP and very low intrinsic GTPase activities. This indicates that in contrast to most other small G proteins, Rnds are not molecular switches and they are supposed to be constitutively active, insensitive to the effects of classical Rho GTPase regulators, including GEFs and GAPs (Fiegen et al., 2002; Foster et al., 1996; Garavini et al., 2002; Govek et al., 2011; Guasch et al., 1998; Nobes et al., 1998). This suggests that the regulatory mechanism of Rnd protein localization is likely to be different from those of other Rho family members. Studies in neurons have provided important insights into the mechanisms that control the activity of the Rnd proteins, and revealed that their expression, localization and phosphorylation control their activity, rather than the GDP/GTP switch (Chardin, 2006). Like most Rho family members, Rnd proteins are post-translationally modified at the C-terminus by addition of a 15-carbon farnesyl group, which is important for their localization to membranes (Roberts et al., 2008).

Subcellular localizations of the Rnd proteins are mostly associated with membranes, and do not seem to be associated with a GDI (Nobes et al., 1998). The Rnd proteins are expressed differently in different tissues: Rnd1 was found in the liver, brain and human myometria during pregnancy; Rnd2 expression is highest in the testis (Chardin, 2006) and Rnd3 has aubiquitously low expression level, which changes with diverse stimuli and conditions (Riou et al., 2010). All three Rnd family members are expressed in the brain, and several studies have shown they affect multiple aspects of neuronal function, through direct effects on RhoA/ROCK signaling and/or through their interaction with plexins (Chardin, 2006; Ishikawa et al., 2003; Oinuma et al., 2003; Püschel, 2007; Riou et al., 2010; Talens-Visconti et al., 2010; Yoshihara et al., 2009).

Like in the case of the classical Rho GTPases, a clear role for Rnd proteins has emerged from studies in many cell types, in actin cytoskeleton remodelling and affecting cell migration, invasion, axon pathfinding, neurite extension and branching (Riou et al., 2010). Rnd proteins play important roles in cell migration during mammalian cortical development. Among the key regulators of cortical neuron migration, the small GTP binding proteins of the Rho family and the atypical Rnd members play important roles in integrating intracellular signaling pathways into changes in cytoskeletal dynamics and motility behavior (Azzarelli et al., 2015a). Interestingly, Rnd proteins might also play a role in neuronal remodelling in the adult brain (Marie-Claire et al., 2007). In the nervous system, Rnd1 and Rnd2, play a role in neurite formation and retraction. Rnd1 has been implicated in process extension in the PC12 pheochromocytoma cell line (Aoki et al., 2000) and neurite extension in hippocampal neurons (Ishikawa et al., 2003) by a Rac-dependent mechanism involving disruption of cortical actin filaments (Aoki et al., 2000), the extension of axons in cultured neurons by regulating microtubule stability (Li et al., 2009), and dendrite development in rat hippocampal neurons (Ishikawa et al., 2006). Morphological changes induced by Rnd1 are mediated by p190 RhoGAP through an antagonistic effect on RhoA (Wennerberg et al., 2003). Rnd1 is also involved in axonal guidance and cytoskeleton collapse by interacting with the cytoplasmic domains of Semaphorin receptors (Oinuma et al., 2012, 2003; Yukawa et al., 2010; Zanata et al., 2002).

It has also been proposed that Rnd2 promotes dendrite branching and inhibits axon growth in differentiating neurons (Fujita et al., 2002; Negishi and Katoh, 2005; Uesugi et al., 2009). Rnd2 regulates neurite outgrowth by functioning as a RhoA activator in contrast to Rnd1 and Rnd3 effects whith usually inhibits RhoA signaling (Tanaka et al., 2006). Rnd2 is expressed by radially migrating cells, which primarily develop to pyramidal neurons, during their stay in the SVZ of embryonic cerebral cortex and hippocampus. These results indicate that Rnd2

functions *in vivo* as a regulator of the migration and morphological changes associated with the development of pyramidal neurons (Nakamura et al., 2006). This hypothesis was recently confirmed indicating that Rnd2 is downstream of a neurogenic program of neuron migration (Pacary et al., 2011).

Neurogenin2 (*Ngn*2), a proneural factor with a prominent role in neurogenesis in the embryonic cortex (Nieto et al., 2001; Schuurmans et al., 2004), coordinates the acquisition of the radial migration properties and the unipolar dendritic morphology characterizing pyramidal neurons (Hand et al., 2005). It was observed that Rnd2 expression is induced directly by *Ngn2* (Chardin, 2006) in newly generated mouse cortical neurons and that it is, indeed, a major effector of Ngn2 function in the promotion of migration. In acute loss of function experiments by in utero electroporation experiments, the lack of Rnd2 results in an accumulation of cells in the VZ and SVZ of the developing cerebral cortex (Heng et al., 2008).

Pacary and collaborators showed that Rnd2 as well as Rnd3 (see below) promote neuronal migration by inhibiting RhoA signaling. Specifically, Rnd2 controls the multipolar to bipolar transition in the IZ during radial migration (Heng et al., 2008; Pacary et al., 2011). This finding was more unexpected because Rnd2 does not interfere with RhoA activity in fibroblasts (Chardin, 2006; Nobes et al., 1998). The exact mechanism by which Rnd2 inhibits RhoA in neurons is currently unknown but it does not involve the interaction with p190RhoGAP and is therefore different from that of Rnd3 (Pacary et al., 2011).

Recently, it was demonstrated that Rnd2 regulates cell migration, including the multipolar to bipolar transition within the embryonic cortex (Heng et al., 2015; Ohtaka-Maruyama et al., 2013). Rnd2 expression is regulated by other transcription factors within the developing cortex including COUP-TFI, which directly represses *Rnd2* expression at the post-mitotic level along the rostrocaudal

axis of the neocortex; so by finely regulating *Rnd2* expression levels (Alfano et al., 2011).

1.11.1.1 Rnd3

Rnd3 was originally defined as a repressor of ROCK1 and initial studies of Rnd3 focused mainly on this inhibitory effect. Together with Rnd1, Rnd3 was subsequently shown to antagonize RhoA/ROCK signaling as a binding partner for p190RhoGAP (GAP for RhoA), that involve actomyosin contractility reduction and controls the actin cytoskeleton formation, myosin light chain phosphatase phosphorylation and apoptosis (Chardin, 2006; Ongusaha et al., 2006; Riento and Ridley, 2003; Riento et al., 2005, 2003).

The understanding of how Rnd3 function is regulated came from the observation that Rnd3 is phosphorylated (Riou et al., 2010). Rnd3 phosphorylation is important in regulating both its localization and stability. Rnd3 can be phosphorylated by at least two kinases, ROCK1 and PKCa. Rnd3 phosphorylation leads to its translocation from the plasma membrane to the cytosol (Madigan et al., 2009; Riento et al., 2005). In addition, phosphorylation regulates the stability of Rnd3 and protects the protein from degradation (Riento et al., 2005).

Rnd3 is involved in migration of fibroblasts and tumor cells (Chardin, 2006; Guasch et al., 1998; Klein and Aplin, 2009; Nobes et al., 1998), regulates cell proliferation in different cell types (Bektic et al., 2005; Poch et al., 2007; Villalonga et al., 2004) and is important for neurite extension in PC12 cells (Talens-Visconti et al., 2010).

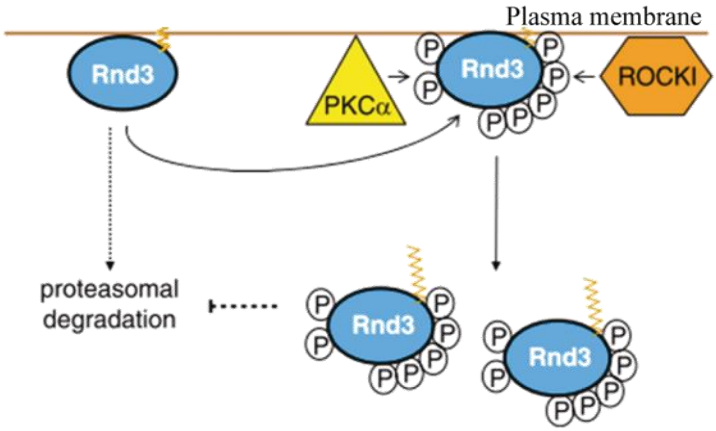

Figure 16. Regulation of Rnd3 by phosphorylation. ROCKI and PKCa can both phosphorylate Rnd3, most likely at the plasma membrane. Phosphorylated Rnd3 subsequently translocates from the plasma membrane to the cytosol and/or internal membranes. Rnd3 is degraded in part by the proteasome, and phosphorylated Rnd3 is more stable than unphosphorylatable Rnd3. Figure adapted from (Riou et al., 2010).

Another main function of Rnd3 is the cell cycle arrest and inhibits the cell proliferation (Poch et al., 2007; Villalonga et al., 2004). Rnd3 is down-regulated in different cancer cell (Ma et al., 2013; Xia et al., 2013; Zhao et al., 2012). This effect seems to be dissociated from its cytoskeletal functions (Riou et al., 2010). In fact, Rnd3-mediated cell proliferation is regulated through Notch signaling regulation via post-translational modification (Y. Tang et al., 2014).

To study the role of Rnd3 *in vivo*, several mice lacking *Rnd3* expression were generated, including a gene trap allele (gt) and a targeted knockout (KO) (Lin et al., 2013; Mocholí et al., 2011). *Rnd3* $^{gt/gt}$ mice are significantly smaller at birth than their heterozygous or wild-type (WT) littermates, show significant postnatal growth retardation and do not survive beyond P29, with anaverage survival of 15 days (Mocholí et al., 2011). Null mutant mice show important structural and behavioral deficits, specially related to the function of the nervous system. They perform worse than control littermates in several motor tests and display delayed

neuromuscular maturation and reduction in the number of spinal motor neurons. *Rnd3* $^{gt/gt}$ mice lack the common peroneal nerve and, consequently, show a complete atrophy of the target muscles (Ballester-Lurbe et al., 2014; Mocholí et al., 2011). Histological analysis of this mutant did not reveal major differences between WT and mutant cortex but hippocampal neurons showed a delayed polarization, decreased number and length of the neurites and reduction in axon outgrowth and delay in the process of neuronal polarization in culture (Peris et al., 2012).

On the other hand, the *Rnd3* KO mice display aqueduct stenosis and development of congenital hydrocephalus caused by an increased Notch signaling and an enhanced proliferation of ependymal cells (Lin et al., 2013), a phenotype that was not described for the *Rnd3* $^{gt/gt}$ mutant. These phenotypic differences among different Rnd3 mutants are some how surprising and require further investigation, but it may be accounted for differences in the genetic background. A further analysis of the targeted *Rnd3* KO at earlier stages than investigated so far (Lin et al., 2013) may also reveal additional defects.

The effects of Rnd3 on neuronal development and the underlying molecular mechanisms have not yet been extensively investigated. Like Rnd2, Rnd3 has also been involved in the radial migration by inhibiting RhoA signaling. However, Rnd2 and Rnd3 seem to control distinct steps of the migratory process, and, in turn, their gene expression is regulated by different proneural factors, by Ngn2 and by Ascl1, respectively (Pacary et al., 2011). The idea is that these proneural proteins integrate the process of neuronal migration with other events in the neurogenic program through the spatiotemporal regulation of Rnd proteins: while Rnd2 is involved in the transition from the multipolar to the bipolar stage in the IZ, Rnd3 is required later during the migration of bipolar neurons within the CP. Ascl1-Rnd3 pathway regulates the migration of cortical neurons by promoting the

disassembly of actin filaments. In cortical neurons, however, this effect seems to be mediated by antagonizing RhoA activity directly though the interacting with the Rho-GAP p190RhoGAP. In contrast, in the case of Rnd2, the mechanism of RhoA inhibition remains to be elucidated *in vivo* (Pacary et al., 2011).

An interesting recent finding was that Rnd3 function can be regulated by extracellular cues which provide mechanisms of how environment influences cytoskeleton rearrangements leading to changes in migration. The Semaphorin Plexin B2 receptor interacts physically and functionally with Rnd3 and stimulates RhoA activity in migrating cortical neurons (Azzarelli et al., 2014). Besides, the migration effects on cortical neurons, reduced levels of Rnd3 also disrupts the apical attachment of RGC, interferes with interkinetic nuclear migration and changes the cleavage plane of their division suggesting a role for Rnd3 in the regulation of cell cycle and the early steps of neurogenesis (Pacary et al., 2013). Therefore, Rnd3 exerts its pleiotropic functions in early steps of cortical neurogenesis by employing distinct mechanisms in RG cells and BP (Pacary et al., 2013). The inhibition of RhoA by Rnd GTPases is one example for the different mechanisms that mediate a crosstalk between GTPases during neuronal migration. The function of Rnd1 and Rnd2 in cortical development remains to be analyzed genetically (Shah and Püschel, 2014).

Finally, very recently was showed that Rnd3 $^{gt/gt}$ display a remarkable postnatal broadening of the SVZ and of the caudal rostral migratory stream (RMS) (Ballester-Lurbe et al., 2014). The SVZ represents an important reservoir of progenitor cells in the adult brain. Cells from the SVZ migrate along the RMS and reach the OB, where they originate different types of interneurons (Doetsch et al., 1999). The lack of Rnd3 expression affected mainly the development of CB-expressing cells in the OB. This adds a new function for Rnd3 on subventricular cells because of its role in proliferation and tangential migration, and indicate that

it plays an important role in neural stem cells development (Ballester-Lurbe et al., 2014).

1.12 FLRTs and neuronal development

The fibronectin leucine rich transmembrane (FLRT) family proteins: FLRT1, FLRT2, and FLRT3, comprise a small family of transmemebrane proteins isolated in a screen for extracellular matrix proteins expressed in muscle. They are conserved among vertebrates but not present in invertebrates such as D. melanogaster and C.elegans. Their structure is comprised of a fibronectin type III domain (FNIII) and 10 leucine-rich repeats (LRR) in the extracellular part. The intracellular part includes around 100 aminoacids with no catalytic activity. FLRT1 is mainly expressed in kidney and brain, FLRT2 is expressed in pancreas, skeletal muscle, brain, and heart; and FLRT3 is expressed in kidney, brain, pancreas, skeletal muscle, lung, liver, placenta, and heart. Moreover FLRT1 and FLRT2 are glycosylated (Lacy et al., 1999).

While the functions of FLRT1 is essentially unknown, different aspects of the function of FLRT2 and FLRT3, in different animal models, have been analyzed in the last past years (Müller et al., 2011; Yamagishi et al., 2011). During *Xenopus* development for instance, FLRT3 can physically interact with FGF receptors and modulate FGF-ERK signalling (Böttcher et al., 2004). This function is dependent on an intact FNIII domain and the cytoplasmic tail of FLRT3 (Böttcher et al., 2004). In a different study, it has been proposed that FLRT3 could mediate homotypic cell adhesion and trigger cell sorting, a function that requires, in this case, the extracellular LRR domains (Karaulanov et al., 2006). More recently, biochemical and bioinformatic analysis through a cell surface binding screen for

FLRT3 partners revealed high affinity interactions between FLRTs and the Unc5 family of Netrin receptors (see below) (Karaulanov et al., 2009).

In the mouse, *FLRT3* KO embryos display defects in ventral closure, headfold fusion and definitive endoderm migration (Maretto et al., 2008), as well as disorganization of the basement membrane which leads to rupture of the anterior visceral endoderm (Egea et al., 2008). This suggests that cell adhesion is affected upon FLRT3 ablation, maybe due to the role of this protein in homotypic interaction and cell sorting. Moreover, FLRT2 and FLRT3 expression partially overlapping in the developing heart. The mouse embryos lacking FLRT2 expression arrest at mid-gestation owing to cardiac insufficiency (Müller et al., 2011). Thus, either FLRT2 or FLRT3 contribute similar functional activities in the epicardium and anterior visceral endoderm, respectively. Consistent with previous reports (Egea et al., 2008; Maretto et al., 2008), it was also demonstrated that as for FLRT3, that FLRT2 expression is independent of FGF signaling (Müller et al., 2011).

In the nervous system FLRT3 promotes neurite growth in dissociated neurons *in vitro*, non-cell autonomously or cell autonomously (Robinson et al., 2004; Tsuji et al., 2004). Interestingly, FLRT3 was recently identified as an endogenous postsynaptic ligand for Latrophilins (LPHNs), specifically LPHN3, the target receptor for the black widow spider venom α-latrotoxin. They interact by the ectodomains (ECD) with high affinity in *trans* suggestingan important role for FLRT3-LPHN3 in glutamatergic synapse development (O'Sullivan et al., 2012).

The role of FLRTs as ligands, acting in *trans* or non-cell autonomously, was also supported by the observation that the ECDs of all three FLRTs undergo cleavage by metalloproteases and are shed from cultured neurons and from the tissue (Yamagishi et al., 2011). The soluble forms of FLRT ECDs are able to bind and

activate Unc5 receptors with certain degree of specificity being FLRT2 is the preferred binding partner of Unc5D and FLRT3 the preferred biding partner of Unc5B. The activation of Unc5 receptors by FLRT ECDs, as it happens upon Netrin binding, triggers a repulsive signaling inducing growth cone collapse and cell sorting. The relevance of this interaction and the repulsive consequences were found *in vivo* for the FLRT2-Unc5D system and the regulation of radial migration in the developing cortex (Yamagishi et al., 2011). During development, FLRT2 is expressed by neurons in the CP while Unc5D is expressed in deeper layers, by the IP allocated in the SVZ. The fact that FLRT ECDs are shed by neurons *in vivo* raised the possibility that they may act as diffusible repulsive ligands and affect the migration of young neurons from the SVZ via its interaction with Unc5D. Indeed, the analysis of KO animals of either *Unc5D* or *FLRT2* showed a premature departure of neurons from the SVZ which in WT conditions is usually inhibited by the ECD of FLRT2 which is shed by cells from the CP (Yamagishi et al., 2011). Interestingly, during normal development of the cortex, down regulation of Unc5D expression is observed in migrating Svet1^{+} cells which is thought to be the mechanism that allows them to be insensitive to FLRT2 ECD and trespass the FLRT2 territory to reach the proper layer in the CP. After reaching upper cortical layers (mainly layer IV), Unc5D is re-expressed but the function of this re-expression in a FLRT2 rich environment is currently unknown. From these studies it was proposed that it is possible that repulsive FLRT2 and attractive Sema3A signals cooperate in guiding projection neurons to their appropriate cortical layers (Yamagishi et al., 2011).

Very recently were presented structural analysis indicate that FLRTs control cortical neuron migration by distinct mechanisms, since distinct FLRT LRR surfaces mediate homophilic adhesion and Unc5-dependent repulsion (Seiradake et al., 2014). FLRTs can affect both adhesive and repulsive functions in the same receiving cell, for example, neurons or vascular cells that co-express FLRT and

Unc5. This separation of adhesive and repulsive functionalities allows FLRTs to regulate the behavior of migrating pyramidal neurons in distinct ways; FLRT2 repels Unc5D^{+} neurons and thereby controls their radial migration, while FLRT3-FLRT3 homophilic interactions regulate their tangential distribution. FLRT3 also controls retinal vascularization, possibly involving combinatorial signaling via FLRT and Unc5 (Seiradake et al., 2014).

Besides these non-cell autonomous functions (in *trans*), FLRTs have been shown to display some cell-autonomous functions *in vivo* as well. FLRT3 was identified as a target gene of Nodal signalling, inhibiting cadherin adhesion in *Xenopus* early development through interaction with the Rho family GTPase Rnd1 (Ogata et al., 2007). During *Xenopus* development PAPC (paraxial protocadherin) and FLRT3 form a functional complex with cadherins for physiological regulation of C-cadherin adhesion, cell sorting, and morphogenesis. PAPC counteracts FLRT3 function by inhibiting the recruitment of the GTPase Rnd1 to the FLRT3 cytoplasmic domain (Chen et al., 2009). Also was shown that FLRT3 functionally interacts with Unc5B and the effecton adhesion is mediated by Rnd1, suggesting that FLRT3, Unc5B and Rnd1 proteins interact to modulate cell adhesion in early *Xenopus* development (Karaulanov et al., 2009). From these studies it is tempting to speculate that FLRT-Rnd could play a role *in vivo* as well in the development of the mouse cortex and could be, like PlexinB (Azzarelli et al., 2014), another transmembrane protein involved in the localization of Rnd activity in specific domains of the developing neuron for proper migration/differentiation.

FLRT3 appears to modulate several developmental processes such as cell growth, cell migration, and axon guidance by interacting in *cis* or in *trans* with distinct transmembrane receptors. Furthermore FLRT3 is also involved in axon pathfinding. A recent study demonstrates that FLRT3 acts as a co-receptor of the Robo1 and that it is required in rTCAs to modulate their Netrin-1 responsiveness.

FLRT3 modulates the degree of responsiveness to Netrin-1 from a neutral response to attraction, which is crucial for their topographic positioning. The absence of FLRT3 produces defects in axon guidance *in vivo.* This result highlight a novel mechanism by which interactions between limited numbers of axon guidance cues can multiply the responses in developing axons, as required for proper axonal tract formation in the mammalian brain. It constitutes a novel mechanism by which crosstalk between axon guidance cues is integrated in developing axons (Leyva-Díaz et al., 2014).

CHAPTER 2: AIMS AND OBJECTIVES

2. AIMS AND OBJECTIVES

Functioning of the cerebral cortex relies on the precisely regulated migration of newly born neurons from different origins to their final position, to ensure the coordinated assembly of circuits involving glutamatergic projection neurons and GABAergic interneurons. The cerebral cortex better illustrates how both types, radial and tangential migration, are integrated during brain development, establishing the basis for the subsequent neural circuitry. Many studies have contributed to understanding the molecular mechanisms that control the tangential migration of interneurons from the basal telencephalon to the cortex. However, the mechanisms controlling their precise integration within the cortex, related with proper receptors, ligands and other unknown extracellular factors, are still limited. In particular, among the molecules involved in interneuron intracortical migration and dispersion along stereotyped routes, mainly chemoattractive factors have been identified.

In this scenario, FLRT proteins are good candidates to contribute to the regulation of intracortical interneuron migration. *In vivo*, FLRTs have been related with neuron migration by different mechanisms of action (Seiradake et al., 2014; Yamagishi et al., 2011). For instance, it was shown that FLRTs have a repulsive function acting as ligands of the Netrin1 Unc5 receptors. *In vivo*, the FLRT2/Unc5D signaling has been implicated in the radial migration of a subset of cortical neurons (Yamagishi et al., 2011). In detail, proteolytic shedding of the FLRT2 ECD by cells in the CP prevents the premature migration of Unc5D-expressing neurons located in the SVZ to the CP in the developing cortex (Yamagishi et al., 2011). In contrast to this FLRT-Unc5 repulsive function, it was reported more recently that FLRT3 protein is implicated in the tangential dispersion of pyramidal neurons in a manner that involves FLRT3-FLRT3 homophilic adhesion (Seiradake et al., 2014). Therefore, FLRTs can regulate the

radial migration of pyramidal neurons through a repulsive effect mediated by Unc5 receptors, as well as their tangential spread, through an adhesive function involving FLRT-FLRT homophilic interactions.

Besides controlling neuron migration, our group has also recently shown that FLRTs have a role in controlling axon guidance *in vivo* (Leyva-Díaz et al., 2014). In this case, it was shown that FLRT3 is a novel co-receptor for Robo1 in rTCAs and modulates Robo1 activity for a proper topographic projection of these axons into the developing cortex (Leyva-Díaz et al., 2014). If this FLRT3-Robo1 signaling could also been involved in the regulation of other processes such as neuron migration are currently unknown.

Finally, the intracellular molecular mechanisms regulating FLRT signaling, cell autonomously (by homophilic cell adhesion or as co-receptors), or non-cell autonomously (acting as ligand for Unc5 receptors), is not very well undertood. In this context, Rnd familly of Rho GPTases, have been suggested to play an important role in FLRT signaling. For instance, FLRT3, Unc5B and Rnd1 proteins interact to modulate cell adhesion in early *Xenopus* development (Karaulanov et al., 2009). This effect might be mediated by a direct interaction of FLRT3 and Rnd1 which controls the levels of C-cadherin at the cell-surface (Ogata et al., 2007). On the other hand, Rnd2 and Rnd3 have been involved in several steps of pyramidal neuron migration in the developing cortex, although the upstream signaling effectors are currently unknown (Pacary et al., 2011). Altogether, it is tempting to speculate that the regulation of Rnd proteins by FLRTs could play a role in controlling different aspects of neuron development *in vivo*.

Considering all these antecedents which reveal that FLRTs are relevant factors during cortex development, it was proposed the hypothesis that FLRTs could

control the tangential migration of cortical interneurons during development. At the intracellular signalling level, it was hypothesized that Rnd proteins could be important regulators of the function of FLRTs *in vivo*.

The specific objectives are:

1.) Analyze the role of FLRTs proteins in the tangential migration of cortical interneurons during development.

1.1.) Determine the expression pattern of FLRT2 and FLRT3 in the routes of interneuron migration in the mouse telencephalon during development, specially around the interneuron migratory streams within the cortex.

1.2.) Determine the laminar distribution pattern and migration progression of tangentially migrating CB^{+} interneurons in WT and nervous system specific *FLRT* KO brains at two different developmental stages, E14.5 and E16.5. This analysis will include the single *FLRT2* or *FLRT3* KOs as well as the double *FLRT2*/*FLRT3* KOs.

1.3.) Analyze the morphological parameters and the cortical lamination (with specific markers) of pyramidal neurons in the developing cortex of the FLRT mutants.

1.4.) Discriminate the cell autonomous from the non-cell autonomous funcion of FLRTs in intracortical interneuron migration *in vivo* by using the palial-specific (cortical pyramidal neurons specific) Cre line, *Emx1-Cre*.

2.) Evaluate the possible functional interaction between Rnd3 and FLRT proteins.

2.1.) Determine the expression pattern of *rnd3* by *in situ* hybridization in the developing mouse brain and compared this pattern with those of FLRTs.

2.2.) Determine the interaction between FLRTs and Rnd3 by *in vitro* assays in heterologous cells.

2.3.) Assess the similarities of the FLRT phenotype in interneuron migration with that of *Rnd3* mutants suggesting a possible functional interaction of the two proteins *in vivo*.

CHAPTER 3: MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, reagents, commercial kits and enzymes

All chemicals and reagents were purchase from Fulka, GE Healthcare, Invitrogen, Merck, Sigma, Serva, Roche, Roth and VWR, 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 purchase from the 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.

3.1.2 Mouse lines and animal housing

Mouse lines *Nes-Cre*$^{+}$;*FLRT3*$^{lx/-}$, *FLRT2*$^{-/+}$, *FLRT3*$^{-/+}$, *FLRT3*$^{lx/lx}$ and *Sox1-Cre*$^{+}$ (Takashima et al., 2007) were previously described (Egea et al., 2008; Yamagishi et al., 2011). *FLRT2*$^{lx/lx}$ line was obtained from EUCOMM. *FLRT2*$^{-/+}$;*FLRT3*$^{-/+}$; *Sox1-Cre*$^{+}$ males were crossed with *FLRT2*$^{lx/lx}$;*FLRT3*$^{lx/lx}$ females in order to obtain a conditional deletion of *FLRT2* and *FLRT3* in the nervous system: *FLRT2*$^{-/lx}$;*FLRT3*$^{-/lx}$;*Sox1-Cre*$^{+}$, here called *FLRT2* and *FLRT3* double KO (DKO) (F2F3-Sox-DKO). Mouse line *Rnd3*$^{+/gt}$ (from Dr. Ignacio Pérez) were crossed to obtain *Rnd3* $^{gt/gt}$ embryos (Mocholí et al., 2011). Brains from *FLRT2*$^{-/lx}$;*FLRT3*$^{-/lx}$;*Emx-Cre*$^{+}$ double conditional mutants and the respective controls were kindly provided by Dr. Daniel del Toro.

3.2 Methods

3.2.1 Molecular biology

3.2.1.1 Generation of labeled riboprobes for *in situ* hybridization

Digoxigenin labeled riboprobes for mouse and human were generated using the plasmids obtained from S. Homma's Lab. For sense and antisense probe synthesis, plasmids were linearized using the respective restriction enzymes. After linearization of 10 µg 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°C. For *in vitro* transcription of RNA, 200 ng of linearized plasmid was used in 20 µl transcription reactions together with 2 µl digoxigenin-RNA labeling mix (Roche), 2 µl transcription buffer, 2 µl DTT 0.1 M, 1 µl RNase inhibitor, 1 µl RNA Polymerase (T3, T7 or SP6) and RNase-free water. After 3 hrs incubation at 37°C, the transcription efficiency was assessed using agarose gel electrophoresis and the RNA was precipitated by addition of 100 µl TE buffer, 10 µl LiCl 4 M and 300 µl EtOH 100% with subsequent centrifugation at 13 000 rpm for 15 min at 4°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°C. For *in situ* hybridization 10 µl/ml prehybridization solution was used.

3.2.2 Tissue culture

3.2.2.1 Cell culture

HEK293T and HeLa cells were grown in DMEN (Invitrogen), 10% FBS, antibiotics (Penicillin and Streptomycin), glutamine and divided every 48-36 hrs. For HEK293T and HeLa cells transfection experiments, cells were cultivated in collagen coated either p60 plates or 24 well plates, respectively. This coating was performed by adding 1 ml of 0.1 mg/ml of collagen in 0.02 N acetic acid. The cells were maintained at 37°C in a 5% CO_2-humidified incubator.

3.2.2.2 Transfection

For transfection of HEK293T and HeLa cells, were cultured in p60 plates and 24 well plates, respectively. The DNA was mixed with OptiMEM: 1 µg DNA per 50 µl OptiMEM and 4 µg DNA per 600 µl OptiMEM, respectively. In other tube the PEI and OptiMEM were mixed: 10 of PEI (1 µg DNA) in 40 µl OptiMEM and 40 of PEI (4 µg DNA) in 600 µl OptiMEM, respectively. Content of both tubes is mixed in one, vortex and kept 10 min at room temperature (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 to 1 or 2 ml total volume, respectively; then added to the cells and incubated 1 hr at 37°C in the 5% CO_2-humidified incubator. After that, the media was replaced by fresh media and kept in the incubator.

3.2.3 Biochemistry

3.2.3.1 Cell and brain tissue lysates

Lysates of cultured HEK293T cells were obtained by placing culture dishes on ice. Then, was removed the media, washed twice with ice-cold phosphate-

buffered saline (PBS) and incubated cells with ice-cold lysis buffer [LB: 20% glycerol, 2% Triton X-100, 40 mMTris pH 7.4, 300 Mm NaCl, 1 mM EDTA, 5 mM NaPP, 1 mM Na3VO4, 5mM NaF and and inhibitors of proteases (Roche)] for 10 min on ice. After the cells scraped, the lysate was rotated for 45 min at 4°C. The cellular debris was pelleted by centrifugation at 13 000 rpm for 15 min at 4°C.

The telencephalon was separated from the rest of the brain and quickly deposited in 2 ml tubes and frozen immediately in liquid nitrogen, then kept at -80 °C. The samples were defrosted quickly and the tissue was homogenized with ice-cold LB in a glass homogenizer. The lysate was incubated rotating for 45 min at 4°C and subsequently treated as cell lysates. The samples were stored at -80°C. The protein concentration was determined by a Colorimetric assay kit (BioRad) to further perform the Western blotting.

3.2.3.2 Immunofluorescence assay

HeLacells were grown on coverslips in 24 well plates and co-transfected with: pcDNA3-FLRT^{HA} (JEN055) and pcDNA3-RhoE^{FLAG} (MDN18) (both N-terminal-tagged), two wells per transfection condition. After 24 hrs one well was incubated with a mix: rbb anti-FLRT3 (home-made antibody raised against the ECD) and anti-rabbit Cy5 (previously incubated 30 min at RT, in order to promote the formation of FLRT3 clusters at the membrane of the transfected cells. After 1 hr in the incubator, all cells were washed with PBS and fixed 30 min with 500 µl 4% paraformaldehyde (PFA). Then, were washed 10 min with ice-cold NH_4Cl 50 mM in PBS. After that, permeabilization was performed during 15 min with 500 µl of 0.1% Triton X-100 in PBS on ice. Then, coverslip was taken out the well and processed directly for immunofluorescence. The coverslip was treated 60 min with 50 µl of blocking solution (BS: 5% donkey serum in PBS), followed by 2 hrs

of incubation with 50 µl primary antibody [rat anti-HA (Roche) and mouse anti-FLAG (Abcam); 1:250] in BS at RT. The cells were washed twice 5 min with PBS and then incubated for minimum 45 min with secondary antibody [donkey anti-rat Cy2 and donkey anti-mouse Cy3 (Jackson ImmunoResearch; 1:500] and DAPI was included in order to visualize nuclei (1:2000) in BS at RT. Cells were finally washed with PBS and the coverslip quickly rinsed with water before mounting with slowfade (Invitrogen). This preparation was sealed with polish nail and stored at 4°C under the dark until the analysis.

3.2.3.3 Western Blotting

Lysates samples were mixed with SDS PAGE, vortexed, centrifuged and boiled for 5 min at 95°C before loading them into the polyacrylamide gel (4% stacking and 12% concentration gel). Electrophoresis was performed in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) using the following parameters: 300 V and 20 mA/gel during 60 min. For transfer, the PVDF (Millipore) membrane was first activated 1 min in methanol, washed 1 min in water and 1 min more in transfer buffer (48 Mm Tris, 39 mM glycine, 0.04% SDS, 10% methanol). This step was performed by semi-dry method, applying 300 mV and 60 mA/membrane current during 60 min. After that, membrane was washed withTris Buffer Saline Tween (TBST: 50 mM Tris, 150 mM NaCl, 0.05% Tween 20) and incubated 45 min with 5% skimmed milk in TBST. Then, membrane was washed three times with TBST and incubated with primary antibody [goat anti-FLRT1, goat anti-FLRT2, goat anti-FLRT3 (R&D System), mouse anti-FLAG (Abcam), rabbit anti-EphA4 (Santa Cruz); 1:1000] prepared in TBST. Incubation was performed 2hrs at RT or overnight at 4°C in a mixer roller. The excess of primary antibody was washed three times with TBST and then the membrane was incubated 45 min with the secondary antibody [anti-goat HRP, anti-mouse HRP, anti-rabbit HRP (Jackson ImmunoResearch); 1:5000] diluted in TBST. The

membrane was washed six times in 30 min approximately. Finally, membrane was incubated 3 min in 2 ml of mix of Chemiluminiscent HRP Substrates (Millipore) and image was taken in BioRadChemidoc. Quantification of band intensity was performed with Image Lab software.

3.2.3.4 Co-immunoprecipitation

After 24 hrs from transfection of HEK 293T with: pcDNA3-FLRT^{HA} (JEN055), pcDNA3-RhoE^{FLAG} (MDN18), pNEGFP-FLRT3ΔC^{GFP} (JEN192), pcDNA3-FLRT1 (JEN025), FLRT2 (JEN079); the cells were harvested with LB (previously described) and centrifuged to collect supernatant. Then, 50 µl of the extract were kept for total cell lysate (TCL) and the rest was incubated 4 hrs with 10µl of anti-FLAG coupled beads (SIGMA). Finally, beads were washed and left with 10 µl of protein loading buffer (reducing).

3.2.4 Animal handling and experiments

Pregnant WT mice maintained on a CD1 background and pregnant females from the crosses described above were used for protein and gene expression analysis. Timed-pregnant dams (day of vaginal plug = embryonic day 0.5) were killed by rapid cervical dislocation and mouse embryos were immediately harvested for different analysis. Mouse embryos from E14.5 to E 16.5 were used in the present study. Tail biopsies were taken from the embryos to obtain DNA as template to perform a genotyping PCR in order to identify the mutant embryos. Animals were maintained in Specific Pathogen Free (SPF) conditions in the Animal house of the University of Lleida under standard conditions and were treated according to the laws and regulations of the European Union and Spanish government.

3.2.5 Genotyping

Embryo's tail obtained in the dissection was washed in PBS and placing in PCR tube to dissolve the tail. DNA was obtained by 15min (three times) boiling steps of sample in 50 mM NaOH at 94°C, and a neutralization step with Tris pH 8.8 1.5 M.

To detect F2F3-Sox-DKO were used the following primers:

FLRT2 mutant:

5'-GACTTCTCTTAACAGTACTTCGCATCACGC-3' (MDN-O-4).

5'-GTTCATGGGGAACTCATCCAACTGGTTGCC-3' (MDN-O-5)

5'-AGTTATATTAAGGGTTCCGGATCAGCAGCC-3' (MDN-O-6)

FLRT2^{lx}:

5'-GTGGAAGGAAGGAATTGTCTCAGG-3' (MDN-O-59)

5'-GGAGCCAGGTTGGCAGGAGTTGGC-3' (MDN-O-60)

FLRT3 mutant:

5'-GCTTATACTACAAGGGTCTCATGTGAACGC-3' (MDN-O-42)

5'-GGCTGCAGGAATTCGATATCAAGCTTATCG-3' (MDN-O-43)

5'-CCGGTACTAAGAAAGACAACTCCATCCTGG-3' (MDN-O-44)

FLRT3^{lx}:

5'-GATATTTGCCAAAGGAGACAGAAAATACTGGC-3' (MDN-O-160)

5'-CTGGGTTCATTGCTGTCTACCAACAAGCAC-3' (MDN-O-80)

Cre allel:

5’-GCCTGCATTACCGGTCGATGCAACGA-3’ (MDN-O-46)

5’-GTGGCAGATGGCGCGGCAACACCATT-3’. (MDN-O-47)

The following PCR program was used in a BioRad T100™ ThermalCycler:

3 min/94°C + 38 x [1 min/94°C + 1 min/63°C + 1 min/72°C] + 20 min/72°C + Infinite x 10°C.

In the case of genotyping the *Rnd3* $^{gt/gt}$, PCR was performed with the following primers following a three primer strategy:

KO-specific primers:

5’-AAATGGCGTTACTTAAGCTAGCTAGCTTGC-3’ (MDN-O-276)

Common primer:

5’-TGAGCTAGGAAGATGCGGATGT-3’ (MDN-O-277)

WT-specific primer:

5’-TTTACACAGTAGGCTGACTC-3’ (MDN-O-278).

The following PCR program was used in a BioRad T100™ ThermalCycler:

3 min/94°C + 38 x [45 seg/94°C + 1 min/58°C + 1 min/78°C] + 20 min/72°C + Infinite x 10°C.

3.2.6 Brain dissection, cryopreservation and cryosection

Brains were dissected under a lens microscope in ice-cold PBS, washed and fixed in 4% PFA 4 hrs (for immunohistochemistry) or overnight (for *in situ* hybridization). Then, the brains were washed subsequently with PBS to remove excess PFA. After that, were cryoprotected in 30% sucrose to avoid any desiccation while storage in -80°C till it sinks to bottom and then embedded in

cryoprotective Tissue Tek. Coronal sections of 20 μm thickness were made in cryostat (Leica CM3000) at -24°C. Air dry for 1 hr at RT and then used or stored at -80°C.

3.2.7 Histology

3.2.7.1 *In situ* hybridization

Serial brain 20 μm cryosections stored at -80°C were defrosted for 30 min at RT. Hybridization was carried out over night in hybridization buffer containing digoxigenin-labeled probe of Rnd3 at 70°C. Sections were rinsed and washed with MABT three times for 20 min at RT. Sections were incubated in blocking solutions for 60 min at RT. Subsequently, sections were incubated over night at 4°C with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) diluted 1:2000 in blocking solution. Sections were then rinsed and washed six times for 20 min with MABT at RT, followed by rinse and wash of 10 min, 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 5 min washes (3 times) with PBT at RT. Sections were postfixed in 4% PFA in PBS for 15 min, rinsed and washed with PBS for 5 min (2 times). Rinsed and washed with water for 5 min (2 times). Air dried under hood. Mounted with glycerol-gelatin and stored at RT.

3.2.7.2 Immunohistochemistry

Slices from E14.5 and E16.5 embryos coronal sections brain were let to dry 30 min at RT. Then, 10 min washes (3 times) with PBS, 10 min wash with 50 mM NH_4Cl and a final wash with PBST (0.1% Triton X-100 in PBS). Sections were permeabilized and unspecific bindings were blocked for 1 hr at RT with blocking solution (BS: 5% donkey serum in PBST). Sections were incubated with primary

antibodies [goat anti-FLRT2, goat anti-FLRT3 (R&D System), rabbit anti-Calbindin (Swant), rabbit anti-Tbr1, rabbit anti-Tbr1, mouse anti-Satb2, rat anti-Ctip2 (Abcam); 1:100] were diluted in BS and incubated overnight at 4°C. After washing 15 min with PBST (3 times), the secondary antibodies [donkey anti-goat Cy3, donkey anti-rabbit Alexa488, donkey anti-mouse Cy3, donkey anti-rat Alexa647 (Jackson ImmunoResearch); 1:300] with DAPI (1:2000) were also prepared in BS and incubated for 2 hrs at RT. The slides were washed 15 min with PBST (3 times), and coverslipped with an aqueous mounting medium Fluoromount-G (SouthernBiotech). Immunofluorescently labeled cryosections were imaged on a Olympus Bx51 fluorescence microscope, and micrographs were uniformly adjusted for levels, brightness, and contrast in Adobe Photoshop.

3.2.8 MGE explant assay

E14.5 WT; *FLRT2* KO (F2-Sox-KO); *FLRT3* KO (F3-Sox-KO) and F2F3-Sox-DKO brains were dissected in ice-cold Hank's Balanced Salt Solution (HBSS) with antibiotics (penicillin and streptomycin). MGE pieces were embebed in a drop, grown in Matrigel matrix and cultured in p35 plates at 37 °C in a 5% CO_2-humidified incubator in neurobasal medium supplemented with B27 serum and L-glutamine (Invitrogen), antibiotics (penicillin and streptomycin). Images were acquired at 24, 48, 72 and 96 hrs using an Olympus 1x71 microscope and analyzed.

3.2.9 Quantitative and statistical analysis

3.2.9.1 Cells intracortical distribution

For the analysis of interneurons migration *in vivo*, the number of CB-expressing cells was quantified. These cells were counted of the dorsal E14.5 and E16.5

cortex at intermediate rostrocaudal levels in six different animals in all cases (n=6).

The total cells were counted manually. To determine the distribution of the cells at E14.5, the cortex was analyzed with a counting box subdivided into 10 equidistant bins that spanned the lower part of MZ (not included) to ventricular surface. Bin 1 roughly corresponds to the beginning of the CP, and bin 10 corresponds to the VZ. The grid was placed systematically in cortical locations approximately in the middle of lateral-medial axis of the embryonic cortex. This distribution also was determined for cytologically distinct zones (CP, IZ, SVZ/VZ) and two tangential streams (SP and IZ/SVZ). At E16.5 the counting box was subdivided in two parts containing the CP and the rest of the cortical layers (MZ not included). The numbers of cells present in each bin were counted and their percentile distributions across all bins were determined for each tissue section.

3.2.9.2 Tangential progression of cells

For quantification of tangential progression, cortical length was measured from the corticostriatal boundary to the cortical hem. The relative streams distance was calculated as the distance of both SP and IZ/SVZ streams respect to cortical length. Also, cortices were segmented into three equidistant counting boxes to count the relative total CB^+ interneurons. The last bin (III) (see Figure 31A) was subdivided in three bins and measured the total CB^+ interneurons in each bin.

3.2.9.3 Layer thickness

The total and relative thickness of different layers of the cortex was measured using the fluorescence nuclear staining as a cytoarchitectonic reference. The relative thickness of cortical layers labeled with differentiation markers (Tbr1,

Tbr2, Ctip2, Satb2) and the respective fluorescence levels were measured using ImageJ software.

3.2.9.4 MGE explants migration

The interneurons migration from the MGE explants was quantified as the migration distance of cells in the half of each explant. The maximum migratory distance away from the explant was determined calculating the average of nine measures: the ratio between the distance from the explant to the furthest neuron migrated from the explant and the explant radius (see Figure 44C). Two tailed unpaired Student' t tests were used to determine statistical differences between groups. Significance was considered $p<0.05$ and $p<0.001$. Error bars were calculated using the standard error of the mean (s.e.m), calculated from standard deviation.

CHAPTER 4: RESULTS

4. RESULTS

4.1 Mendelian analysis of the *FLRT2* and *FLRT3* double knockout progeny

To begin addressing the objectives derived from the hypothesis of the present thesis, a conditional DKO for *FLRT2* and *FLRT3* in the nervous system, using the *Sox1-Cre* line, was created (Takashima et al., 2007). The breeding strategy was to cross a male $FLRT2^{+/-};FLRT3^{+/-};Sox1\text{-}Cre^{+}$ with a female $FLRT2^{lx/lx};FLRT3^{lx/lx}$ obtaining the offspring that includes all genetic possibilities (Figure 17A). All the litters produced were genotyped by PCR from tail biopsies of E14.5 embryos (see Material and Methods for the PCR genotyping conditions and primers). This data was analyzed by ANOVA to see if the crossing results complies with the terms of the Mendelian distribution and, as observed, the DKO genotype of offspring is according with the theoretical ratio expected (~12.5%) (Figure 17B). Only a control group gave significative differences, the reason for which is unknown (Figure 17B). In all cases "control group" refers to control littermates.

4.2 Migrating Calbindin^{+} interneurons follow three principals tangential routes

To begin the study, was analyzed the normal distribution of CB-expressing cortical interneurons in WT embryos at E14.5, after the initial cohort of interneurons have entered the cerebral cortex (E12.5). By E13.5 interneurons have crossed the corticostriatal boundary and have migrated approximately half the length of the lateral cortical wall (Myers et al., 2014). Thus, at E14.5 is the beginning of interneurons tangential migration peak. In mice, CB is a well known marker for tangentially migrating interneurons in the cortical rudiment at the earliest stages of cortical histogenesis (Anderson, 1997).

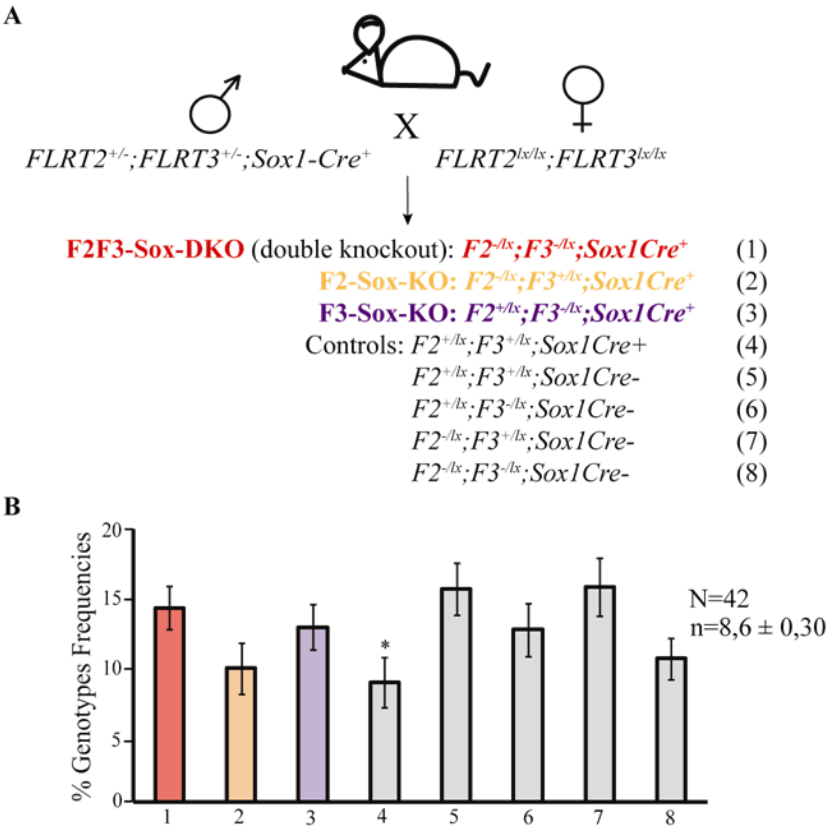

Figure 17. Generation and Mendelian analysis of *FLRT2* and *FLRT3* DKO mice. (A) Analysis of all progeny derived from mating *FLRT2*$^{+/-}$;*FLRT3*$^{+/-}$;*Sox1-Cre*$^{+}$ and *FLRT2*$^{lx/lx}$;*FLRT3*$^{lx/lx}$ parents. (B) Distribution of animals generated. "N" value represent the total number of litters analyzed and "n" the average of animals per litter. No significant differences were obtained by ANOVA analysis. All data are presented as mean ± s.e.m.

Embryos from E14.5 pregnat females were dissected to obtain the brain. These embryonic brains were coronally sectioned and the cerebral cortex was sampled at three rostrocaudal locations spanning sections containing the MGE until before the caudal-most end of the telencephalon. By this, the analysis covers the majority of GABAergic migrating interneurons mainly originating in the MGE of the embryonic ventral telencephalon and discard the CGE-derived cells (Figure 18D). Then, the spatial distribution of WT interneurons within the cortex, in the rostrocaudal and mediolateral axes of the cortex (Figure 18A, B, C), was

analyzed. At this stage most CB^+ interneurons were found forming three medially oriented large migratory streams: one coursing a superficial route through the MZ, another one, more prominent, in the lower IZ/SVZ and a smaller fraction of interneurons migrating through the SP (Figure 18E).

In order to quantify the normal radial distribution during tangential migration of CB^+ interneurons, a binning analysis across the developing wall was performed, using a fixed area box positioned in the cerebral wall (as indicated in panels A-C of Figure 18). This box was subdivided into ten equidistant bins along the radial axis of the lateral cortex (Figure 18E), and the percentage of CB^+ cortical interneurons appearing in bins 1 (above the MZ) through 10 (VZ) for each tissue section was determined.

The results for this quantifications indicate that, in WT embryos, interneurons were predominantly distributed in the SP stream (bins 2-3) and IZ/SVZ stream (bins 6-7) (Figure 18F). Same results were obtained with this distribution was also determined grouping bins for cytologically similar zones: CP (bin 1), SP stream (bins 2-3), IZ (bins 4-5), IZ/SVZ stream (bins 6-7) and SVZ/VZ (bins 8-10) at this stage (Figure 18F').

4.3 FLRT2 and FLRT3 expression in embryos the mouse developing telencephalon

It is well known that FLRT2 and FLRT3 regulate radial migration of a subset of projection neurons (Yamagishi et al., 2011) and that the absence of FLRT3 produces defects in axon guidance *in vivo* in the developing thalamocortical projections in the forebrain (Leyva-Díaz et al., 2014).

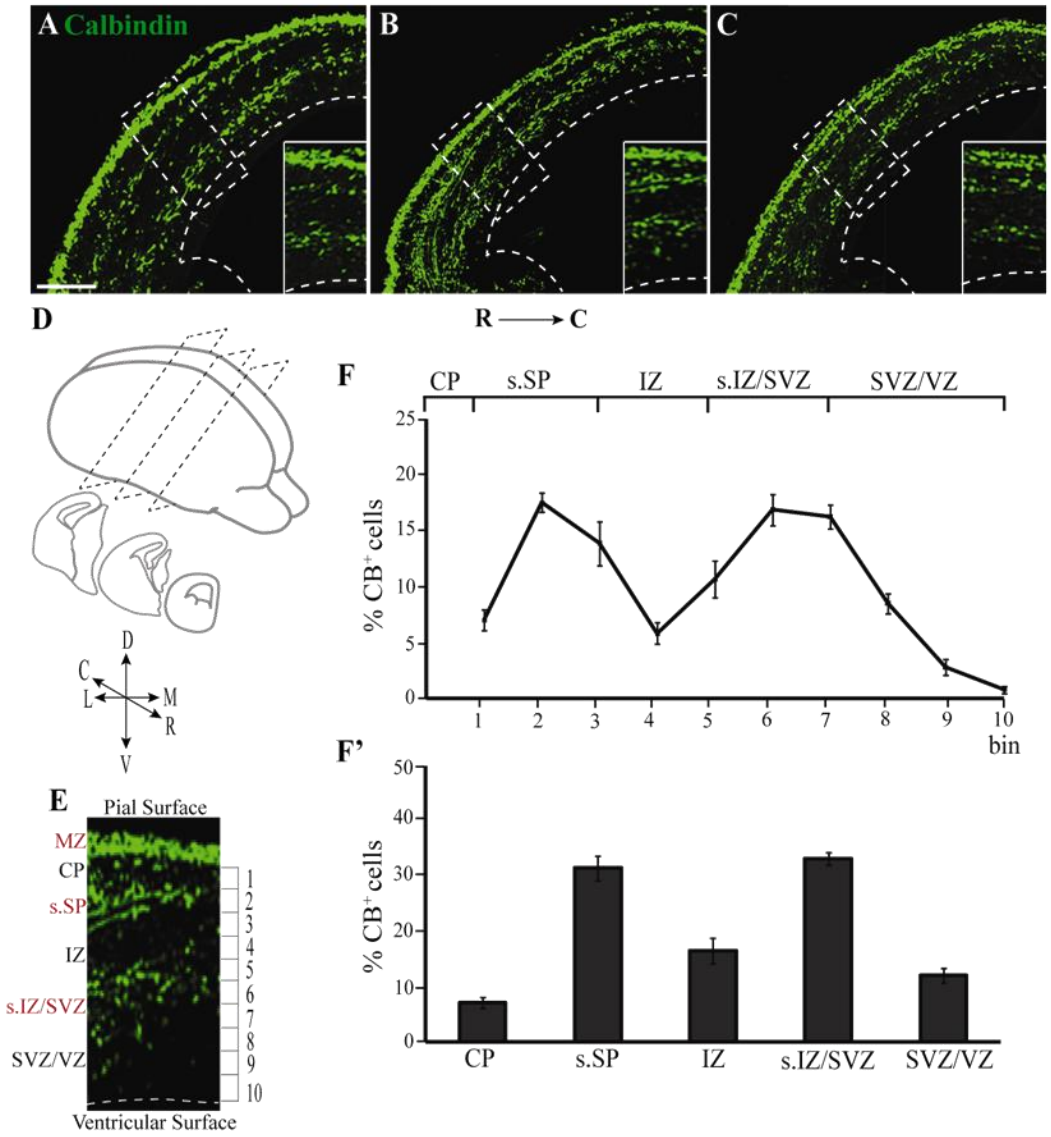

Figure 18. Normal distribution pattern of tangentially migrating CB$^+$ interneurons in the cerebral cortex at E14.5. Immunohistochemistry for CB$^+$ interneurons expression in coronal sections through the rostral (A), intermediate (B) and caudal (C) telencephalic levels of WT brain as shown in squeme (D). Insets show a higher magnification of the boxed regions in each panel. (E) Boxed area taken from the (B) panel showing the laminar distribution of CB$^+$ interneurons compared to 10 equidistant bins from the CP (avoidinig the MZ) to the ventricular surface. As indicated the main migratory streams are observed in the MZ, SP and IZ/SVZ (red). (F) Quantification of the number of interneurons in each bin respect to the total in each selected area (values are given in percentage). Numbers in ordinates identify bins for quantification, from the CP to the VZ. (F') Percent distribution of CB$^+$ interneurons for cytologically distinct zones: CP (bin 1), s.SP (bin 2 and 3), IZ (4 and 5), s.IZ/SVZ (6 and 7), SVZ/VZ (8, 9 and 10). All data are presented as mean ± s.e.m (n=3). Abbreviations: CB, calbindin; R, rostral; C, caudal; M, medial; L, lateral; D, dorsal; V, ventral; MZ, marginal zone; CP, cortical plate; s.SP, stream subplate; IZ,

intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone; WT, wild type. Scale bar, 200μm.

However, the extent to which FLRT2 and FLRT3 signaling influences the migration of cortical interneurons is unknown. To evaluate the potential involvement of FLRT2 and FLRT3 signaling in interneuron migration, the expression pattern of FLRT2 and FLRT3 was mapped by immunofluorescence in the embryonic telencephalon at E14.5.

Coronal sections were labeled with anti-FLRT2 and anti-FLRT3 antibodies raised against their ECDs. FLRT2 is widely expressed in the developing forebrain, but appears to be enriched in the nascent CP and in the mantle region of the LGE (van den Berghe et al., 2013; Yamagishi et al., 2011), where the newly generated postmitotic neurons reside (Figure 19A). In the case of FLRT3, prominent expression is evident in the thalamus, in the boundary between the LGE and MGE, and in the IZ of the developing cortex (van den Berghe et al., 2013; Yamagishi et al., 2011) (Figure 19B). Both expression patterns are specific since the signal dissapear in knockout tissue (see below). This expression analysis shows that both of proteins are expressed in different regions traversed by tangentially migrating interneurons suggesting that FLRT2 and FLRT3 may play a role in the regulation of interneuron migration. To test this hypothesis a double labeling of FLRT2 or FLRT3 in conjunction with CB was performed (Figure 19A', B'). From this analysis, striking observations were made in the developing cortex. Interestingly, these results revealed that CB^+ interneurons in the MZ and IZ/SVZ, at E14.5, avoid the cortical areas with enriched expression of FLRT2 and FLRT3, the CP and IZ, respectively. For the CB^+ interneurons in the SP, these avoid the FLRT3 expression in the IZ and avoid the high expression of FLRT2 in the CP.

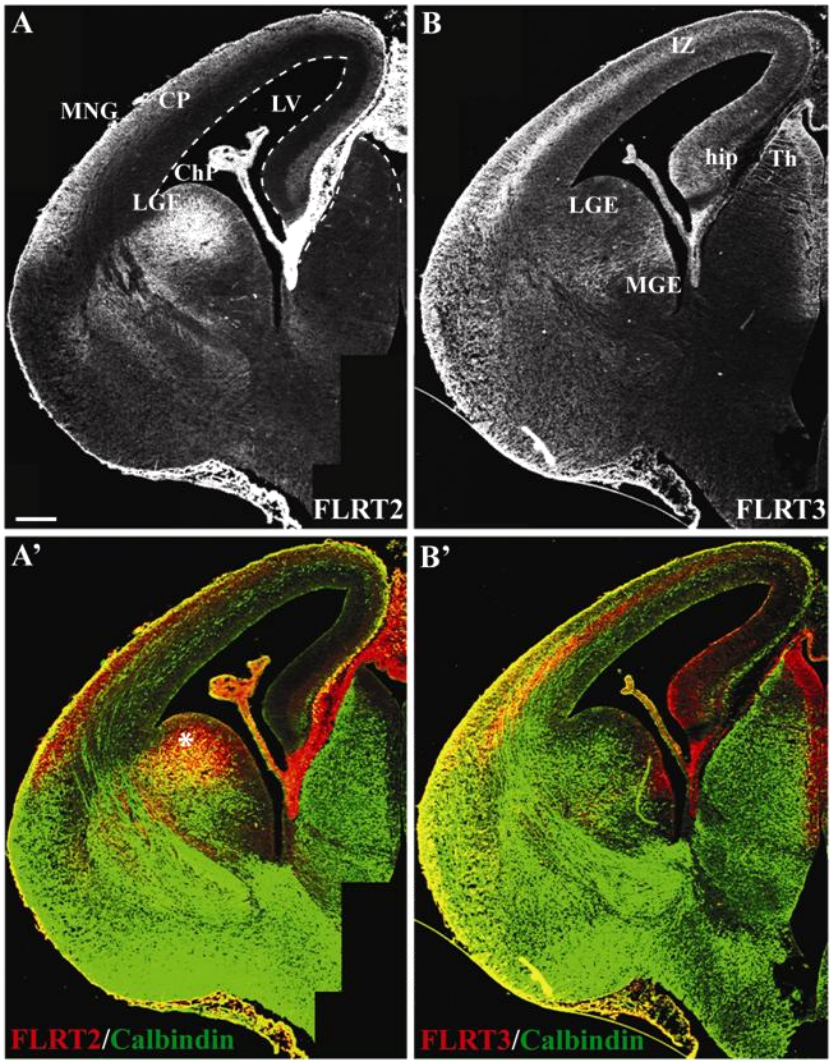

Figure 19. FLRT2, FLRT3 and CB expression in the developing brain. Overview pictures of coronal section of E14.5 telencephalon. (A) Within the brain, FLRT2 immunoreactivity is strongly detected in the CP and LGE. Strong FLRT2 immnoreactivity was also detected in the choroid plexus and meninges (indicate meninges in the figure). (B) FLRT3 is expressed in the IZ, in the boundary between LGE and MGE, hippocampus and thalamus. Sections in panels A and B were co-labelled with CB (A' and B', respectively). The three streams of tangential migrating CB^+ interneurons (MZ, SP and IZ/SVZ) follow trajectories that do not overlap with the regions of high FLRT2 or FLRT3 expression in most cortical regions. * some segregation between the FLRT2 territories (LGE) and CB^+ interneurons Abbreviations: CB, calbindin; CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; ChP, choroid plexus; hip, hippocampus; Th, thalamus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MNG, meninges. Scale bar, 200μm.

However, they do migrate through a region that still contains detectable amounts of FLRT2, since FLRT2 protein expression expands into the IZ region (this issue will be discussed in the discussion section) (Figure 20A, B). By contrast, in the ventral telencephalon migrating interneurons from the MGE or CGE traverse the LGE along the permissive corridor in the SVZ (Flames et al., 2004). In this transit they encounter FLRT2 in the LGE, and as it happens in the cortex, some segregation also exists between the FLRT2 territories and CB^+ interneurons. Thus, in the developing cerebral cortex, FLRT2 and FLRT3 are expressed in the migratory route of the CB^+ interneurons; thereby these proteins may have a functional role during interneuron tangential migration.

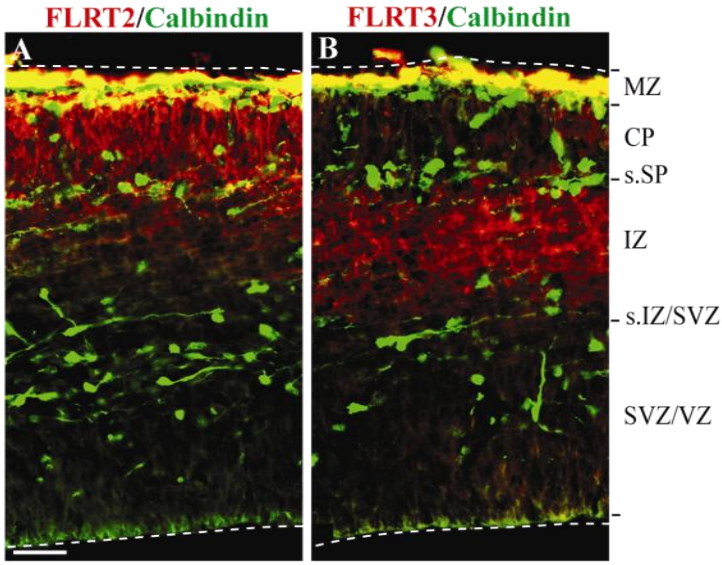

Figure 20. Detail of the laminar distribution of the tangentially migrating CB^+ interneurons in the WT cerebral cortex. High-magnification pictures of the E14.5 dorsal cortex. The CB^+ interneurons follow three principals routes that avoid the FLRT2 cortical expression in the CP (A) and the FLRT3 expression in the IZ (B). The interneurons forming the SP stream migrate through a region that contains detectable amounts of FLRT2. The dashed lines show the demarcation of the Pial surface (top) and Ventricular surface (bottom). Abbreviations: CB, calbindin; MZ, marginal zone; CP, cortical plate; s.SP, stream subplate; IZ, intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone; WT, wild type. Scale bar, 20μm.

4.4 Interneurons distribution in the *FLRT3* nervous system specific knockout

To examine whether the absence of FLRT3 function could modulate the migratory behavior of CB^+ interneurons, brains from *FLRT3* nervous system specific knockouts were analyzed. For this study, a first set of animals were obtained by removing *FLRT3* using the *Nes-Cre*$^+$ mouse line (Tronche et al., 1999), which inactivates FLRT3 in the entire embryonic CNS already at ~E10.5 (see generation of *FLRT3*^{lx} conditional alleles in Yamagishi et al., 2011). In order to obtain these animals a similar crossing protocol as depicted in Figure17 was used. The mutant *Nestin-Cre*$^+$*;FLRT3*$^{lx/-}$ mice, here referred as "F3-Nes-KO", were compared to several other genotypes of the rest of the progeny that were used as controls.

Six E14.5 F3-Nes-KO embryos and six controls were coronally sectioned, and the lateral cortical wall was sampled at three rostrocaudal locations, as previously described (Figure 18). The Figure 21 shows representatives pictures of the immunohistochemical labeling for CB^+ interneurons at rostral (Figure 21A, A'), intermediate (Figure 21B, B') and caudal (Figure 21C, C') locations in the dorsal cortex of the F3-Nes-KO and control embryos. The immunofluorescence pictures do not show any obvious defect in the distribution of CB^+ migrating interneurons in the absence of *FLRT3*.

The quantitative analysis shows that the total number of CB^+ interneurons is the same in the F3-Nes-KO and control embryos suggesting that they reach the dorsal telencephalon normally (Figure 22). Moreover, the binning analysis demonstrated that F3-Nes-KO mutant animals displayed a cortical bin distribution closely matching control embryos, with no significant differences at rostral (Figure 23A, A'), intermediate (Figure 23B, B') and caudal (Figure 23C, C') locations. Therefore, the conclusion from this part of the study is that the number and CB^+ interneuron distribution is intact and unperturbed in the absence of *FLRT3*.

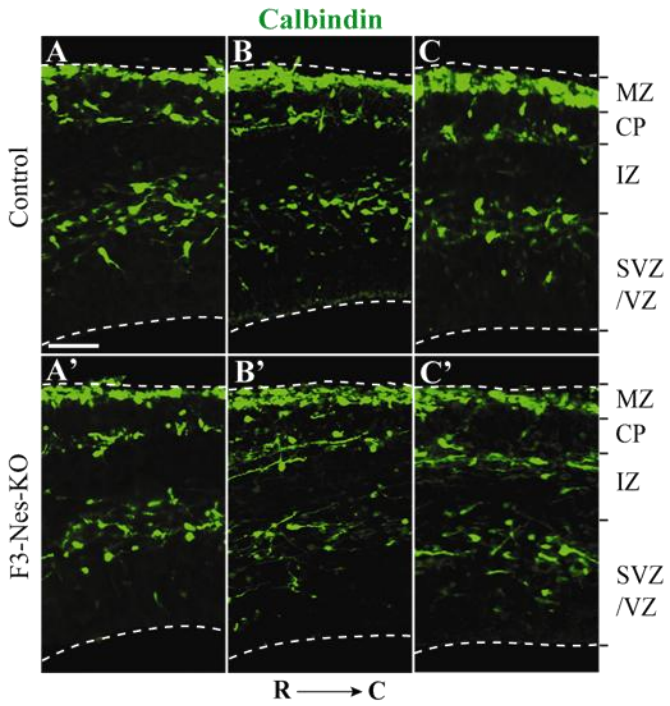

Figure 21. Normal laminar distribution of the tangentially migrating CB$^+$ interneurons in the cerebral cortex of F3-Nes-KO animals. Representative images of the cortical region stained with CB, at three different rostro (R)-caudal (C) levels in F3-Nes-KO (bottom panels) and control (upper panels) animals. The three principal routes of CB$^+$ interneurons in the mutants are similar to controls. The dashed lines show the demarcation of the Pial surface (top) and Ventricular surface (bottom). Abbreviations: CB, calbindin; MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone. Scale bar, 50μm.

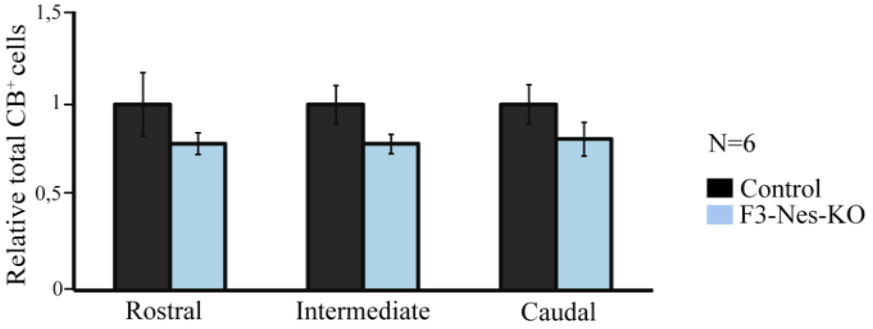

Figure 22. Total number of CB$^+$ interneurons within the cortex is similar in F3-Nes-KO than in controls. Relative total CB$^+$ interneurons from F3-Nes-KO and control embryos at rostral,

intermediate and caudal telencephalic levels (controls took value "1" in each region and values in mutant animals were expressed as fold induction respect to each control). All data are presented as mean ± s.e.m. (n=6 per genotype, p>0.05, two tailed Student' t test for each region). Abbreviations: CB, calbindin.

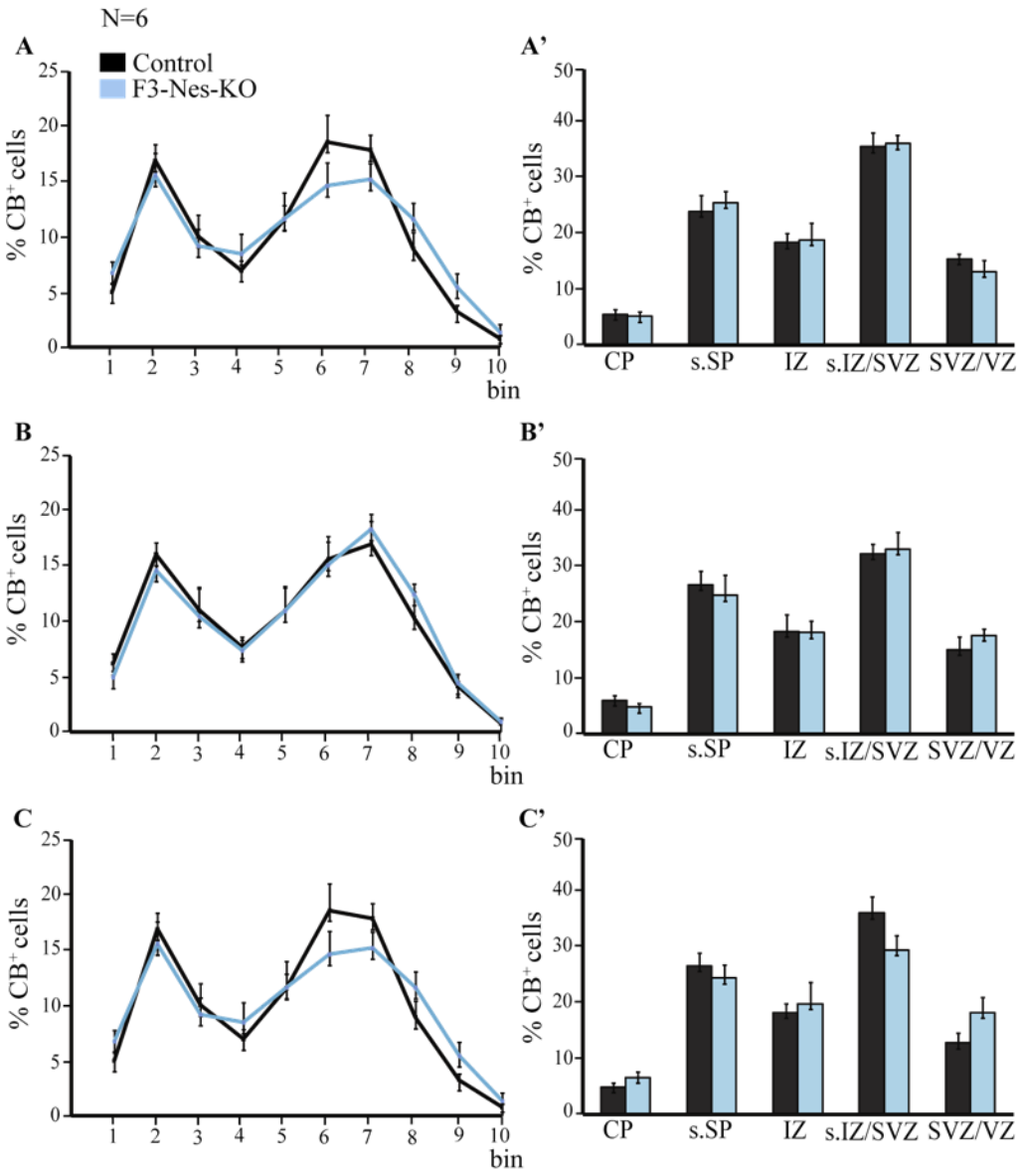

Figure 23. Distribution patterns of tangentially migrating CB$^+$ interneurons in the F3-Nes-KO cerebral cortex at E14.5. Distribution of CB$^+$ interneurons from F3-Nes-KO (blue lines) and control (black lines) embryos (6 animals, each), as described in Figure 18. In left graphs, numbers

in ordinates identify bins for quantification, from the CP (bin 1) to the VZ (bin 10) while in right panels, ordinates depict cytologically distinct zones at rostral (A, A'), intermediate (B, B') and caudal (C, C') telencephalic levels. All data are given as percentage and represented as mean ± s.e.m. (n=6 per genotype, $p>0.05$, two tailed Student' t test for each pair of data). Abbreviations: CB, calbindin; CP, cortical plate; s.SP, stream subplate; IZ, intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone.

4.5 Normal CB^+ interneuron distribution in the *FLRT2* single knockout animals

Next, the functional relevance of *FLRT2* during interneuron migration *in vivo*, was assessed. In this case, the Nestin-Cre transgene was located in the same chromosome and in close vicinity to the *FLRT2* gene and therefore was difficult to combine both genes in a single animal. For this reason, these experiments were performed using a different Cre line instead, the *Sox1-Cre* line which Cre recombination occurs as early as E9.5 (Guo et al., 2000), a bit earlier than the *Nestin-Cre* approach. FLRT3 was also included in this analysis in order to confirm the results obtained with *Nestin-Cre* line and to create DKOs in case some kind of functional compensation existed between FLRT2 and FLRT3. The crossing protocol used for these experiments is the one depicted in Figure 17 and the progeny resulting of this breeding included the single mutants for *FLRT2* (*FLRT2*$^{-/lx}$;*FLRT3*$^{+/lx}$;*Sox1-Cre*$^{+}$; here referred as "F2-Sox-KO") and for *FLRT3* (*FLRT2*$^{+/lx}$;*FLRT3*$^{-/lx}$;*Sox1-Cre*$^{+}$; here referred as "F3-Sox-KO"). The double mutants (hereafter referred as "F2F3-Sox-DKO") were also obtained in the same way but will be discussed in a different section.

Foremost, it was confirmed that both *FLRT2* and *FLRT3* genes were efficiently recombined from the entire telencephalon as verified in the KO animals by the lack of the characteristic immunoreactivity pattern observed in controls (Figures 19 and 24). Efficient recombination of both genes simultaneously and ablation of

their expression in neuronal tissue was even observed in the F2F3-Sox-DKO at two developmental stages, E14.5 (Figure 24) and E16.5 (Figure 25).

Interestingly, FLRT immunoreactivity was still observed in non-neuronal tissue in the KO brains; for example FLRT2 expression in the meninges (connective tissue membranes cover all the nervous system) and in the choroid plexus (ventriclular vascular structure) (Figure 24A', 25A') while FLRT3 was still present in medial habenula (Figure 24B', 25B'). This immunfluorescence approach was also validated by biochemical methods using a Western blot analysis (Figure 24C). For these experiments, lysates prepared from the whole telencephalon at E14.5, where the meninges were removed because of the FLRT2 abundant expression in this tissue, were obtained from the single and DKO animals. Then, the samples were subjected to a lectin pull-down for the enrichment of glycosylated transmembrane proteins and precipitates analyzed by Western blot with specific antibodies against FLRT2 or FLRT3. As observed in Figure 24C, the levels of FLRT2 and FLRT3 in the mutant brains were entirely absent in the corresponding genotypes, even the DKO.

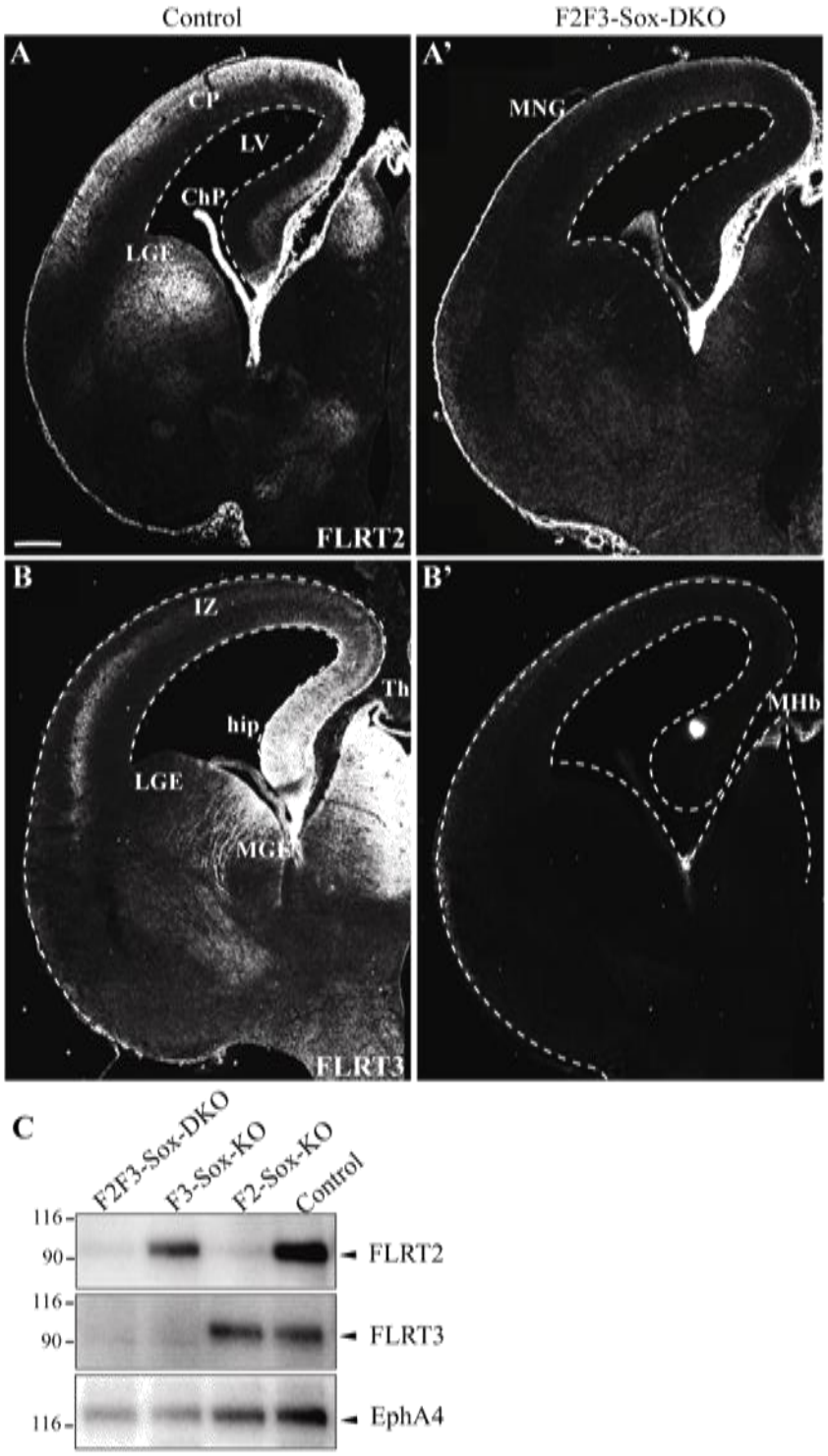

Figure 24. Efficient recombination of *FLRT2* and *FLRT3* in the developing brain driven by *Sox1-Cre* line at E14.5. Overview pictures of E14.5 telencephalon coronal sections. (A) FLRT2

immunoreactivity is mainly detected in the CP, LGE, ChP and MNG in a control section. (B) FLRT3 is strongly expressed in the IZ of the developing cortex, in the boundary between LGE and MGE, hip and Th. In the F2F3-Sox-DKO brains, FLRT2 (A') and FLRT3 (B') immunoreactivity is completely absent in neuronal tissue while in not neuronal tissue, FLRT expression is still maintained: for instance, expression of FLRT2 in the MNG and ChP (A') or expression of FLRT3 in the MHb. (C) FLRT protein expression is strongly reduced in total cell lystes from the telencephalon of the *FLRT* KOs. Protein lysates from single KOs (F2-Sox-KO and F3-Sox-KO) or the F2F3-Sox-DKO and from controls were obtained from the whole telencephalon and subjected to a lectin pull down in order to enrich the transmembrane protein fraction. Samples were then blotted with antibodies against FLRT2 (upper panel), FLRT3 (middel panel) and EphA4 (lower panel) as loading control. Note the absence of FLRT2 and FLRT3 proteins in F2 or F3 singles KOs, respectively and in the F2F3-Sox-DKO. In these experiments, the meninges were removed from the tissue because of the high FLRT2 expression in this tissue that is not abolished using the neuron specific *Sox1-Cre* line and could therefore interfere with the result. Abbreviations: MNG, meninges; CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; ChP, choroid plexus; hip, hippocampus; Th, thalamus; MHb, medial habenula; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Scale bar, 250μm.

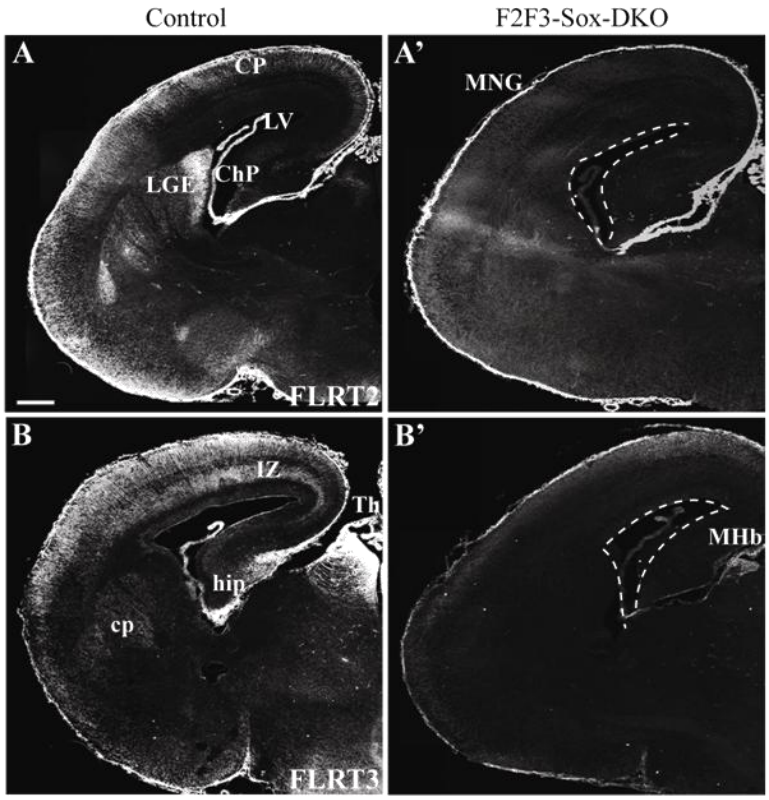

Figure 25. Efficient recombination of *FLRT2* and *FLRT3* in the developing brain driven by *Sox1-Cre* line at E16.5. Overview pictures of the E16.5 telencephalon coronal sections. (A) FLRT2 immunoreactivity is detected in the CP and LGE in the control. (B) FLRT3 is expressed in

the IZ, in cells migrating through the CP and in the upper layers of the CP, the CA3 region and dentate gyrus of the hip, cp and th. FLRT2 (A') and FLRT3 (B') immunoreactivity is absent in F2F3 DKO. FLRTs immunoreactivity is present in not nervous tissue, in the case of FLRT2 in the MNG (A') and FLRT3 in the MHb and CP (inespecific antibody labeling). Abbreviations: MNG, meninges; CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; ChP, choroid plexus; hip, hippocampus; Th, thalamus; MHb, medial habenula; LGE, lateral ganglionic eminence; cp, caudate putamen. Scale bar, 200μm.

These control experiments indicate that Sox1-Cre drives a very robust expression of Cre in the nervous system. It is important to emphasize that the above immunoflurescence control was performed for most of the brains analyzed in the present study in order to validate our PCR genotyping strategy and confirm efficient recombination. Therefore, these results evidenced that the selected mice for the present analysis were truly knockout for *FLRT2*, *FLRT3* or both.

Similar to the study with the *Nestin-Cre* line, six E14.5 knockout embryos for *FLRT2* or *FLRT3* using the *Sox1-Cre* line were collected and coronally sectioned. After that, CB staining was performed and the lateral cortical wall was sampled as previously described to check for interneuron migration defects. The Figure 26 shows representative pictures of the immunohistochemical labeling for CB^+ interneurons in the dorsal cortex for *FLRT2* (Figure 26B) and *FLRT3* (Figure 26C) single mutants compared to controls (Figure 26A). In this case only the intermediate sections of the telencephalon were analyzed since we considered that there were not differences in the distribution from rostral to caudal locations (see Figure 18).

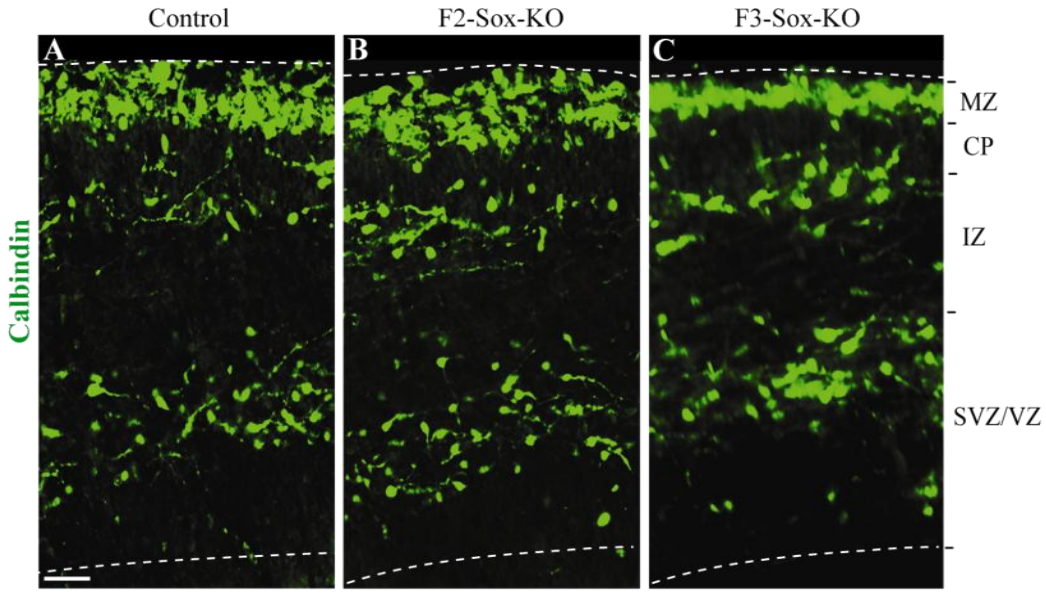

Figure 26. Normal laminar distribution of the tangentially migrating CB$^+$ interneurons in the developing cortex of *FLRT2* and *FLRT3* single KOs. The CB$^+$ interneruons follow three principals routes in the E14.5 dorsal cortex of the *FLRT2* KO (F2-Sox-KO) (B) and in the *FLRT3* KO (F3-Sox-KO) (C) similar to control (A). The dashed lines show the demarcation of the Pial surface (top) and Ventricular surface (bottom). Abbreviations: CB, calbindin; MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone. Scale bar, 50μm.

As expected, and according to previous results with the *Nestin-Cre* line, the distribution of CB$^+$ cells showed no obvious defects in the F3-Sox-KOs respect to control (Figure 26). In addition, the absence of *FLRT2* did not affect either the ditribution pattern of CB$^+$ interneurons in the developing cortex (Figure 26). In order to quantify these images, a similar analysis was performed as previously described in Figure 23 where bin distributions were averaged across sections. This analysis revealed that there are not differences in the total CB$^+$ cells reaching the dorsal cortex and confirmed that there are no differences in the laminar distribution through the different migratory streams respect to controls (Figure 27 for F2-Sox-KO and Figure 28, for F3-Sox-KO). In summary, all these results indicate that the single *FLRT2* or *FLRT3* mutants did not display deficits in the

migration and positioning of interneurons within the cortex, so the loss of one of these proteins did not compromised the migration of cortical interneurons *in vivo*.

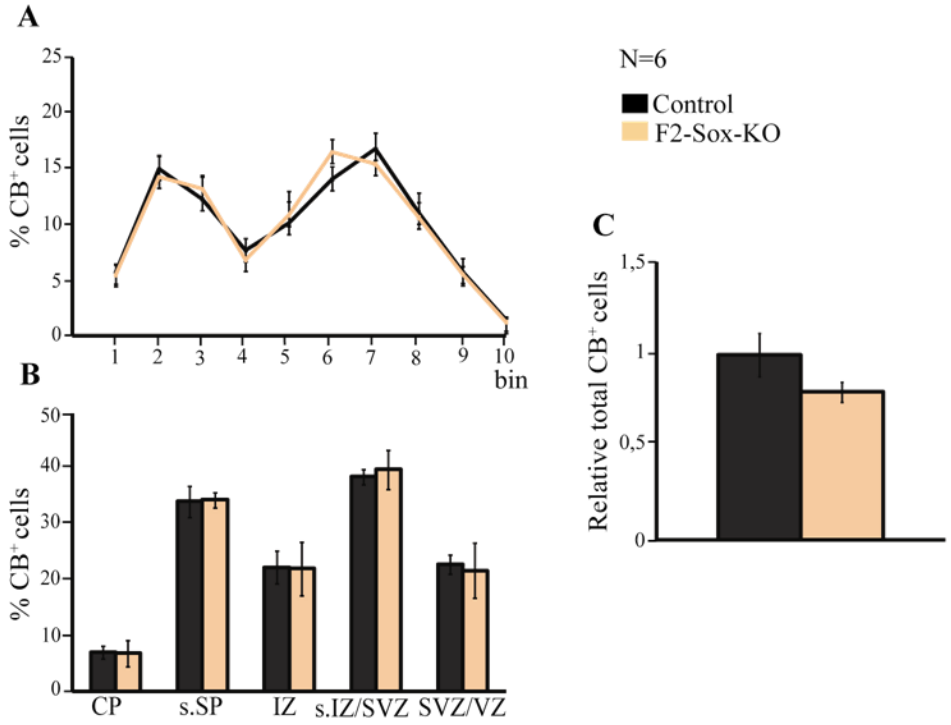

Figure 27. Distribution pattern of tangentially migrating CB$^+$ interneurons in the *FLRT2* KO cerebral cortex at E14.5. Percent distribution of CB$^+$ interneurons in each bin (A) or in each cortical layer (B) across the cortical wall in *FLRT2* KO (F2-Sox-KO, orange labels) and control embryos (black labels) (6 animals, each). (A) Numbers in ordinates identify bins for quantification, from the CP (1) to the VZ (10). (B) Percent distribution of CB$^+$ interneurons for cytologically distinct zones. (C) Relative amount of total CB$^+$ interneurons in the cortex where the total number of CB$^+$ cells in controls was given the value "1" and the value found in the *FLRT2* KO tissue was referred as fold induction respect to control. All data are presented as mean ± s.e.m. (n=6 per genotype, p>0.05, two tailed Student' t test for each pair of data). Abbreviations: CB, calbindin; CP, cortical plate; s.SP, stream subplate; IZ, intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone.

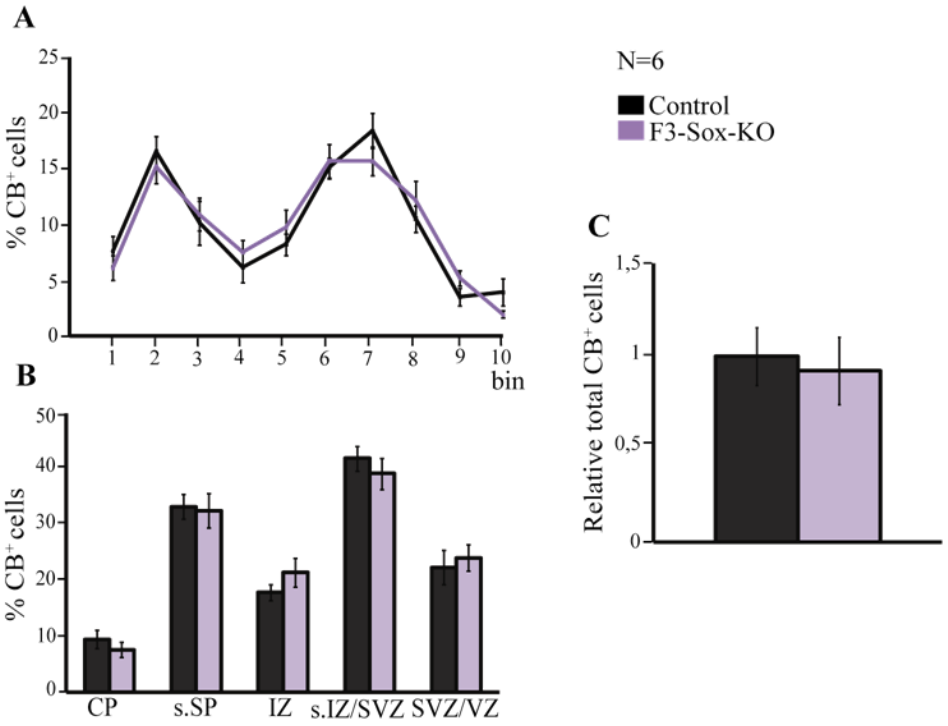

Figure 28. Distribution patterns of tangentially migrating CB$^+$ interneurons in the *FLRT3* KO cerebral cortex at E14.5. Percent distribution of CB$^+$ interneurons in each bin (A) or in each cortical layer (B) across the cortical wall *FLRT3* KO (F3-Sox-KO, purple labels) and control embryos (black labels) (6 animals, each). (A) Numbers in ordinates identify bins for quantification, from the CP (1) to the VZ (10). (B) Percent distribution of CB$^+$ interneurons for cytologically distinct zones. (C) Relative amount of total CB$^+$ interneurons in the cortex where the total number of CB$^+$ cells in controls was given the value "1" and the value found in the *FLRT3* KO tissue was referred as fold iniduction respect to control. All data are presented as mean ± s.e.m. (n=6 per genotype, p>0.05, two tailed Student' t test for each pair of data). Abbreviations: CB, calbindin; CP, cortical plate; s.SP, stream subplate; IZ, intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone.

4.6 Loss of both *FLRT2* and *FLRT3* disrupts specifically the intracortical distribution of interneurons

The previous data shows that the distribution during tangential migration of CB$^+$ cortical interneurons is not affected by the absence of *FLRT2* or *FLRT3* suggesting that either *FLRT2* or *FLRT3* alone not do not affect tangential distribution and progression of cortical interneurons *in vivo*. However, we

observed that both proteins are coexpressed around the SP migratory stream of interneurons, specifically, underneath the SP stream (Figure 20). This observation suggests that both proteins, FLRT2 and FLRT3, could eventually cooperate in the migration of the SP interneurons and that the lack of any of the two genes in the single KO animals could be compesanted by the presence of the other gene. To test this hypothesis, double *FLRT2* and *FLRT3* KO (*FLRT2*$^{-/lx}$*;FLRT3*$^{-/lx}$*;Sox1-Cre*$^{+}$ or "F2F3-Sox-DKO") were generated as previously described (see also Figure 17).

To evaluate the redundant role of these two proteins during tangential migration, brains from F2F3-Sox-DKO deficient embryos were processed and analyzed as previously performed, based on CB staining. In the F2F3-Sox-DKOs, the CB^{+} interneurons invaded the lateral cortical rudiment by tangential migration and as they enter the cortical rudiment they navigate through the three characteristic migratory streams (SP, MZ and lower SVZ/IZ). However, it was very interesting to notice that CB^{+} interneuron distribution seemed to be more disorganized in the mutants; in particular, with a tendency to observe more CB^{+} interneurons in the IZ of the F2F3-Sox-DKO compared with controls. In addition, the lateral to medial progression distance of CB^{+} interneurons through the SP stream was reduced in the F2F3-Sox-DKO cortices (Figure 29).

With the images taken from the CB immunofluorescence of six independent F2F3-Sox-DKOs and sibling controls, at E14.5, a similar quantification was performed as previously described for the single mutants. This analysis first revealed that the total number of CB^{+} cells in the lateral neocortex of the F2F3-Sox-DKO was not different from controls, indicating that interneurons arrive normally from the subpallium to the cortex in the mutant embryos (Figure 30C).

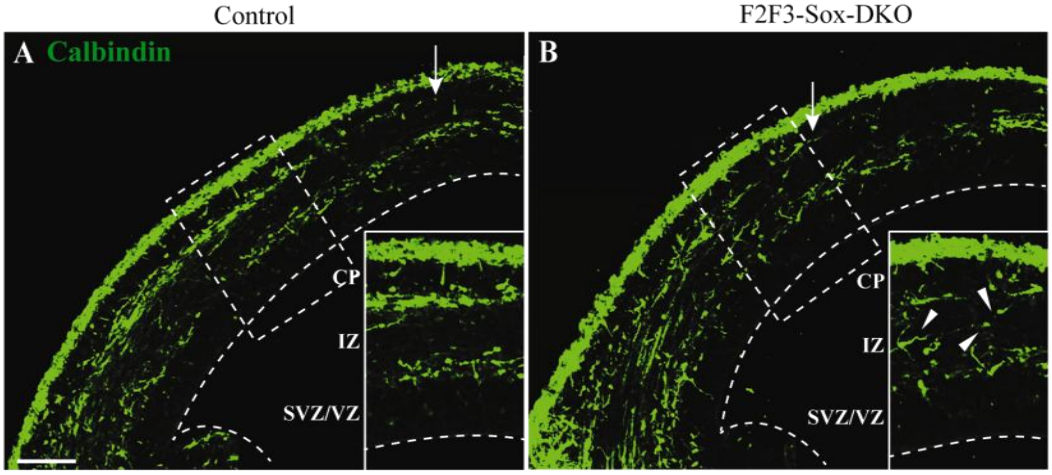

Figure 29. Interneuron distribution phenotypes in the double *FLRT*2 and *FLRT3* KOs. Representative coronal sections at comparable rostro-caudal levels through the telencephalon of E14.5 control (A) and F2F3-Sox-DKO (B) embryos showing immunofluorescence for CB. Insets show a higher magnification image of the boxed areas. Arrowheads in B inset show the ectopic CB^+ neurons located in the IZ of the F2F3-Sox-DKO compared to control. The arrows point to the front of the SP migratory stream which in the case of the F2F3-Sox-DKO brains is shorter than in controls. Abbreviations: CB, calbindin; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone. Scale bar, 100μm.

However, the binning analysis of the CB^+ migrating interneurons within the cortex confirmed our observations and that important difference exist between F2F3-Sox-DKO and controls. Specifically, when compared statistically, distributions of CB^+ migratory interneurons within bins 1-10 were significantly altered between F2F3-Sox-DKO and controls with the largest shifts occurring in bins 2-3 and 4-5 (Figure 30A). These bins correspond to the SP and IZ, respectively, indicating that a significant reduction of the number of CB^+ interneurons in the SP stream exits (Figure 30A, control: 31.4%; F2F3-Sox-DKO: 24.1%, n=6) with a concomitant increase of CB^+ cells in the IZ (Figure 30B, control: 16.49%; F2F3-Sox-DKO: 24.31%, n=6). Thus, complete loss of both *FLRT2* and *FLRT3* leads many migrating interneurons fail to maintain their normal route of migration through the SP stream. Instead, many interneurons were found to be abnormally located within the IZ. From these results, it seems as if

interneurons deviate from their normal route of migration within the SP stream and tend to accumulate within the IZ in the mutant embryos (Figure 30A, B and see discussion). In any case, this abnormal intracortical distribution of CB^+ interneurons in the double *FLRT2* and *FLRT3* KOs highlights therefore the significance of FLRT proteins in the proper migration of interneurons during cortical development.

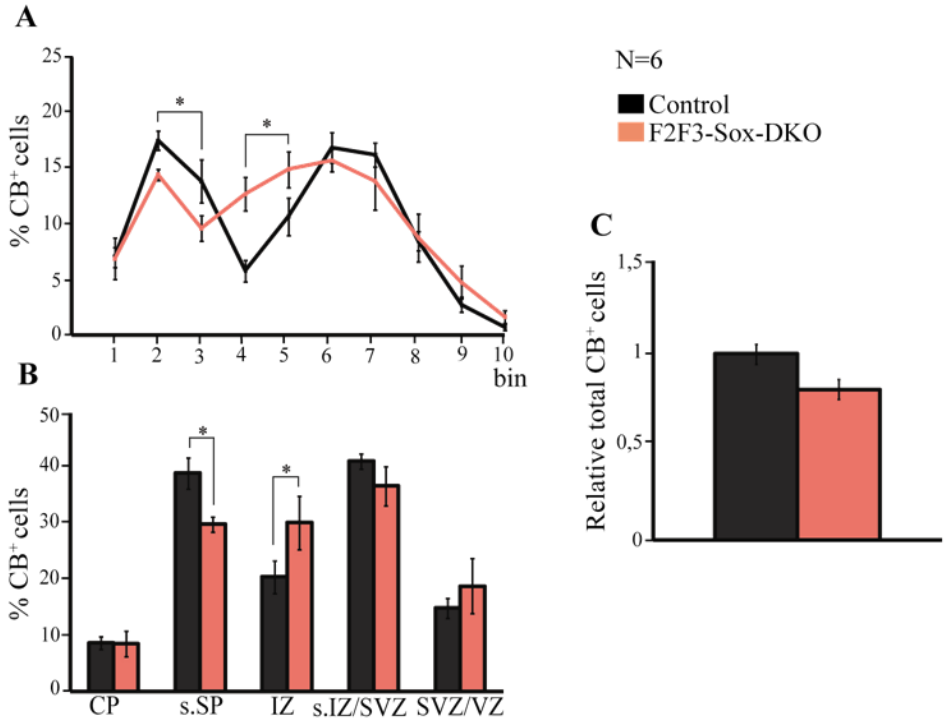

Figure 30. Distribution patterns of tangentially migrating CB^+ interneurons is altered in the cerebral cortex of *FLRT2* and *FLRT3* DKO at E14.5. Percent distribution of CB^+ interneurons in each bin (A) or in each cortical layer (B) across the cortical wall of *FLRT2* and *FLRT3* double KO (F2F3-Sox-DKO, red labels) and control embryos (black labels) (6 animals, each). (A) Numbers in ordinates identify bins for quantification, from the CP (1) to the VZ (10). (B) Percent distribution of CB^+ interneurons for cytologically distinct zones. (C) Relative amount of total CB^+ interneurons in the cortex where the total number of CB^+ cells in controls was given the value "1" and the value found in the F2F3-Sox-DKO tissue was referred as fold induction respect to control. All data are presented as mean ± s.e.m. (n=6 per genotype, *$p<0.05$, two tailed Student' t test for each pair of data). Abbreviations: CB, calbindin; CP, cortical plate; s.SP, stream subplate; IZ, intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone.

Another interesting observation was that the CB^+ interneurons were in general more loosely organized in F2F3-Sox-DKO cortices, leading to frequent gaps between them, mainly within the SP stream. Considering this observation, we aimed to determine the extent of progression of interneuron tangential migration within the streams in the dorsal telencephalon. First, to ensure that potential differences in brain size did not influence interpretation of our results, we measured relative cortical length from every section that was analyzed. This analysis showed no statically significant differences in cortical length between mutants and controls, indicating that the cortex is well shaped and no important structural differences exist within the two group of embryos (Figure 31B, see also Figure 32A, A').

Next, the distance progressed by the SP and IZ/SVZ streams, starting from the pallial-subpallial boundary to the foremost tangentially migrating CB^+ neurons detected, was quantified. Although no differences in cortex size were observed, the stream distance value was expressed relative to the cortical length (measured from the pallial-subpallial boundary to the dorsal-most aspect of the cortex, the cortical hem) (Figure 31A). Interestingly, the distance progressed by CB^+ interneurons within the SP stream in the F2F3-Sox-DKO embryos was significantly smaller to that seen in controls: interneurons in the SP stream migrated 70.26% of the cortical length, whereas in the F2F3-Sox-DKO mutants CB^+ interneurons migrated only 48.07% of the cortical length (n=6) (Figure 31C).

Importantly, this defect was specific for the SP stream interneurons since no significant changes were detected in the advancement of the interneurons in the IZ/SVZ stream in the double mutants compared to controls. At this point we considered the possibility that this phenotype was already present in any of the single *FLRT* mutants and that one or the other molecule would be responsible for this effect. However, and in contrast to F2F3-Sox-DKO embryos, tangential

progression of CB^+ interneurons in the SP stream was completely unperturbed in neither *FLRT2* nor *FLRT3* single mutants (Figure 31C).

To better characterize this effect in migration progression, the cortical rudiment was subdivided into three equidistant areas: the lateral cortocostriatal boundary (area I), a middle position (area II) and the medial cortical arch (area III) (Figure 31D). Then, the percentage of CB^+ interneurons found in each of these cortical areas was determined for each section and averaged across all embryos of the same genotype. Since placement of equidistant areas was done with respect to the length of each cortical hemisphere, our sampling strategy accounts for variations in cortical length that might occur between sections. As shown in Figure 31E, there was a shift in the proportion of CB^+ interneurons in the mutant embryos where it was observed an increase in the lateral parts (areas I and II) with a concomitant decline in medial parts (area III). These differences were statistically significant in area III (Figure 31E, control: 33.89%; F2F3-Sox-DKO: 23.48%, n=6).

Further, the area III was subdivided in three internal bins corresponding morphologically with the SP stream, IZ/SVZ stream and SVZ/VZ part, to analyze if the reduction observed in this area affects specifically any of these layers. As seen in Figure 31F, the total number of the most advanced CB^+ interneurons, corresponding to area III, was significantly and specifically reduced in the SP stream (Figure 31F, control: 9.89; F2F3-Sox-DKO: 4.39, n=6).

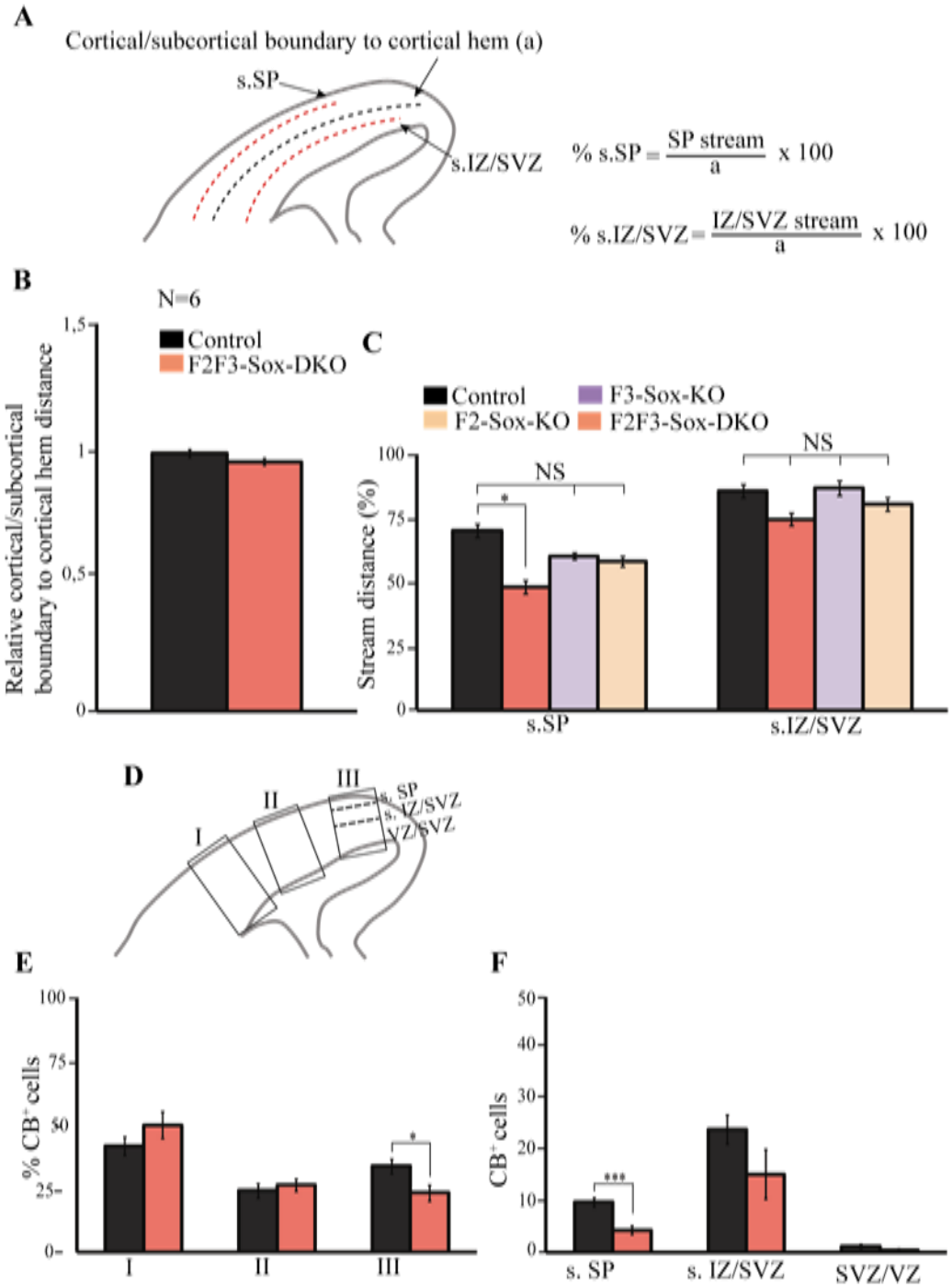

Figure 31. Reduced CB$^+$ interneuron tangential migration in *FLRT2* and *FLRT3* DKO. (A) The extent of interneuron tangential progression in the F2F3-Sox-DKO and control littermates at E14.5 in the dorsal cortex was measured as indictated where tangential interneuron progression of the SP and IZ/SVZ interneuron streams within the cortex was measured and quantified as a

relative distance respect to the cortical/subcortical boundary to cortical hem distance (total cortical lenght, value "a"). Values were expressed as percentage of the total distance. (B) Relative total cortical lenght (value "a" in A panel) comparing controls (black bar) and F2F3-Sox-DKOs (red bar) where the total lenght in controls was given the value "1" and the value found in the *FLRT2* and *FLRT3* DKO tissue was referred as fold induction respect to control. (C) Relative stream distance (SP, left bars; IZ/SVZ, righ bars) as measured in A showing a significant reduction of the tangential migration distance through the s.SP in the F2F3-Sox-DKO (red bar) respect to the controls (black bar). No statistical differences (NS) in the progression of this SP stream were found in the single *FLRT2* or *FLRT3* mutants (orange and purple bars, respectively). NS where found in the progression of the IZ/SVZ stream of any of the genotypes analyzed. (D) Tangential interneuron migration was also measured in three equidistant areas from the lateral to the medial part of the cortex between the cortical/subcortical boundary (area I) to the cortical hem (area III), as indicated. The area III was further subdivided in three bins: SP stream, IZ/SVZ stream and SVZ/VZ to analyze intra-area distribution differences. (E) Percentage of CB^+ interneurons in the three areas depicted in D (ordinate axis) in controls (black bars) and mutants (red bars). (F) Total CB^+ interneurons in controls (black bars) and mutants (red bars) in the three bins in which area III was subdivided as shown in panel D. All data are presented as mean ± s.e.m. (n=6 per genotype, *$p<0.05$, ***$p<0.001$, two tailed Student' t test for each pair of data). Abbreviations: CB, calbindin; CP, cortical plate; s.SP, stream subplate; IZ, intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone.

Taken together, these data demonstrate that F2F3-Sox-DKO interneurons developed abnormal and perturbed distribution of CB^+ interneurons, especially in those located in the SP stream. The results suggest that these SP interneurons are not longer restricted to their stream and since total numbers are not different, it seems as if they get redistributed into other cortical compartments with an apparent shift of CB^+ cells toward the IZ at the expense of the integrity of the SP stream. This redistribution may account for the reduced advancement observed of the SP stream (both, in distance and number of cells) during tangential progression. Thus, FLRT2 and FLRT3 cooperate in maintaining interneuron streams and therefore are important regulators of tangential interneuron migration during cortex development.

4.7 Normal cortical structure and development of the F2F3-Sox-DKO

The above data indicate that interneuron distribution is altered in the F2F3-Sox-DKO cortex. In order to assess if the observed phenotypes were specific or were due to a general defect in cortex development we performed the following analysis. First, in the inspection of the gross brain morphology at E14.5, differences in the appearance and size of the cerebral hemispheres of the F2F3-Sox-DKO respect to controls were not detected suggesting that cortex develops normally in the mutants (Figure 32A, A'; see also Figure 31). At this point, the effect of the lack of *FLRT2* and *FLRT3* in the normal corticogenesis was addressed more specifically by the expression of well acknowledged cortical layers markers. For that, coronal sections from mutant and control embryos were immunostained for a panel of layer-specific markers: Tbr1, Satb2, and Ctip2; to label developing cortical post-mitotic projection neurons.

At E14.5, higher Tbr1 expression was observed in the CP and SP. Cells in the IZ expressed lower levels of Tbr1 protein, as has been shown previously (Hevner et al., 2001). Ctip2 is highly expressed by cells in the CP at E14.5, but not by cells in the VZ or SVZ (Arlotta et al., 2005). Satb2 cells are present in the upper part of IZ and CP (Britanova et al., 2006) (Figure 32C-E). All these markers, showed a normal expression pattern in the *FLRT2* and *FLRT3* DKO deficient cortex (Figures 32C'-E') indicating that corticogenesis and lamination was not defective and that differentiation of pyramidal cells occurs normally in these mutant embryos. Quantification of these images was performed taking in consideration the pixel intensity (Figures 33G) and the thickness of each stained region (Figure 33D-F) and no significant differences were obtained between controls and DKOs brains. Accordingly, the double mutants did not exhibit significant reductions in the layers thickness or in the cortical width (Figure 33A, B).

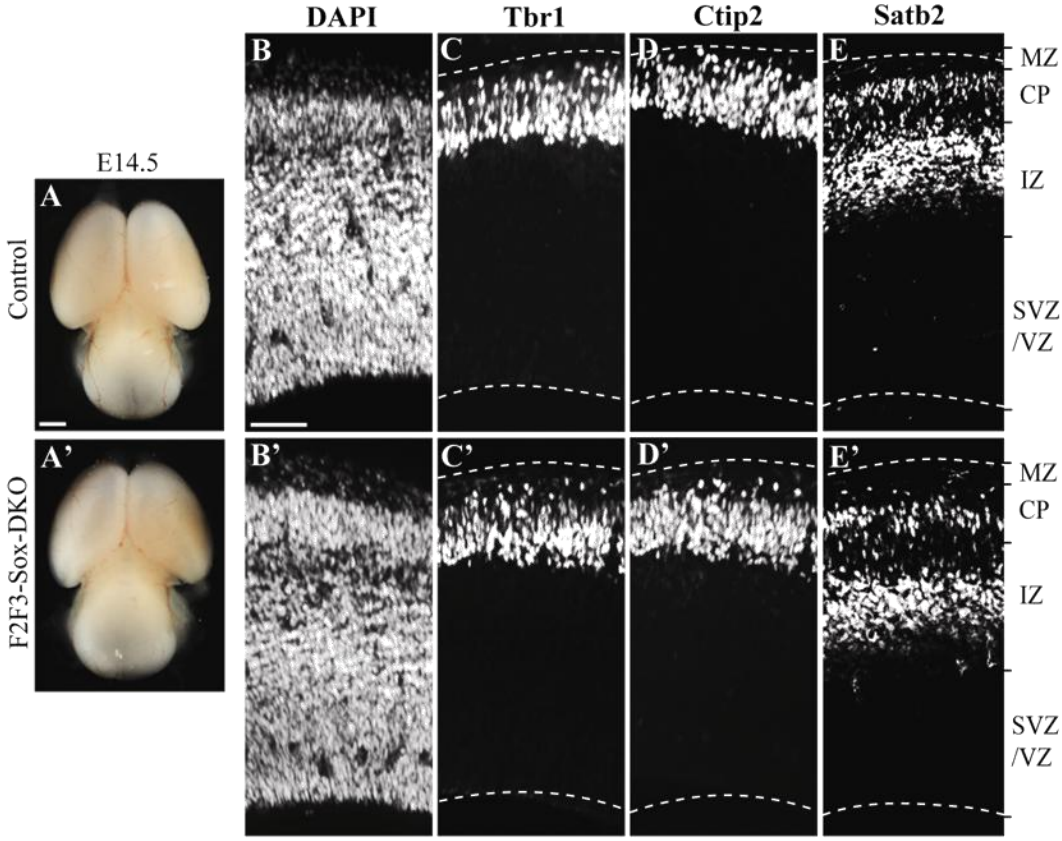

Figure 32. Normal brain structure and cortical lamination in the FLRT2 and FLRT3 DKO at E14.5. (A, A') Dorsal views of freshly dissected E14.5 brains showing normal diencephalon appearance of F2F3-Sox-DKO (bottom) compared with control (top). (B, B') DAPI staining (nuclear staining) of representative coronal sections through the cortex showing normal cortical layer organization in F2F3-Sox-DKOs (B') compared to control (B). (C, C', D, D', E, E'). Representative images of immunofluorescence stainings against the indicated markers (Tbr1, Ctip2 and Satb2) on coronal sections through the cortex of F2F3-Sox-DKOs (C'-E') and control mice (C-E) showing the preservation of cortical layer organization in the double mutant. The dashed lines show the demarcation of the Pial surface (top) and Ventricular surface (bottom). Abbreviations: MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone. Scale bar, 0.5mm (A, A') and 50μm (B-E').

All these results suggest that the laminar allocation and the differentiation of projection neurons is not affected by the absence of *FLRT2* and *FLRT3* at this stage and therefore rule out the possibility that abnormal distribution of CB^+ interneurons in the F2F3-Sox-DKO is an indirect effect, as a consequence of the misslocation of projection neurons.

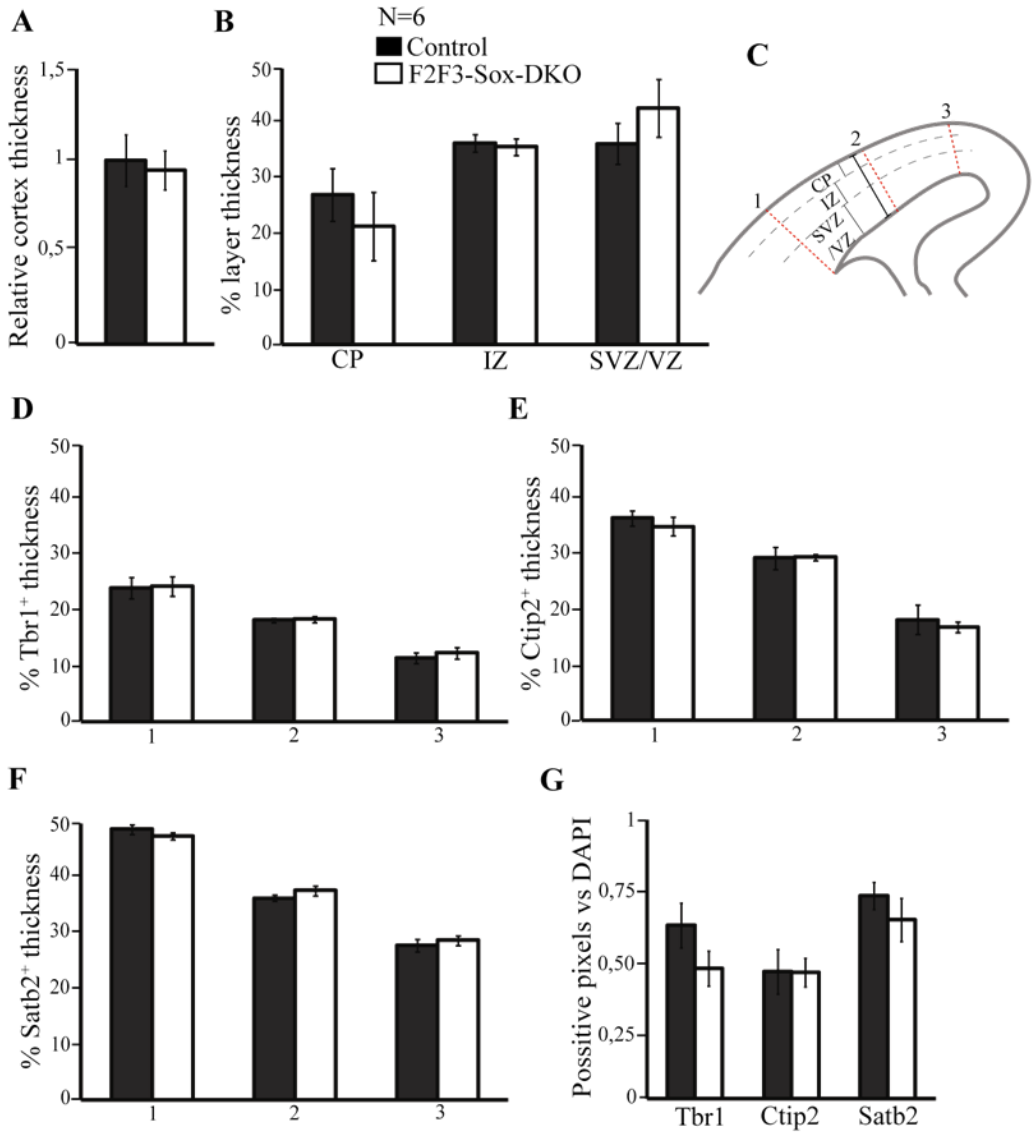

Figure 33. Quantification analysis of the layer-specific markers in F2F3-Sox-DKO cerebral cortex at E14.5. (A, B, C) Relative total cortical thickness (A) or relative thickness of each specific layer (B), based on DAPI staining and measured in the middle position through the mediolateral axis (position 2 in panel C). Control, black labels; DKO, withe bars. Values in A are expressed as fold induction respect to control, where cortical thickness took vaue "1". Values in B are expressed as percentage respect to the total thickness of the cortex. (D, E, F) Percentage of the thickness of $Tbr1^+$ (D), $Ctip2^+$ (E) and $Satb2^+$ (F) layers respect to total cortical thickness in the three positions (1 to 3) along the mediolateral axis depicted in C. (G) Quantification of $Tbr1^+$, $Ctip2^+$ and $Satb2^+$ staining (positive pixels) in the whole cortical wall respect to DAPI intensity. All data are presented as mean ± s.e.m. (n=6 per genotype, *$p<0.05$, two tailed Student' t test for each pair of data). Abbreviations: CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone.

4.8 Analysis of interneuron migration in the F2F3-Sox-DKO at E16.5

Next, we explore further the relevance of FLRT2 and FLRT3 in CB^+ interneuron migration in the cortex at later stages of development. For this purpose, mutant embryos were collected at E16.5. The CB interneuron labeling at this stage revealed that still some positive cells can be detected. However, the stream pattern is different from the one observed at E14.5. Firstly, the three migratory streams are not as well defined probably because at this moment, many interneurons have started the radial migration towards the CP. At this stage, the tangential migration of CB^+ cells is happening mainly through the MZ and lower SVZ (Figure 34A). However it cannot be discarded the presence of the SP stream of migrating interneurons, because in these pictures the prominent label of thalamo-cortical and corticofugal projections, may mask the SP CB^+ cells. In contrast to E14.5, in the case of the double *FLRT2* and *FLRT3* KO brains, the overall density of CB-immunoreactive interneurons seemed to be less compared to controls, although the differences are not significatives (Figure 34 and see also Figure 35A).

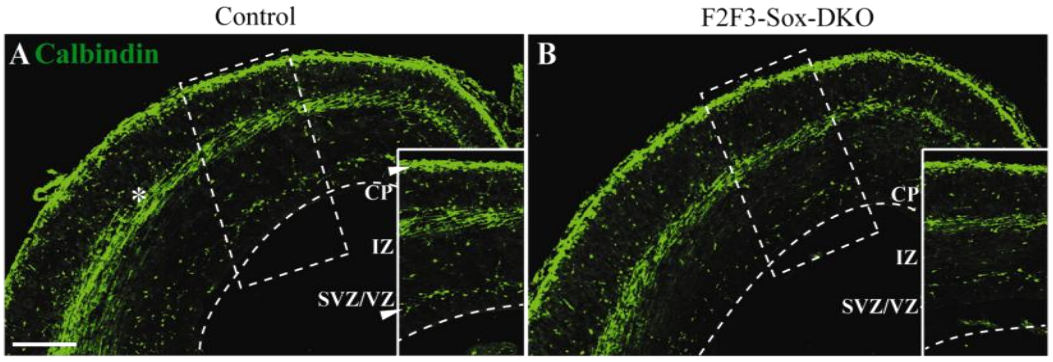

Figure 34. CB staining in E16.5 brains in control and F2F3-Sox-DKO. Representative images of coronal sections through the cortex of controls (A) and F2F3-Sox-DKOs (B) at E16.5 stained with CB. Comparable rostro-caudal levels are shown. Insets show a high magnification of the boxed area in each panel. In inset of panel A, arrowhead point to the interneuron streams still identifiable at this statge, through the MZ and the lower IZ, respectively. Asterisk in panel A marks an unspecific staining of the CB antibody which probably are thalamo-cortical and corticofugal projections. Abbreviations: CB, calbindin; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone. Scale bar, 200μm.

The quantification of these types of images revealed that, although the DKO mutants displayed a decrease in the total CB^+ cells in the DKO relative to controls mice, the difference was not statistically significant (Figure 35A). A similar distribution analysis was performed at this stage as it was done at E14.5 but due to the reduced amount of CB^+ cells, in this case, only two regions spanning the width of the cortex were considered: the CP region and the rest, the IZ/SVZ/VZ region (Figure 35C). The early cortical GABAergic neurons (E13.5) only invade the CP after E15.5, and also at E16.5, and therefore the number of CB^+ interneurons present in the CP substantially increased at this stage, compared with those found at E14.5 (López-Bendito et al., 2008). The results obtained in this analysis, indicate that, compared to controls, the F2F3-Sox-DKOs exhibit a small but significant reduction in the number of CB^+ interneurons within the CP (Figure 35B, control: 64.89%; F2F3-Sox-DKO: 59.71%, *n*=6). It seems as if this difference is due to an accumulation of CB^+ interneurons in the mutants, in the region comprising the IZ, SVZ and VZ since an increase of the number of these cells, although not significant, is observed in the mutants (Figure 35B).

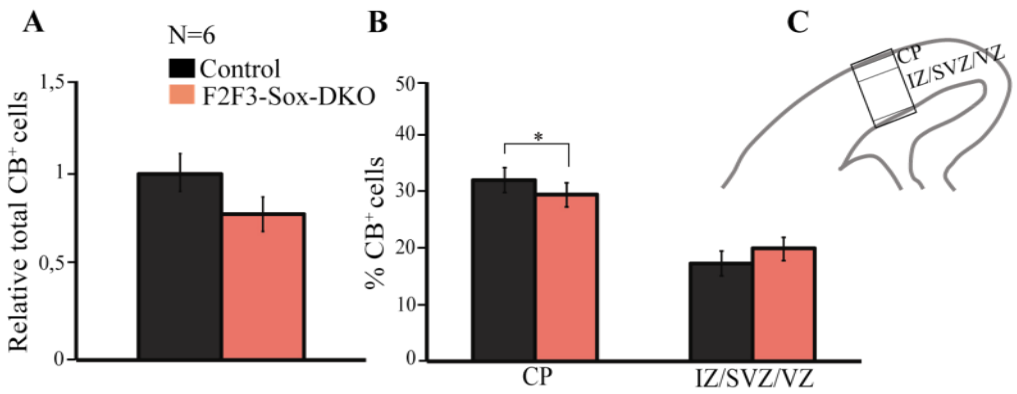

Figure 35. Distribution of cortical CB^+ interneurons is affected in the F2F3-Sox-DKOs at E16.5. (A) Relative total number of CB^+ interneurons where in controls (black bar), the mean value was equal to "1" and the value for the mutants (red bar) was expressed as fold induction. (B) Distribution of CB^+ interneurons for the two cytologically distinct zones depicted in panel C (CP and the rest, IZ/SVZ/VZ), for F2F3-Sox-DKOs (red bars) and control embryos (black bars). Values are expressed as percentage respect to the total number of CB^+ in the boxed area. All data are presented as mean ± s.e.m. (n=6 per genotype, *p<0.05, two tailed Student' t test for each pair

of data). Abbreviations: CB, calbindin; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

In summary, the analysis of CB^+ interneuron distribution at these two different developmental stages reveals important differences in their intracortical distribution when the expression of FLRT2 and FLRT3 are deleted. At E14.5, it was observed a specific effect on the distribution of SP stream interneurons at the same time that these interneurons progressed, tangentially, a shorter distance within the cortex. At E16.5, instead, it was observed a significant reduction of CB^+ internerons within the CP which may suggest a defect in radial invasion of this region in the mutants. It is possible that these observations reflect independent functions of FLRT proteins but it is tempting to speculate that, indeed, the reduction of CB^+ interneurons in the SP stream at E14.5, may affect the switching from tangential-to-radial oriented migration towards the CP at E16.5, leading to the perturbation of laminar interneuron organization in the F2F3-Sox-DKOs.

4.9 FLRT2 and FLRT3 are required for proper layering of projection neurons at late stages of brain development

Similarly to E14.5 stage, the F2F3-Sox-DKO brains were analyzed for morphological parameters and layer specific markers in the cerebral cortex later in development. The relative cortical length (measured as indicated in Figure 36A), cortical thickness, as well as the thickness of each histological layer based on DAPI staining, were measured and no significant differences were noticed between F2F3-Sox-DKOs and control groups (Figure 36B, C, D). According to these results, the gross morphology of the cortex of these mutant mice is normal at these late developmental stages.

Next, sections were stained for Tbr1, Tbr2, Ctip2 and Satb2 layer-specific markers to perform a more accurate analysis of cerebral cortex formation in the mutants, since these four markers are related with neurogenesis and neuronal differentiation. Particularly, Tbr1 labels layer 5/6 (Hevner et al., 2006), Tbr2 staining is localized in the SVZ (Arnold et al., 2008), Ctip2 is expressed in the layer 5 (Arlotta et al., 2005), and Satb2 is expressed in the upper layers of the cerebral cortex (Britanova et al., 2008) (Figure 37). The immunofluorescence staining shows a similar distribution of the $Tbr1^+$ and $Ctip2^+$ cells in the F2F3-Sox-DKO compared to controls (Figure 37B, B', D, D'). However, the $Tbr2^+$ area seem to be expanded in the F2F3-Sox-DKOs suggesting that some $Tbr2^+$ cells are located ectopically above the SVZ in the mutants. On the other hand, the $Satb2^+$ area is also expanded with many $Satb2^+$ cells located at deeper layers in the F2F3-Sox-DKOs (Figure 37C, C', E, E').

The relative thickness and pixel intensity of $Tbr1^+$, $Tbr2^+$, $Ctip2^+$ and $Satb2^+$ region was measured in regard to the entire cortical thickness (in three cortical areas) or the the DAPI intensity, respectively (Figure 38). No statistical differences were found in the case of $Tbr1^+$ and $Ctip2^+$ stained regions (Figure 38 C, E); however, for $Tbr2^+$ and $Satb2^+$ layers, the area of positive staining was significantly wider in the F2F3-Sox-DKOs, compared to controls (Figure 38D, Tbr2; control: 37.75%, F2F3-Sox-DKO: 42.29%, n=6) (Figure 38F, Satb2; control: 38.10%, F2F3-Sox-DKO: 44.82%, n=6). Interestingly, and in contrast to the distribution area, the quantification of fluorescence (pixels intensity) revealed no significant differences between control and F2F3-Sox-DKO in any case (Figure 38A). These results suggest that there is not a change in cell fate of the Tbr2 and Satb2 population of neurons in the F2F3-Sox-DKOs but rather a migration defect of these neurons.

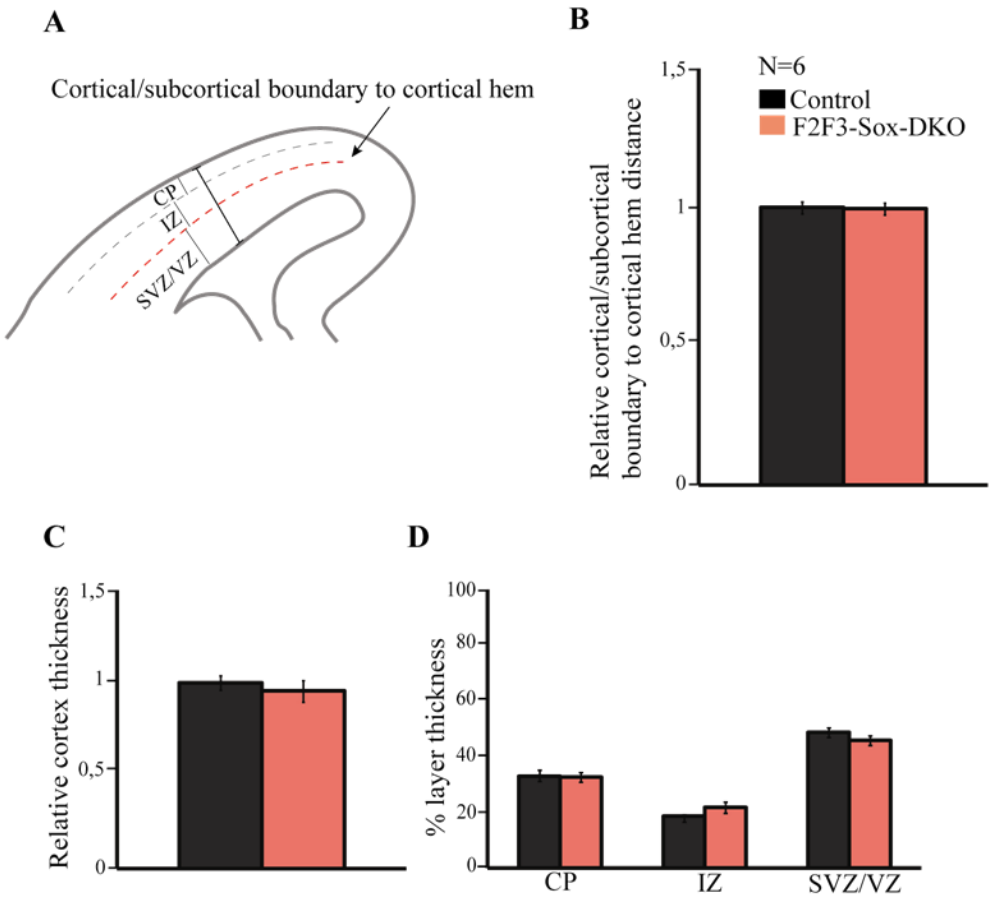

Figure 36. Morphological analysis of the F2F3-Sox-DKO cerebral cortex at E16.5. (A,B) The distance between the cortical/subcortical boundary to the cortical hem (red dotted line in A) was measured in six different animals for each group, F2F3-Sox-DKO (red bar) and controls (black bar). The values were expressed relative to controls as fold induction where controls took the value "1". (C) Cortical thickness was measured as in A, taking in consideration the distance between the pial and ventricular surfaces in a middle position through the mediolateral axis of the cortex as shown in B. (D) Specific layer thickness (CP, IZ and SVZ/VZ based on DAPI staining) was measured in a middle position through the mediolateral axis of the cortex as shown in B and values were given in percentage respect the total cortical thickness. All data are presented as mean ± s.e.m. (n=6 per genotype, $p>0.05$, two tailed Student' t test for each pair of data). Abbreviations: CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone.

Previously, was proposed that FLRT2 and FLRT3 functions in the E14.5 dorsal cortex regulate interneuron migration without having a detectable effect on the position of cortical projection neurons, however, at E16.5 it was found a

premature migration of the $Tbr2^+$ cells and a delay in the $Satb2^+$ cells failed to migrate properly towards upper layers. Particularly, in two of the four examined mice was observed that effect which means a penetrance percentage of 50%. Although this was only a first approach, this observation suggests that it could be a problem in the cerebral cortex upper layers expressing Satb2. Furthermore, the conserved distribution of the other differentiation markers, Tbr1 and Ctip2, point out a specific effect on the upper layers.

4.10 Migrating cortical interneurons express low levels of FLRT2 and FLRT3

From the previous experiments, it seems clear that FLRT2 and FLRT3 are not required for interneuron migration intrinsically and that the phenotypes we observed in the double *FLRT2* and *FLRT3* KO is due to the action of FLRTs as ligands to some receptor expressed in interneurons. Nevertheless, we wanted to study the expression of FLRT2 and FLRT3 in the migrating interneurons of the SP stream, the ones that are significantly more affected in the mutants. For this, high-magnification immunofluorescence images double stained with FLRT2 or FLRT3 antibodies (those that recognize the ECD) and CB were analyzed.

Considering the immunofluorescence results, the expression of FLRT2 and FLRT3 by the vast majority of tangentially oriented CB^+ interneurons in the cortical wall was relatively small (although detectable) compared with their expression in the projection neurons and in the extracellular compartment (Figure 39).

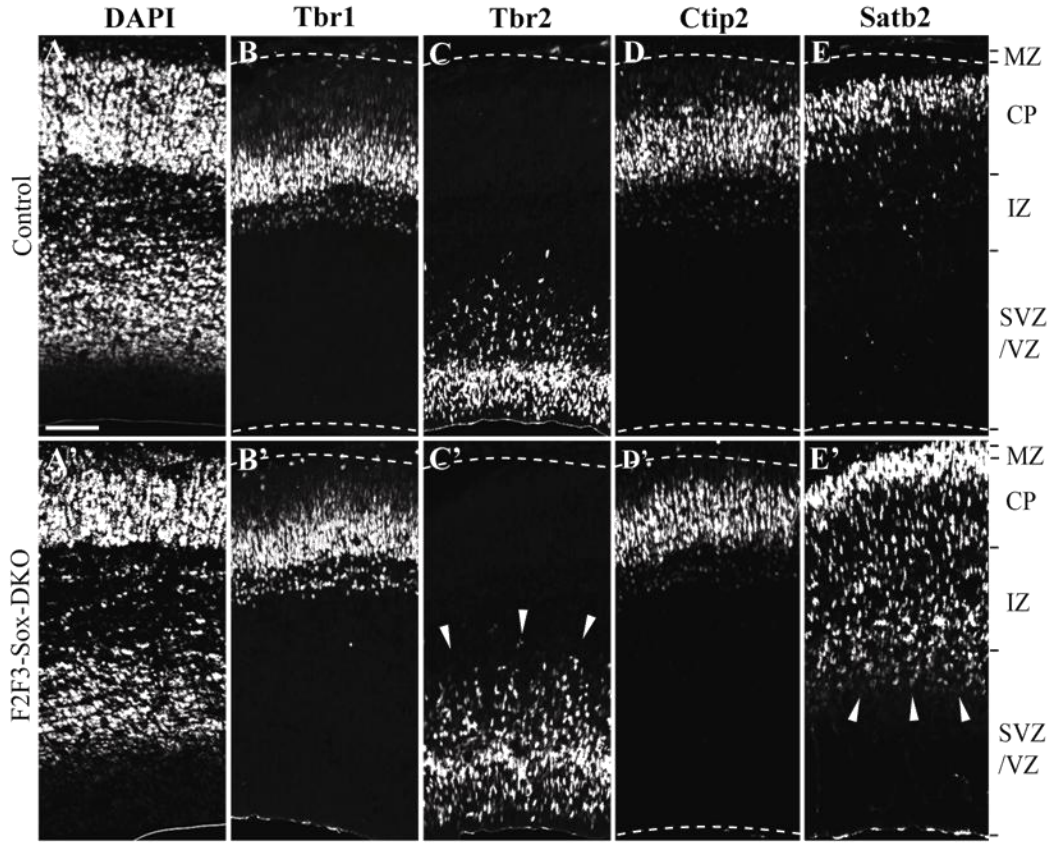

Figure 37. Marker analysis of cortical lamination of the F2F3-Sox-DKO at E16.5. Representative images taken from coronal sections of the developing brain of F2F3-Sox-DKOs (bottom panels) and control littermates (upper panels). (A, A') DAPI staining. (C, C', D, D', E, E') Immunofluorescence against Tbr1 (C, C'), Ctip2 (D, D') and Satb2 (E, E'). Arrowheads in C' point to ectopic postive cells, outside of the SVZ. Arrowheads in E' point to ectopic positive cells in deep layers of the developing cortex. The dashed lines show the demarcation of the Pial surface (top) and Ventricular surface (bottom) Abbreviations: MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone. Scale bar, 50µm.

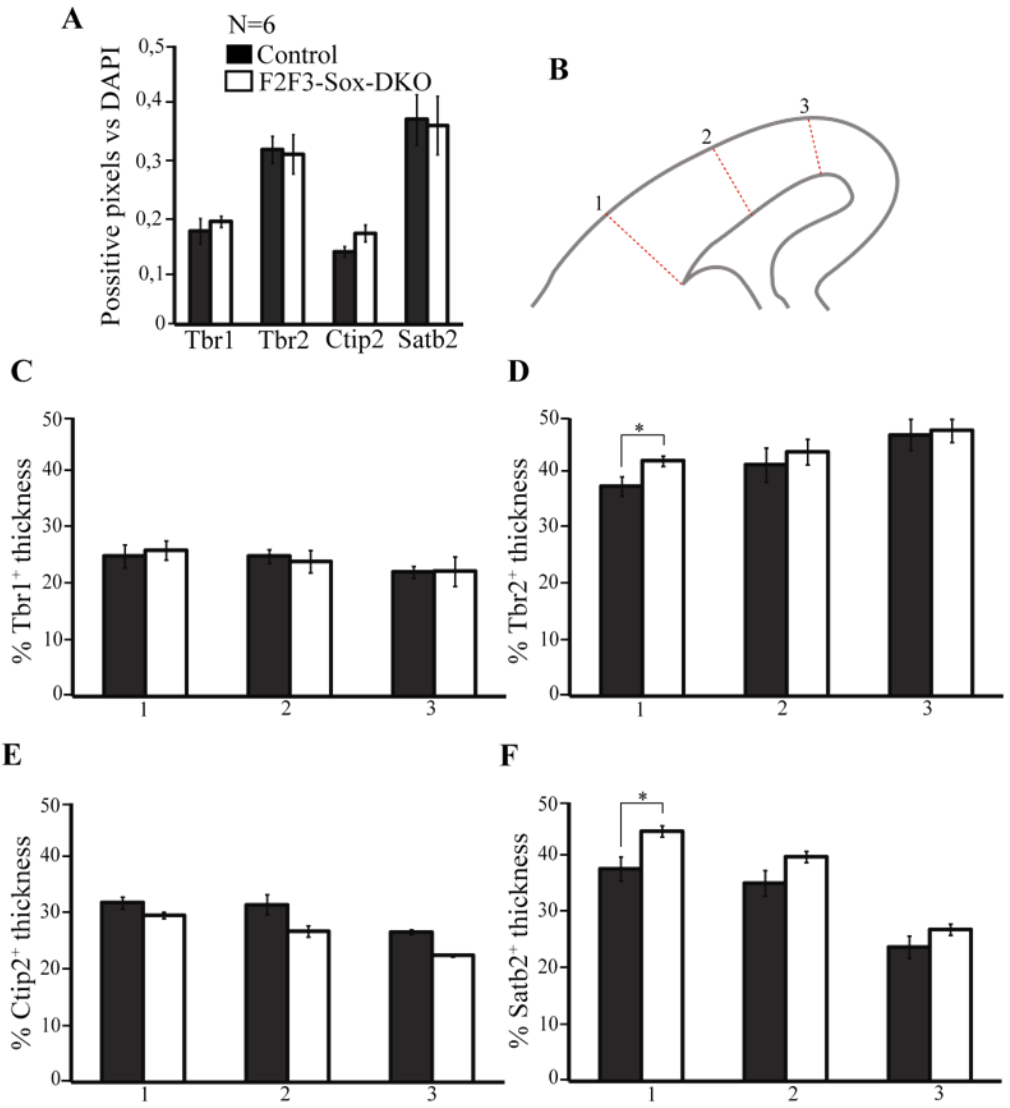

Figure 38. Quantification of the staining of the layer-specific markers in F2F3-Sox-DKO cerebral cortex at E16.5. (A) Relative pixel intensity of the indicated stainings related to DAPI, in controls (black bars) and mutants (white bars). (B) Scheme of the position of the sections analyzed in panels C-F. (C-F) Relative thickness of the staining of Tbr1 (C), Tbr2 (D), Ctip2 (E) and Satb2 (F) along the mediolateral axis, at the indicated positions (1-3) in panel B. All data are presented as mean ± s.e.m. (n=6 per genotype, *$p<0.05$, two tailed Student' t test for each pair of data). Abbreviations: CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone.

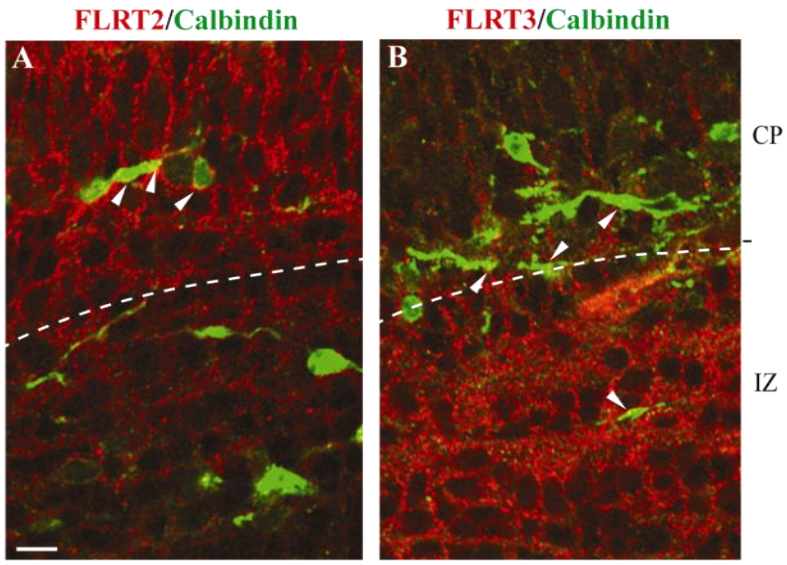

Figure 39. FLRT2 and FLRT3 expression in CB$^+$ interneurons of the SP stream. (A,B) Representative high magnification images of the SP stream in the cortex at E14.5 (coronal sections) stained with CB (green) and FLRT2 (red in A) or FLRT3 (red in B). Arrowheads show the overlapped staining between the FLRTs and CB. Dashed line indicates the boundary between the CP and the IZ. Abbreviations: CB, calbindin; CP, cortical plate; IZ, intermediate zone. Scale bar: 10 μm.

4.11 Abnormal interneuron laminar distribution in F2F3 DKO using *Emx1-Cre* line

The previous analysis revealed that FLRT2 and FLRT3 function are required for the migration of cortical interneurons. Since we used Cre lines that recombine in the whole nervous system, the abnormal distribution of migrating interneurons that was observed could be caused by the loss of FLRT2 or FLRT3 in the cortical rudiment (non-cell autonomous function), in migrating interneurons (cell-autonomous function), or in both.

In order to distinguish among these possibilities, the migratory behavior of CB^+ interneruons was analyzed in animals where FLRT2 and FLRT3 were deleted specifically from cortical pyramidal neurons. This specific deletion was achieved by using a well-established Cre line, *Emx1-Cre* line (Guo et al., 2000). Emx1 is a transcription factor which expression starts around E9.5 and is exclusively confined to the pallial progenitor populations in the developing and adult cerebral cortex and hippocampus (Guo et al., 2000; Simeone et al., 1992). Although the Nestin-Cre-mediated recombination commences as early as E10.5, complete recombination is achieve only perinatally; in contrast to Emx1-Cre where about 90% of recombination is achieved by as early as E12.5 (Liang et al., 2012).

As it was done for *Sox1-Cre* and *Nestin-Cre* lines, before beginning the analysis, it was ensured the efficiency of the *FLRT2* and *FLRT3* genes deletion in the F2F3-Emx-DKO with *Emx1-Cre* line. As shown in Figure 40, at E14.5, FLRT2 and FLRT3 staining with specific antibodies demonstrated, indeed, that both genes are effectively deleted specifically in the cerebral cortex, whereas other areas, such as the subpallial telencephalon and thalamus remain stained with the expected pattern (Figure 40). As expected, deletion of *FLRT2* and *FLRT3* did not affect the gross morphology of the cortex and the relative cortical/subcortical boundary to cortical hem distance was similar in the mutants compared to controls (Figure 43A).

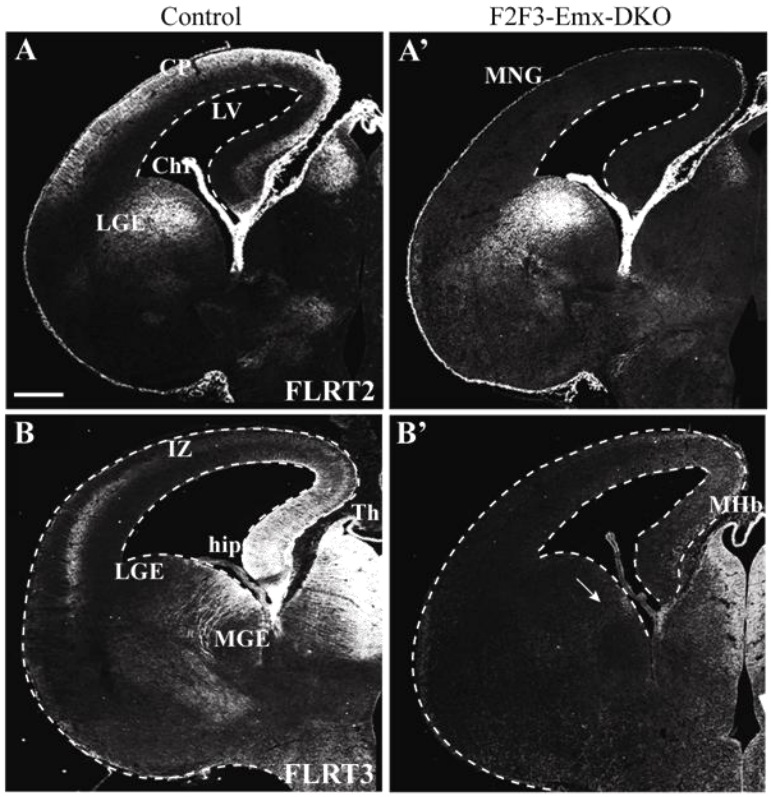

Figure 40. Efficient recombination in the developing cortex of *FLRT2* and *FLRT3* driven by *Emx1-Cre* line. Overview representative pictures at E14.5 of coronal sections of the developing telencephalon. (A, B) FLRT2 (A) and FLRT3 (B) immunoreactivity in control brains show the expected pattern of expression as previously observed in Figure 3. (A', B') FLRT2 (A') and FLRT3 (B') immunoreactivity in F2F3-Emx-DKO. Expression is absent in the cortical region but remains in extracortical regions suchs as the basal telencephalon and thalamus. The arrow indicates the expression of FLRT3 in the LGE-MGE boundary is still present in the F2F3-Emx-DKO. Abbreviations: MNG, meninges; CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; ChP, choroid plexus; hip, hippocampus; Th, thalamus; MHb, medial habenula; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Scale bar, 250μm.

Next, it was asked how the conditional and specific deletion of *FLRT2* and *FLRT3* exclusively in the cortex, but not in the migrating interneurons, affects the migration/distribution of CB^+ neurons. For this, a similar analysis as the one previously performed for the F2F3-Sox-DKO embryos (Figures 29 and 30), was performed. Interestingly, the results showed that in these mutants CB^+ interneurons were found to distribute abnormally within the cortex of the F2F3-

Emx-DKO mutants embryos in a patteern that resemble very closely that observed in F2F3-Sox-DKO mutant embryos (compare Figure 41 and Figure 29). As in the case of F2F3-Sox-DKOs, the total number of CB^+ cells that reached the dorsal cortex was not significantly different between control and conditional F2F3-Emx-DKOs (Figure 42C). However, the CB^+ cortical interneurons were significantly disorganized compared to control littermates, as they were no strictly confined to the migratory streams, especially in the SP stream, and substantially more interneurons were found into the IZ (Figure 41 and Figure 42A, B). The bin distribution analysis of CB^+ interneurons showed statistically significant differences between F2F3-Emx-DKO and controls with the largest shifts occurring in bins 2-3 and 4-5 corresponding to the SP and IZ regions respectively (Figure 42A, B). The proportions of CB^+ interneuron located in the SP stream (bins 2-3) were diminished in the F2F3-Emx-DKO compared with control cortices (Figure 42B, control: 33.45%; F2F3-Emx-DKO: 24.72%, *n*=4). Moreover, the percentage of CB^+ interneurons in the IZ (bins 4-5) was elevated in conditional double mutants compared with controls (Figure 42C, control: 11.60%; F2F3-Emx-DKO: 18.77%, *n*=4).

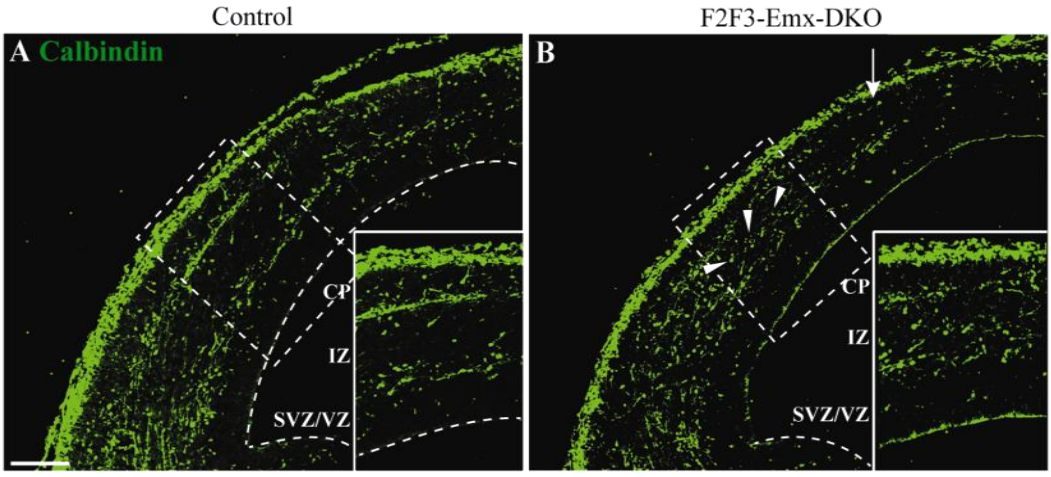

Figure 41. Abnormal interneuron distribution in double *FLRT2* and *FLRT3* KOs using *Emx1-Cre* line. Representative images of coronal sections through the telencephalon of E14.5 of

control (A) and F2F3-Emx-DKO brains (B) showing immunofluorescence for CB in the cortex. Comparable rostro-caudal levels are shown. Insets show a magnification of the boxed area in each image. The arrowheads in B point to ectopically localized interneurons in the IZ region of the F2F3-Emx-DKO brains. The arrows show reduced extent of the migration front through the SP stream in the F2F3-Emx-DKO. Abbreviations: CB, calbindin; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone.Scale bar, 100μm.

In addition to perturbed laminar distribution, the tangential progression of CB^+ interneurons through the SP stream was also altered, as they accumulated laterally and diminished medially within the F2F3-Emx-DKO cortices (Figure 43B). In brains of control groups, interneurons in the SP stream migrated (69.43%; *n*=4) of the cortical length, whereas in the F2F3-Emx-DKO mutants CB^+ interneurons migrated only (55.26%; *n*=4) of the cortical length. In contrast, there were not a significant reduction in the advancement of the IZ/SVZ stream in these double mutant compared to control.

Collectively, these results are reminiscent to those found in the F2F3-Sox-DKO embryos, strongly suggesting that the dispersion of cortical interneurons during tangential migration is regulated by extracellular FLRT proteins and rule out the possibility of a cell-intrinsic requirement for both FLRT2 and FLRT3 signaling in interneurons during early development. Therefore, FLRT2 and FLRT3 may not be required by cortical interneurons to sense or respond to environmental cues located in the cerebral cortex, but rather they act as molecular guiding cues for migrating interneurons; in other words, act non-cell autonomously. These results also indicate that FLRT2 and FLRT3 are required when cortical interneurons enter into the cerebral rudiment and not before, while interneurons migrate through the ventral telencephalon.

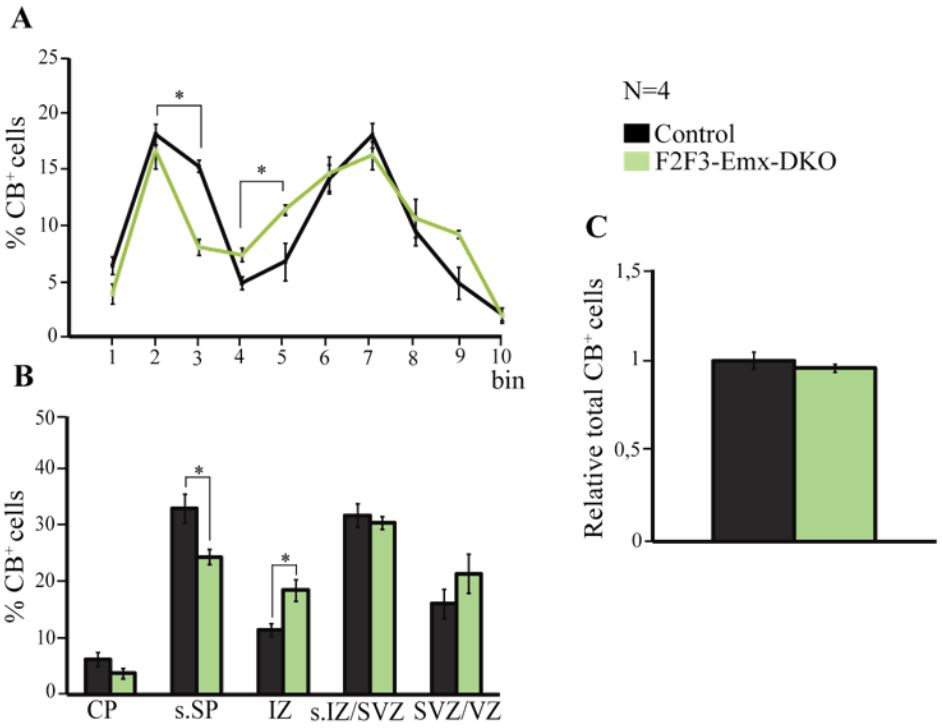

Figure 42. Distribution patterns of tangentially migrating CB$^+$ interneurons is altered in the cerebral cortex of F2F3-Emx-DKO brains at E14.5. Percent distribution of CB$^+$ interneurons in each bin (A) or in each cortical layer (B) across the cortical wall of *FLRT2* and *FLRT3* double KO (F2F3-Emx-DKO, green labels) and control embryos (black labels). (A) Numbers in ordinates identify bins for quantification, from the CP (1) to the VZ (10). (B) Percent distribution of CB$^+$ interneurons for cytologically distinct zones. (C) Relative amount of total CB$^+$ interneurons in the cortex where the total number of CB$^+$ cells in controls was given the value "1" and the value found in the F2F3-Emx-DKO was referred as fold induction respect to control. All data are presented as mean ± s.e.m. (n=4 per genotype, *p<0.05, two tailed Student' t test for each pair of data). Abbreviations: CB, calbindin; CP, cortical plate; s.SP, stream subplate; IZ, intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone.

Thus *FLRT2* and *FLRT3* deletion in the embryonic cortex, disrupts an established molecular mechanism for interneuron migration in which FLRTs act as ligands for a yet unknown interneuron receptor which signaling is necessary for the proper migration and distribution of a subset of interneurons (especially those in the SP stream) during develpoment.

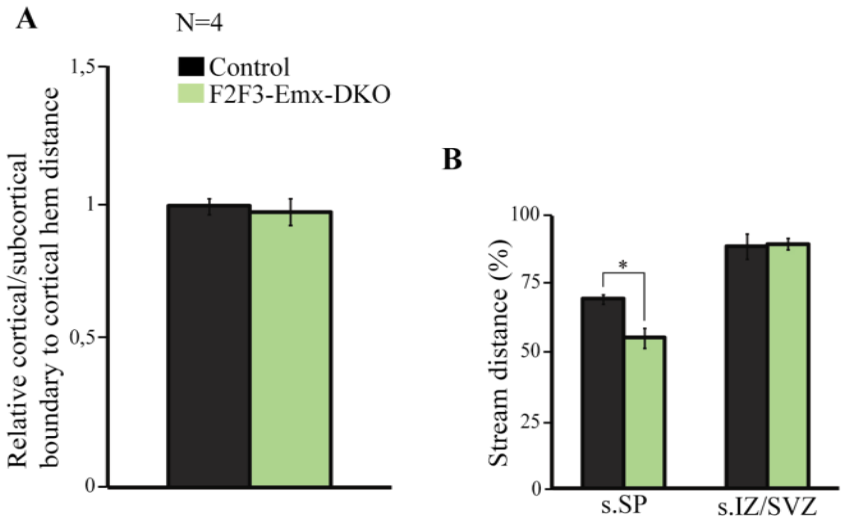

Figure 43. Shorter SP stream of CB$^+$ interneurons in F2F3-Emx-DKO at E14.5. (A) Relative cortical length, where the total length from cortical/subcortical boundary to cortical hem in controls was given the value "1" and that in the mutants was referred as fold induction. (C) Significant reduction of the relative tangential migration distance through the SP stream, but not in the IZ/SVZ stream, in the F2F3-Emx-DKO respect to the controls (tangential migration distance was measured as explained in Figure 31A).All data are presented as mean ± s.e.m. (n=4 per genotype, *p<0.05, two tailed Student' t test). Abbreviations: CB, calbindin; s.SP, stream subplate; s.IZ/SVZ, stream IZ/SVZ.

4.12 *FLRT2* and *FLRT3*-deficient interneurons are intrinsically able to migrate from MGE explants in culture

The results presented above demonstrate that cortical interneuron normally reach the dorsal cortex in the absence of *FLRT2* and *FLRT3*, since the total number of CB$^+$ interneurons are the same in controls and mutants. Moreover the results indicate that the effect of FLRTs is non-cell autonomous, suggesting a ligand function of these proteins through a yet-unknown receptor. These observations could mean that interneurons in the double mutants have not an apparent intrinsic defect in motility due to the lack of FLRT2 and FLRT3 expression. In order to better address this aspect, the intrinsic capacity of double *FLRT2* and *FLRT3*

deficient interneurons was tested in culture. In detail, explants of MGE tissue from WT and from the single, F2-Sox-KO and F3-Sox-KOs as well as from the F2F3-Sox-DKOs, obtained from E14.5 brains (Figure 44C), were cultured *in vitro* in a 3D Matrigel according to (Wichterle et al., 1999). In this assay, interneurons spontaneously migrate out of the explant and it is possible then to measure the migration distance to assess if there is any intrinsic migration defect (Figure 44A-C).

Migratory outgrowth in our experiments was measured blind to genotype for several days in culture (2, 3 and 4 DIV) as shown in quantifications of Figures 44D. Similar migration distance compared to controls was achieved by all the mutant explants analyzed, even in the case of the F2F3-Sox-DKOs. However, it was noticed that during these DIV, just a slight increase of interneuron migration was observed, suggesting that they already reached maximum migraton distance after 48 hrs (Figure 44D). At this point in an independent set of experiments, comparison of migration distance between mutants and controls was performed at earlier time points, after 1 and 2 DIV. In this case, a clear increase in migration distance was observed at 2 DIV, compared to 1 DIV. However, neither in this case it was observed statistically differences in the explant growth between mutants and controls (Figure 44E).

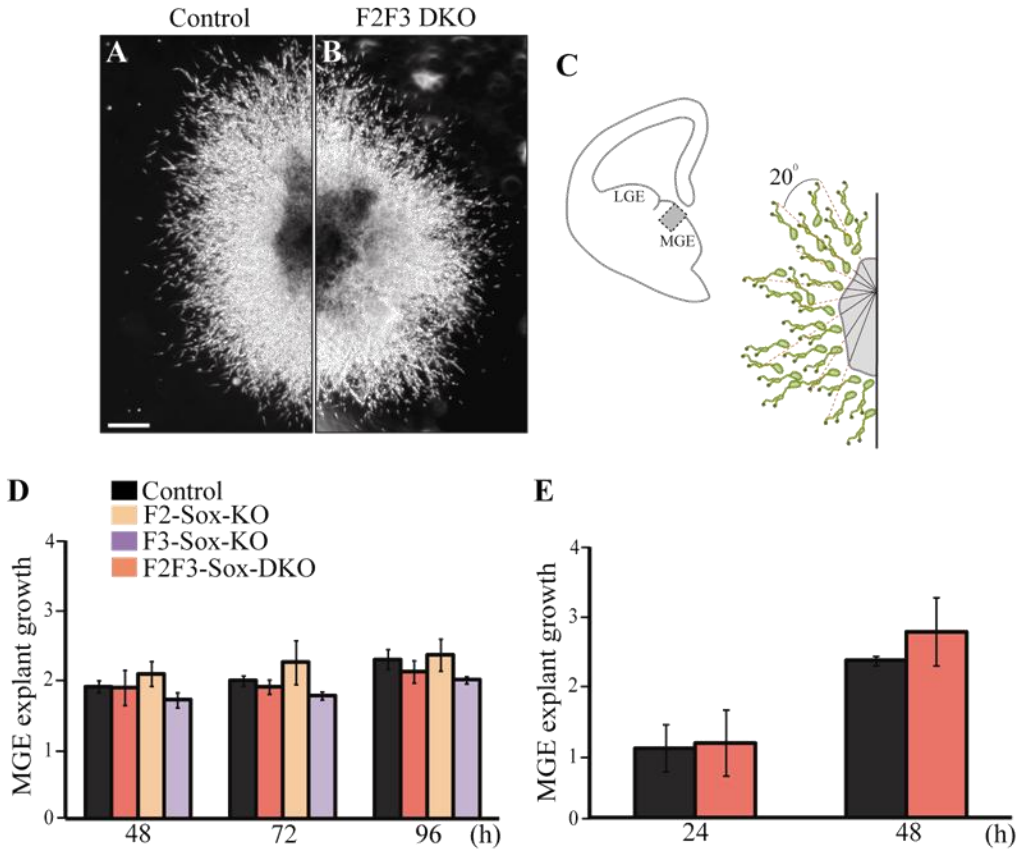

Figure 44. The *FLRT2* and *FLRT3*-deficient interneurons have not intrinsic motility problems *in vitro*. (A, B) Representative images of MGE explants from control (A) and F2F3-Sox-DKO (B) showing the radial dispension of interneurons from the explants (48h in culture). Only half of an explant is shown. Images are in phase-contrast and cells that migrated out formed bright (white) regions surrounding the explants (in black). (C) Schematic representation of how the explant was obtained (grey small box in the MGE region, left cartoon) and of the experimental design for distance quantification. Arbitrary lines were taken every 20o angle to systematically quantify the migration distance and obtain an average distance throughout the entire explant. (D, E) Quantification of migration of MGE cells from the edge of the MGE explants at the indicated times in culture (h). The migration was quantified by measuring the distance from the edge of the explant to the most foremost migrating cell relative to the radius of the explant (in other words, ordinates express the times that interneurons migrate the radius distance of the explant). Values reflet the average among at least nine measures, each 20° distance, to cover at least the half explant area, as described in C. All data are presented as mean ± s.e.m. (n=4 per genotype, $p>0.05$ for each pair of data, two tailed Student' t test). Abbreviations: MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence. Scale bar, 100 μm.

In summary, the normal dispersion of interneurons from mutant MGE explants suggests that FLRT2 and FLRT3 are not required intrinsically for cell motility and

migration. These results, together with the observation that the cells arrive correctly to the cortical rudiment during early development in the F2F3-Sox-DKO mice, confirm the idea of a non-cell autonomous action of FLRTs in interneuron migration.

4.13 Relationship between FLRTs and Rho GTPases in brain development

RhoGTPases, as key cytoskeleton dynamic regulators, are candidates to control the accurate migration of neurons from their origin to the final destination. In the nervous system, Rnd3 has been involved in neurite outgrowth (Talens-Visconti et al., 2010) and in radial migration of cortical neurons (Pacary et al., 2011). In this regard it is noteworthy that FLRT and Rnd proteins have been individually implicated in neurite outgrowth regulation (Aoki et al., 2000; Chardin, 2006; Karaulanov et al., 2009; Robinson et al., 2004; Tsuji et al., 2004) and migration (Pacary et al., 2011; Yamagishi et al., 2011). Moreover, Karaulanov and collaborators provide evidence that FLRT3 and Unc5B functionally interact in modulating cell adhesion during early *Xenopus* development and that the Unc5B effect on adhesion is mediated by Rnd1 (Karaulanov et al., 2009). Finally, FLRT3 has been shown to regulate gastrulation in *Xenopus* embryos through the regulation of the surface expression of cadherin, a mechanism that involves Rnd function and the binding of Rnd to FLRT3 (Chen et al., 2009; Ogata et al., 2007). All these data suggest that Rnd3 and FLRT3 might function together *in vivo* during brain development.

4.13.1 Rnd3 expression in the developing brain

Previous studies have shown the expression pattern of *FLRT2* and *FLRT3* in the developing forebrain by *in situ* hybridization (Yamagishi et al., 2011). In

particular, *FLRT3* expression in the cerebral cortex is detected in the IZ, in cells migrating towards the CP and in the upper layers of the CP at E15.5 (Yamagishi et al., 2011) (Figure 45D). To verify if there is any spatiotemporal co-expression of *FLRT3* and *Rnd3*, the distribution of *Rnd3* mRNA at E15.5 was analyzed by in situ hybridization. *Rnd3* was strongly expressed in the SVZ of the GE, specially in the LGE (Figure 45A-C). *Rnd3* was also expressed in other regions including the cerebral cortex (mainly confined to CP and SVZ/VZ), hippocampus, and thalamus (Figure 45A-C). Comparison of the expression pattern of *FLRT3* and *Rnd3,* revealed that their expression overlapped in similar regions like for instance, the cerebral cortex, thalamus and hippocampus suggesting that they could be related functionally during brain development.

4.13.2 FLRT3 and Rnd3 interact by co-immunoprecipitation assays in transfected cells

Next, the possible relationship between FLRT3 and Rnd3 was addressed by biochemical and cellular methods in heterologous cells. Co-immunoprecipitation experiments were carried out in HEK293T cells transiently transfected with *FLRT3* and *Rnd3* cDNAs (Figure 46). As a control, a deletion mutant of *FLRT3* in which the entire intracellular domain (ICD) was substituted by EGFP (FLRT3ΔC-GFP), was used. As observed in Figure 30A, FLRT3 and Rnd3 are able to co-immunoprecipitate. The amount of co-immunoprecipitated FLRT3 was approximately 3.4 times weaker in the case of FLRT3ΔC-GFP (Figure 46A, A'), suggesting that the interaction is specific and requires an intact ICD region in FLRT3. This result also suggests that interaction between FLRT3 and Rnd3 happens in *cis* and not through a putative triple complex with endogenous Unc5 proteins. Interestingly, co-immunoprecipitation with either FLRT2 or FLRT1 was not detected in these experiments, when $Rnd3^{FLAG}$ was immuniprecipitated (Figure 46B, C) which supports the specificity of the interaction and the preference of Rnd3 to bind only FLRT3.

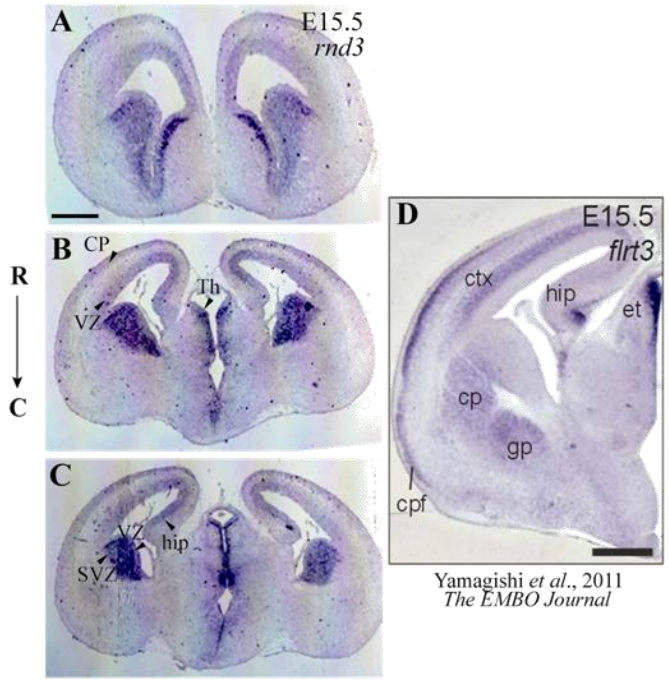

Figure 45. *Rnd3* and *FLRT3* expression in the developing mouse cortex at E15.5. (A-C) Representative images of in situ hybridization analysis of the expression of Rnd3 on coronal brain sections from rostral to caudal (R-->C) at E15.5. *Rnd3* expression is detected in the cerebral cortex (CP, SVZ/VZ), in the basal telencephalon (VZ, SVZ), the th and the hip as indicated. *FLRT3* expression is detected in the cerebral cortex at this stage in the indicated areaas (from Yamagishi et al., 2011). Abbreviations: CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; Th, thalamus; hip, hippocampus. Scale bar: 250 μm.

4.13.3 FLRT3 with Rnd3 co-localize in transiently transfected HeLa cells

The interaction between FLRT3 and Rnd3 was also examined by co-localization using immunofluorescence methods in transfected HeLa cells. HeLa cells were chosen in this assay to avoid artifacts due to the massive protein expression that occurs in HEK293T cells. When HeLa cells were cotransfected with FLRT3 and Rnd3, many of the spots where FLRT3 was localized at the plasma membrane, as

revealed by confocal imaging, also contained Rnd3, at basal levels (Figure 47A-C).

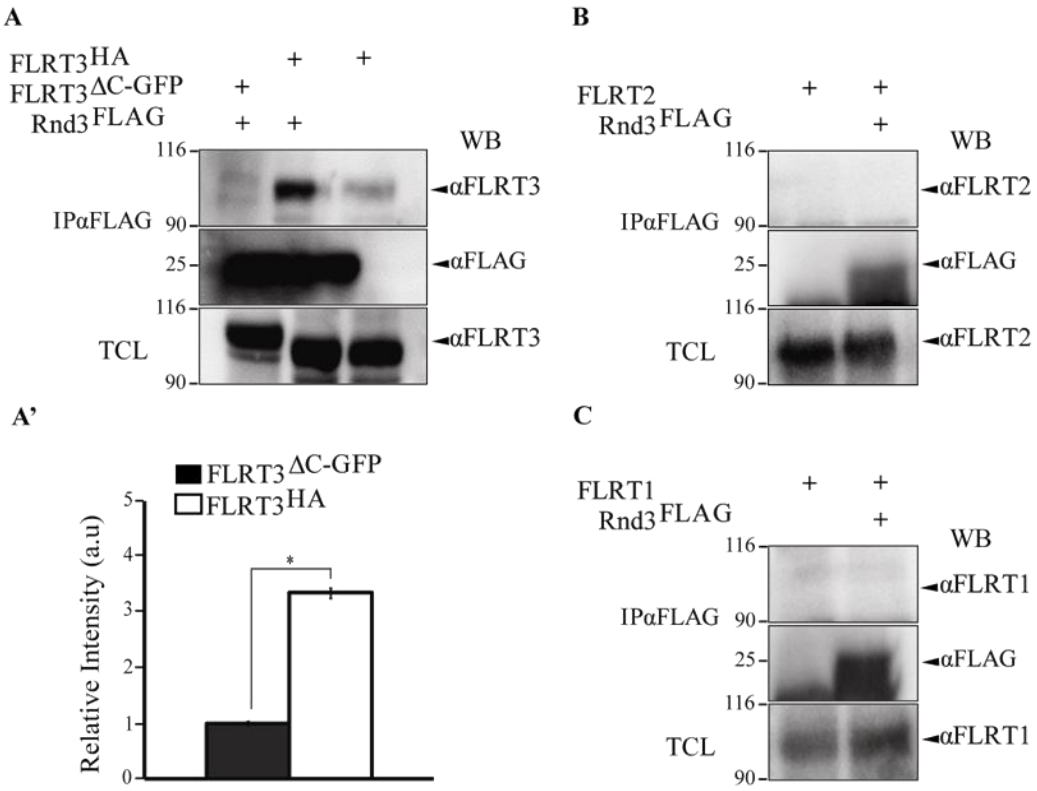

Figure 46. FLRT3 and Rnd3 interact specifically *in vitro* in tranfected HEK293T cells. (A-C) HEK293T cells were transiently transfected as indicated, with HA-tagged FLRT3, FLRT3ΔC-GFP, FLAG-tagged Rnd3, FLRT1 or FLRT2. Protein lysates were immunoprecipitated with anti-FLAG beads (to pull-down Rnd3) and the immunoprecipitates were analyzed by Western blot with the antibodies against FLRT3, FLRT2, FLRT1 and FLAG as indicated on the right side of the each panel. The lower panel shows the levels FLRT proteins in the total cell lysates (TCL). The arrowheads on the right side of the panels indicate the position of the specific proteins and the reference molecular weight is shown on the left side. (A') Quantification of the data shown in (A) about the relative amount of FLRT3 full-length compared with FLRT3ΔC-GFP co-immunoprecipitated with Rnd3 (the amount of FLRT3ΔC-GFP was taken as value "1"). The data are presented as mean ± s.e.m. (n=3, *$p<0.05$, two tailed Student' t test). Abbreviations: IP, immunoprecipitation; TCL, total cell lysates; WB, western blotting.

Next we asked if we could increase the interaction by clustering the surface FLRT3 with a home-made antibody raised against the ECD (see Figure 47E-F and Materials and Methods). After this sort of stimulation, cells were fixed and processed for immunofluorescence detection. In this case, the amount of co-localization between the two proteins increased around 20% after induction with the antibodies (Figure 47A'-C' and D). These results suggest that FLRT3 binds to Rnd3 in mammalian cells and that the interaction is inducible upon FLRT3 clustering, a situation that can resemble better the *in vivo* regulation of the activity of FLRT proteins.

4.13.4 Interneuron entry into the cortical rudiment requires Rnd3 signaling

To investigate the role of Rnd3 during interneuron migration, the distribution of CB^+ interneurons in the *Rnd3* $^{gt/gt}$ mutant brains (see introduction for details of this transgenic line) at E14.5 was examined in coronal sections by a similar bin analysis as previously performed for the F2F3-DKOs. Of notice, the first observations of these sets of staining showed that in the mutant brains fewer CB^+ cells were found in the cortex respect to controls (Figure 48). In addition, these mutant embryos displayed severe axon guidance phenotypes specially affecting the trajectory of the TCAs, a phenotype that is currently being analyzed in our laboratory.

Quantification of these images demonstrated that, indeed, the total number of CB^+ cells arriving he lateral cortex was significantly lower in the *Rnd3* mutants as compared to controls (Figure 49C). Among the CB^+ cells that reached the cortex, their laminar distribution by binning analysis was also examined. Interestingly, it was observed a phenotype with a similar tendency to that observed in our previous analysis in the *FLRT2* and *FLRT3* DKOs.

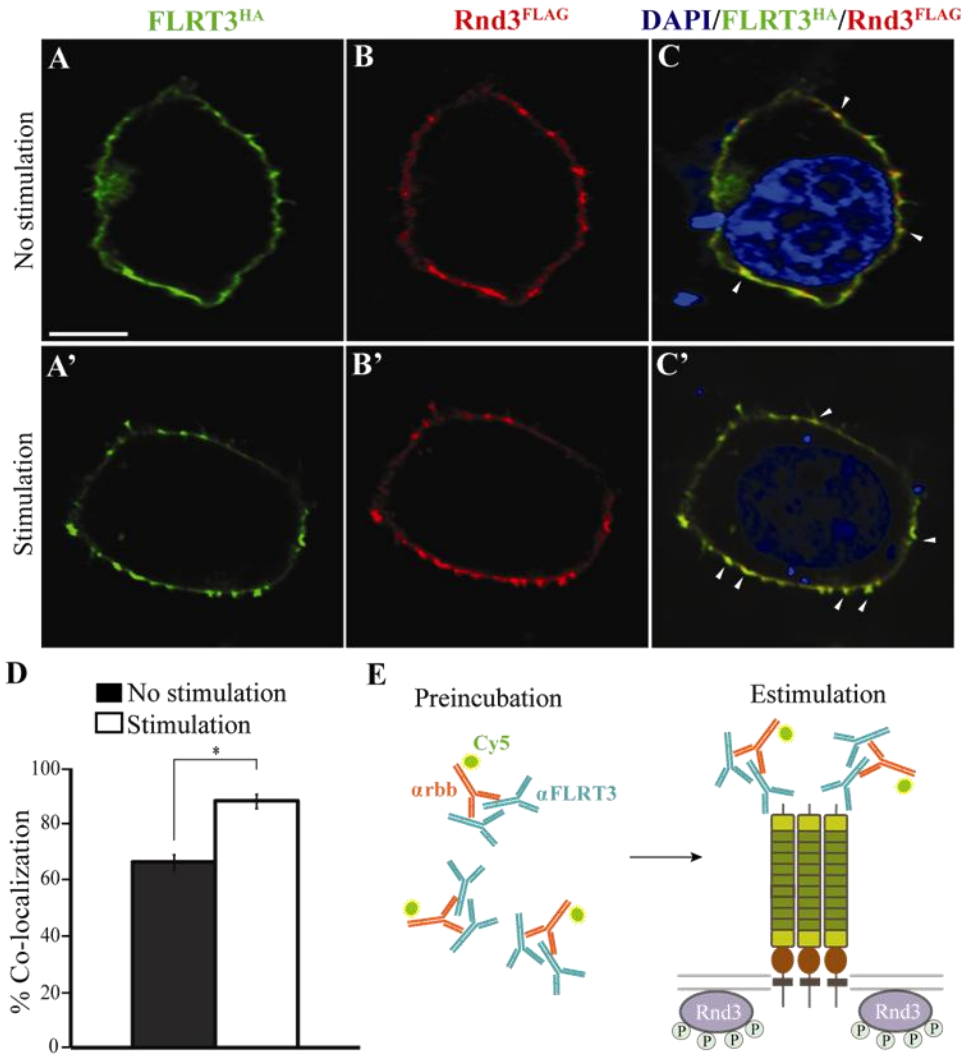

Figure 47. Co-localization of FLRT3 and Rnd3 in the HeLa cells is induced upon FLRT3 clustering. (A-C, A'-C') Representative confocal images of transiently transfected HeLa cells with HA-tagged FLRT3 and FLAG-tagged Rnd3, as dindicated; unstimulated (A-C) or stimulated with anti-FLRT3 antibodies (A'-C'). Localization of FLRT3 is shown in green (A, A'), Rnd3 in red (B, B') and the merged images with DAPI in blue (C, C'). (E) Scheme of the stimulation protocol used in HeLa cells to precluster anti-FLRT3 antibodies with a fluorescently-coupled secondary antibody. (D) Percentage of co-localization FLRT3 and Rnd3, before (black bar) and after stimulation (white bar) with the FLRT3 antibodies. Data are presented as mean ± s.e.m. (n=3, *$p<0.05$, two tailed Student' t test). Scale bar: 10 μm.

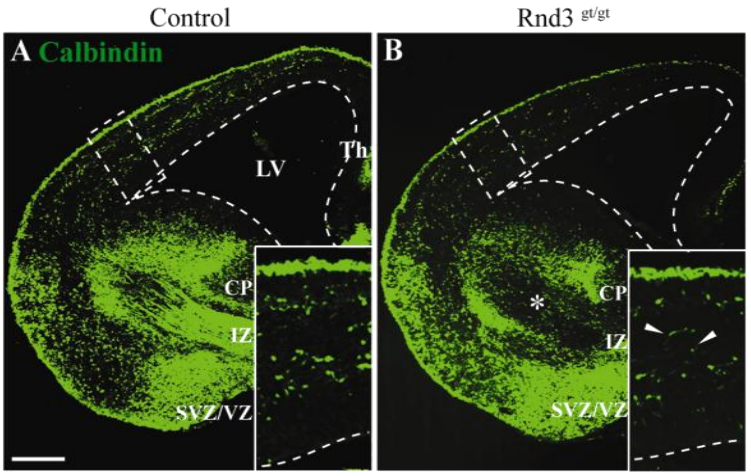

Figure 48. *Rnd3* mutant mice exhibit deficient tangential migration towards the cerebral cortex. Representative images of coronal sections through the telencephalon of E14.5 control (A) and Rnd3 $^{gt/gt}$ (B) showing immunofluorescence for CB in interneurons. Comparable rostro-caudal levels are shown. The total number of CB^+ cells in the dorsal cortex is reduced in the Rnd3 mutants. Arrowheads in B inset show the ectopic CB^+ neurons located in the IZ of the Rnd3 $^{gt/gt}$ compared to control. The asterisk shows the absence of TCA that are unspecifically labelled with CB in controls. Abbreviations: CB, calbindin; CP, cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone; LV, lateral ventricle; Th, thalamus. Scale bar, 200µm.

The quantification revealed that a reduced proportion of CB^+ cells was observed in the SP stream with a concomitant increase in the IZ (Figure 49A, B). Although the differences were not statistically significant, it is important to mention that these are preliminary results obtained from only two animals for each group. Collectively, these results confirmed that Rnd3 is necessary for the correct migration of CB^+ interneurons towards the cortex and that, perhaps, an interaction with FLRT proteins, directly or indirectly, through the Unc5 receptors, is involved in this process, especially in the intracortical distribution of interneurons.

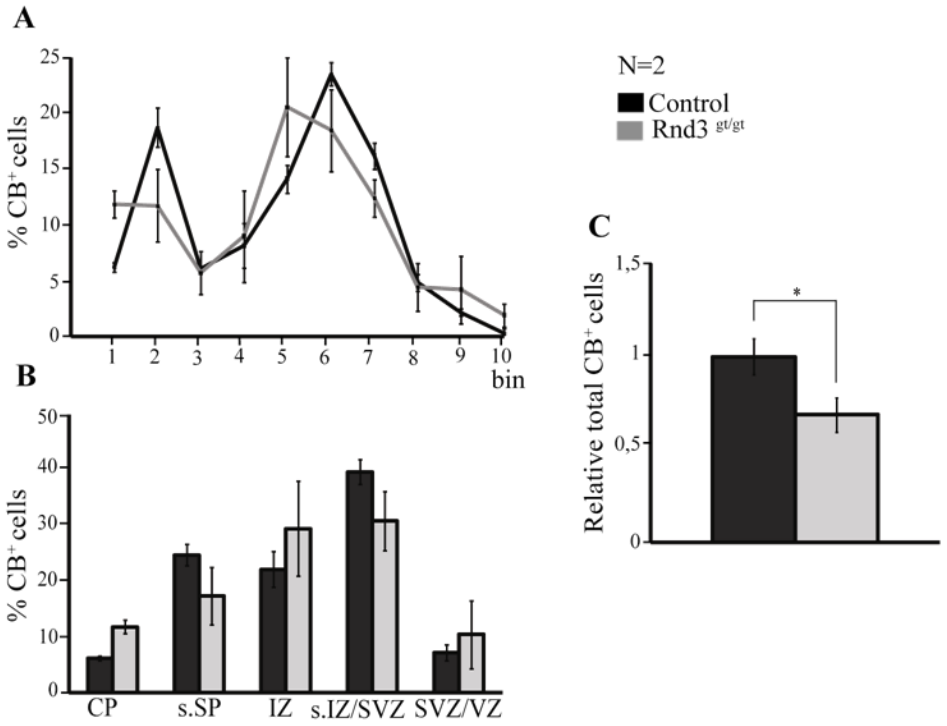

Figure 49. Total number of CB$^+$ interneurons in cortex and their intracortical distribution seem to be altered in the cerebral cortex of the *Rnd3* mutants at E14.5. Percent distribution of CB$^+$ interneurons in each bin (A) or in each cortical layer (B) across the cortical wall in Rnd3 $^{gt/gt}$ (grey labels) and control embryos (black labels). (A) Numbers in ordinates identify bins for quantification, from the CP (1) to the VZ (10). (B) Percent distribution of CB$^+$ interneurons for cytologically distinct zones. (C) Relative amount of total CB$^+$ interneurons in the cortex where the total number of CB$^+$ cells in controls was given the value "1" and the value found in the Rnd3 $^{gt/gt}$ tissue was referred as fold induction respect to control. All data are presented as mean ± s.e.m. (n=2 per genotype, $p<0.05$, two tailed Student' t test for each pair of data). Abbreviations: CB, calbindin; CP, cortical plate; s.SP, stream subplate; IZ, intermediate zone; s.IZ/SVZ, stream IZ/SVZ; SVZ/VZ, subventricular zone/ventricular zone.

CHAPTER 5: DISCUSSION

5. DISCUSSION

The understanding of the integrated cellular and molecular mechanisms involved in the migration as well as the specification of interneurons is based on decades of research. Questions relating to elucidate the molecular mechanisms controlling the preferential tangential migration of interneurons are beginning to be addressed overthe past few years. Many signals responsible for the guidance of cortical interneurons from the GE toward the cortex have been more described in comparison with the molecules controlling the guidance of interneurons within the developing cortex (Evsyukova et al., 2013; Marín et al., 2010). However, it seems that chemoattractive cues preferentially mediate the tangential dispersion of interneuron throughout the cortex than the chemorepellent molecules. So far, only one molecule, the chemokine Cxcl12, a potent chemoattractant for MGE-derived cells, is responsible for maintaining the streams of migrating interneurons within the MZ and SVZ. The interaction between the Cxcl12 and its interneuron receptors Cxcr4 and Cxcr7 are the main mechanism that control the normal laminar and regional distribution preventing the prematurely invasion into the CP (Abe et al., 2014; Li et al., 2008; López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011; Stumm et al., 2003; Tiveron et al., 2006; Wang et al., 2011).

Having observed that FLRT2 and FLRT3 are a novel family of chemorepellent cues for radial migrating neurons (Yamagishi et al., 2011) and also that they are expressed in the cortical layers adjacent to the trajectories of cortical interneurons, it was proposed to investigate the function of these proteins in the tangential migration of interneurons during the developing cortex. Here we show that the single genetic disruption of *FLRT2* or *FLRT3* is not sufficient to disrupt the pattern of tangential migration during development, as they navigate through the normal intracortical routes. However, simultaneous deletion of both genes, affects the correct lamination and intracortical distribution of interneurons leading to a

specific phentoype affecting mainly the SP stream: a decreased number of cells migrating through this stream and an increase of interneurons within the IZ which may lead the decrease number of migrating cells in the CP at posterior stages (E16.5). Given the fact that *FLRT2;FLRT3*-deficient interneurons migrate normally *in vitro* and also that F2F3-Emx-DKOs displayed very similar phenotypes, strongly suggest that cortical interneurons do not have an intrinsic requirement for *FLRT2* and *FLRT3 in vivo.* Therefore, these proteins may act cell non-autonomously, as ligands for unknown receptors that ultimately control interneuron tangential trajectories throughout the cerebral cortex. Thus, the reported phenotypes demonstrate a novel and essential role for FLRT2 and FLRT3 as guidance cues for migrating interneurons within the cortex. Finally, in the current study we are showing preliminary results related to the function of Rnd3 in the signaling mechanisms downstream of FLRTs. Rnd3 is a protein that regulates the actin cytoskeleton organization and is therefore a potential candidate to regulate neuron migration.

5.1 Effects of *FLRT2* and *FLRT3* ablation in the cortical interneurons migration through the SP stream

During the last past years, functional studies of the FLRT (FLRT1-3) proteins have revealed different functions in different systems including the mature and developing brain, early embryogenesis and vascular development (Egea et al., 2008; Leyva-Díazet al., 2014; Maretto et al., 2008; Müller et al., 2011; Ogata et al., 2007; O'Sullivan et al., 2012; Yamagishi et al., 2011). In the developing brain, FLRTs have been specifically related to the migration of excitatory neurons by different mechanisms: by a repulsive effect triggered by FLRT2-ECD on Unc5D expressing neurons in the SVZ, that control the timing of radial migration towards the CP and by an adhesive function triggered by FLRT3 homotypic interactions

that regulate tangential dispersion of excitatory neurons (Seiradake et al., 2014; Yamagishi et al., 2011). All these results demonstrate the relevance of FLRT proteins in radial and tangential patterns of migration of pyramidal neurons during development. However, so far no published work has particularly focused to elucidate the function of FLRT proteins during migration of cortical interneurons. We hypothesized that, given the important differences existing between the migration of these two types of neurons, a function of FLRTs in interneuron migration would be relevant to understand their development and might reveal new mechanisms of action of these proteins.

Most cortical inhibitory interneurons arise from MGE, CGE and POA within the ventral telencephalon, and migrate tangentially (orthogonal to the radial glial scaffold) into the developing neocortex (Anderson et al., 1997, 2001; Batista-Brito and Fishell, 2009; Faux et al., 2012; Gelman and Marín, 2010; Kriegstein and Noctor, 2004; Lavdas et al., 1999; Marín and Rubenstein, 2001; Miyoshi et al., 2010; Nery et al., 2002; Wichterle et al., 1999, 2003; Yozu et al., 2005). They migrate toward the cortex in response to a combination of chemoattractive and chemorepulsive cues that prevent interneurons entering the striatum (Marín et al., 2010; Métin et al., 2006). Once in the cortex, they follow three long and tortuous tangential migratory streams, located at the MZ, SP and IZ/SVZ (Hernández-Miranda et al., 2010; Marín et al., 2013; Marín and Rubenstein, 2003; Métin et al., 2006). Final areal laminar positioning of interneurons involves a shift between tangential and radial migration and depends on the ventral germinal zone of origin, interactions with developing axonal fibers and RG scaffold, and local interactions with projection neurons (Evsyukova et al., 2013). So far, only chemoattractive cues, like Cxcl12 (discussed below), have been suggested to be involved in the maintenance of the tangential streams of migration and downregulation of this signal seems to be key for the tangential-to-radial

migration shift in the later steps of interneuron development (Meechan et al., 2012; Sanchez-Alcañiz et al., 2011; Wang et al., 2011). Although chemorepellent cues have been proposed to work together with chemoattractive signals in the cortex, to date, no evidence for these signals has been provided.

FLRT2 and FLRT3 expression pattern by immunofluorescence revealed that they are expressed in an interesting pattern within the cortical layers of the brain at E14.5. Particulary, FLRT2 is mainly expressed in the CP, whereas FLRT3 is expressed in the IZ. Interestingly, the *in situ* hybridization pattern of FLRT2 mRNA is confined to the CP (Yamagishi et al., 2011), whereas FLRT2 immunoreactivity was detected in the CP and more apical regions such as the IZ. This can be because of the anti-FLRT2 antibody was raised against the ECD and therefore it is labelling as well the soluble ECD of FLRT2 into the IZ. The co-labeled of FLRTs and CB, showed that the interneuronal population reaching the pallium and disperse tangentially through determined routes (through the MZ, SP and IZ/SVZ) actively avoid the CP and the IZ, FLRTs enriched zones. This observation pointed to the idea that FLTRs could function as inhibitory cues to maintaining the distribution of these cells, considering the antecedents of FLRTs as chemorepellent molecules that control the neuronal radial migration (Yamagishi et al., 2011).

To start addressing this hypothesis *in vivo*, single and double *FLRT2* and *FLRT3* mutant mice, using the *Nes-Cre* and *Sox1-Cre* lines in order to conditionally delete the expression of these genes during the nervous system development, were generated. Embryos from these mutants were obtained at different developmental stages and brain sections stained for CB. The results of this analysis evidenced that the single abolishment of *FLRT2* or *FLRT3* do not affect the laminar distribution of CB^+ interneurons within the cortex and they follow the established

normal tangential routes found in control embryos. However, when double mutant mice were analyzed, the results showed two significant phenotypes related to the intracortical tangential dispersion pattern of interneurons: first, less CB^+ interneurons were found in the SP stream and more CB^+ interneurons were detected in the IZ; second, the progression of the SP stream into the cortex was significantly reduced in the mutant brains. These effects were specific because the IZ/SVZ stream was not affected. Our interpretation of all this data is that interneurons, mainly transiting through the SP stream, are unable to keep their normal trajectory in the absence of *FLRT2* and *FLRT3* once they enter the cortical rudiment. Instead, they deviate and acquire an abnormal laminar distribution with a tendency to invade the IZ resulting in the shifted regional distribution. As a consequence, less interneurons proceed in the SP stream which is significantly smaller in the mutants.

Interestingly, the interneurons deviation from tangential route only appear when both genes were removed at the same time, since the single mutants did not show disrupted migration. This suggests that the function of *FLRT2* and *FLRT3* in interneuron migration is redundant and that both cooperate in keeping the tangential migration of the SP stream and explain why the single deletion of any of the two genes do not have any effect. So far, individual functions have been assigned to either *FLRT2* or F*LRT3* in the developing nervous system, but our findings are the first genetic evidence that both genes can cooperate functionally *in vivo*.

It is worth noting that, despite the defects observed in the intracortical dispersion of interneurons in the absence of *FLRT2* and *FLRT3*, interneurons reach the mutant cortex in normal numbers; which has been also observed in the *Cxcl12*, *Cxcr4* and *Cxcr7* mutant mice (Abe et al., 2014; Li et al., 2008; López-Bendito et

al., 2008; Sánchez-Alcañiz et al., 2011; Tiveron et al., 2006; Wang et al., 2011). This observation reinforces the idea that the mechanisms driving the migration of interneurons from the subpallium to the cortex and those controlling their intracortical migration are different (Marín, 2013). It is surprisingly that the interneurons do not seem to invade the CP in the F2F3-DKO. While it is true that FLRTs function affects the laminar allocation of interneurons, they are not sufficient to maintain these cells away from the CP, as in the case of Cxcl12; suggesting that necessarily other molecules are ensuring that interneurons do not prematurely invade the CP, what it is known as a highly controlled process.On the other part, there is no reduced neurogenesis in the basal forebrain progenitors pools as was not found significant decrease in the total number of interneurons arriving and migrating toward the dorsal cortex, away from the GE in the F2F3-DKO when compared with controls. Thus, the observed phenotype not is exactly a tangential progression delay, if is considering that the total number reaching is the same; it is more a stack in a most lateral part because of interneuron deviate from tangential route, affecting more their advancement through the SP stream.

5.2 Regulation of the interneurons migration through the SP stream

One of the most striking observations of the present study is the fact that the ablation of *FLRT2* and *FLRT3* has revealed a specific mechanism of regulation of migration for the SP stream of interneurons. Revisiting the expression pattern of FLRT2 and FLRT3 at the protein level (see Figure 20 in Results section) this is not surprising since both, FLRT2 and FLRT3 protein expression, overlap specifically only in the SP stream. Indeed, several lines of evidence suggest that the interneurons are not distributed randomly across these streams. Thus, the specific guidance requisites that support the migration of interneurons through each of these routes are partially divergent, and so it is likely that the interneurons

that travel through each of these routes express a different complement of guidance receptor (Marín et al., 2013).

The SP is a transient cortical structure that forms during mammalian brain development (Allendoerfer and Shatz, 1994). SP neurons are a heterogeneous population of neurons that are among the earliest generated in the cerebral cortex (Allendoerfer and Shatz, 1994; Hoerder-Suabedissen and Molnár, 2013; Price et al., 1997; Rakic, 1974; Robertson, 2000) and has numerous developmental functions (Hoerder-Suabedissen and Molnár, 2015). A recent study identified a further source of tangentially migrating SP neurons (rostro-medial telencephalic wall), although these are non-GABAergic (Pedraza et al., 2014). All this demonstrate that the SP zone is a complex scenario where converge various neuronal components that might be involved in the migration through this interneuron stream, that or could be the cause or otherwise could be affected by this defect in the F2F3-DKO mice.

The mechanisms that control the preferential interneuron migration through the migratory streams are beginning to be elucidated. Most of the studies have been focused on the larger streams, the MZ and the SVZ/IZ ones but little is known about the migration mechanisms occurring in the less populated, SP stream. Here is proposed that interneurons avoid the cortical layers where is expressed FLRT2 and FLRT3 as they first enter the developing cortex, and the suppressed expression of two proteins leads a misplacement of interneurons migrating through the SP stream suggest that different mechanisms should mediate the highly stereotyped dispersion of interneurons throughout the embryonic cortex. All of this, pointing the idea that the Cxcl12 and the Cxcr receptors are not the hierarchical dominating mechanism that controls the GABAergic interneurons dispersion specifically in the SP stream. These results suggest that FLRTs can be

an entry point to analyze the molecular mechanisms that control the interneuron migration through this specific route.

5.3 Late developomental consequences of *FLRT2* and *FLRT3* ablation on the distribution of cortical interneurons and pyramidal layer formation

At this point, was necessary asked whether the interneurons accumulated in the IZ and decreased progress through the SP stream is transient or sustained. Then was followed the progression of phenotype over different developmental stages, in this case E16.5.

It is well known that switching from tangential to radial migration during CP invasion of interneurons, is a temporally regulated process. These cells actively avoid the CP~48 hrs after reaching the pallium, starting their invasion after E15.5, suggesting that a change in the cortical environment around this stage may coordinate the entry of migrating interneurons into the CP (López-Bendito et al., 2008). For that, was interesting to analyze the number of cells located in this lamina. The results at E16.5 showed a significantly less CB^{+} interneurons in the CP respect to the control mice. In the F2F3-DKO animals is affected the laminar distribution of interneurons since the radial migration toward more superficial layer is delayed compared with control. This could be explained because the redistribution of interneurons toward the IZ at previously stage, delay the rising of these cells to the CP. Is tempting to speculate that these interneurons have left their proper context in the SP stream, where they are prepared to receive the necessary signals that propel them to invade the CP.

Ablation of *FLRT2* and *FLRT3* did not affect the normal cortical development at E14.5 stage, as the gross brain morphology and lamination of postmitotic neurons

were normal. The cortical layers markers (Tbr1, Ctip2 and Satb2) were still present in their appropriate cortical layers in the F2F3-DKO embryos. Surprisingly, at E16.5 was noted that, compared with cortices of control, cortices of F2F3-DKO showed abnormal subsequent migration and lamination of $Satb2^{+}$ and $Tbr2^{+}$ postmitotic neurons.

Satb2 is a gene that is expressed predominantly in young upper layers neurons but not in SVZ progenitors. Its expression pattern suggests that Satb2 may be involved in the control of early aspects of upper layers neuron specification (Britanova et al., 2008). $Satb2^{+}$ late born cortical neurons start their migration immediately after mitotic cycle exit and arrive in the CP early in developing at E14.5-E15.5 (Britanova et al., 2008). In F2F3-DKO $Satb2^{+}$ neurons failed to migrate to the superficial CP and instead settled in the deeper CP and IZ. The $Tbr2^{+}$ cells are confined to the SVZ and they are rarely found in the IZ or CP. The results show that in the F2F3-DKO is observed a premature departure of these cells, toward upper layers compared with controls. Previous results described a Tbr2 phenotype in the $Unc5D^{-/-}$ embryos that could be related with the lack of *FLRT2*. Although these results are preliminary, because more animals need to be analyzed at this stage, they suggest that these subpopulations of $Satb2^{+}$ and $Tbr2^{+}$ cells are influenced by the presence of FLRT2 and/or FLRT3.

The F2F3-DKO conditional mutants survive into adulthood. To evaluate the outcome of *FLRT2* and *FLRT3* supression on interneuronal organization *in vivo* is necessary analyze the positioning of interneurons in the postnatal or adult cerebral cortex, when active interneuronal migration comes to an end. Since cortical interneurons include different subtypes that are classified according to their origin, morphology, and function, will be necessary analyze whether different subgroups of interneurons displayed neuron layered defects for CB^{+}, CR^{+}, SOM^{+}, NPY^{+}, and

PV^+ cortical cells. This could allow the study of the postnatal consequences of the abnormal intracortical migration of embryonic interneurons found in the F2F3-DKO, which underlines the relevance of this process in the development of inhibitory circuitries in the cerebral cortex. Thus, further immunolabelled subpopulations of interneurons in the motor, somatosensory, and visual cortices, will indicate the influence of FLRTs in the appropriate migration and positioning of distinct subsets of interneurons within the developing cerebral cortex.

5.4 Non-cell autonomous action of FLRT2 and FLRT3 regulating intracortical interneuron migration through the SP stream

Analysis of interneuron distribution in the F2F3-Emx-DKO shows a very similar defect previously developed by the F2F3-Sox-DKO; where the interneurons failed to migrate into the SP stream and invading the IZ. Thus, mice with conditional loss of *FLRT2* and *FLRT3* only in the cortical layers phenocopy the interneuron defects observed these proteins are not expressed neither in the cortex nor in the interneurons, establishing that the migration phenotype is probably non cell-autonomous.

The previous results indicated that, independently of its possible function of FLRT2 and FLRT3 if they could be expressed in the interneurons, FLRT2 and FLRT3 presence in the cortex are both indispensable for their correct intracortical migration. However, a preliminary exploration was assessed by co-labelled FLRTs and CB interneruons by immunofluorescence. The pictures showed that interneurons do express FLRT2 and FLRT3 although the levels are not speciallyhigh. It is worth to note that is necessary to demonstrate the expression of FLRTs in interneurons in order to interpret our current working model by other means. For example, analyze gene expression of FLRTs in the cortical

interneurons labeled with GFP using for instance a *Nkx2.1-Cre* line together with a GFP reporter, that have been previously FACS sorted.

Apart, but continuing with the FLRTs cell-autonomous signaling question, was checked the intrinsic capacity of interneurons migration by *in vitro* explants assay. Apparently, FLRT2 and FLRT3 not affect the intrinsically migratory capacity of interneurons, as the total CB^+ cells transiting in the dorsal cortex were not decreased in the F2F3-DKO cortices. That means that the tangential migration of cortical interneruons from the subpallium to the dorsal cortex is not affected in the F2F3-DKO, suggesting that this phase of the migration of cortical interneurons does not depend on FLRTs signaling.

The F2F3-deficient MGE-derived interneurons showed a normal radial propagation and migration compared with the controls. Thus, the capacity of MGE-derived interneurons to ove into the cerebral cortex in the F2F3-DKO mouse model is not compromised and did not disrupt CB^+ interneurons migration. Therefore, changes in interneuron migratory trajectories are not related with interneuron motility during midgestation. These studies point to cell autonomous mechanisms through which FLRT2 and FLRT3 control neuronal migration.

FLRT2 and FLRT3 are expressed in the cerebral cortex layers during development, and the suppression of their expression in the whole telencephalon results in the interneurons migration disruption. To establish whether the observed phenotype are based on the cell-autonomous functions of FLRT2 and FLRT3 in the interneurons, were selectively removed these two proteins in the dorsal telencephalon but not in the interneurons arriving from the subpallium and transiting through the tangential streams using Cre recombination regulated by the Emx1 promoter.

5.5 Possible scenarios of FLRT function

Currently it is not known the exact mechanisms by which FLRT2 and FLRT3 regulate SP stream interneuron migration. Defects in the intracortical interneuron distribution have been observed in other mutants, with other mutations in both extracellular cues and receptors (Liodis et al., 2007; López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011), and also during interneuron migration and placement (Meechan et al., 2012; Myers et al., 2014); that resembles to the defects observed in the F2F3-DKOs. Considering these data, we can envision several scenarios of FLRT2 and FLRT3 action in the context of interneuron migration.

5.5.1 FLRTs and Cxcl/Cxcr interaction

It is known that interneurons that travel through each of these intracortical routes express a different set of guidance receptors that support the different trajectories (Marín, 2013). It has been widely reported that interneurons seek the cortical MZ and SVZ/IZ, where their migration depends on Cxcl12 emanating from meningeal cells and pyramidal cell progenitors (Sessa et al., 2010; Tanaka et al., 2009; Tiveron et al., 2006; Zarbalis et al., 2012). Cxcl12 (also known as Sdf1) is strongly expressed in the MZ and SVZ, and to a minor extent, by cells in the SP (Daniel et al., 2005; Stumm et al., 2003, 2007; Tiveron et al., 2006; Tham et al., 2001). Cxcl12 via Cxcr4 and Cxcr7 interneurons receptors, facilitates the neurons trajectories through these permissive and attractive territories. Cxcl12, Crcx4 and Cxcr7 mutants show similar interneruons positioning defects. The number of cells transiting through the MZ and IZ/SVZ streams decreasing and increase in the lower CP/SP stream with a premature invasion of the CP (Abe et al., 2014; Li et al., 2008; López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011; Stumm et al., 2003; Tiveron et al., 2006; Wang et al., 2011). The regulation of CP invasion by GABAergic interneurons is a key event in cortical development, because it

directly influences the coordinated formation of appropriate glutamatergic and GABAergic neuronal assemblies. This sophisticated fine-tuning mechanism to ensure the waiting period preceding the CP invasion also serve to give GABAergic interneurons the time required to disperse tangentially and colonize those regions of the cortex that are located furthest away from their origin in the subpallium (Sánchez-Alcañiz et al., 2011). However, it has been shown that the timing of exit from the migratory streams correlates with the loss of responsiveness to Cxcl12 as an attractant (Li et al., 2008). This is consistent with the cellular function attributed to Cxcl12, which minimizes the potential of interneurons to sense cues outside the tangential streams by reducing their branching frequency (Lysko et al., 2011). Although CP does not contain a non-permissive activity for MGE-derived interneurons; an unknown factor present in the CP that only becomes perceptible by GABAergic interneurons after they have stopped responding to Cxcl12. That implies that additional cues, either on the routes of migration or expressed by pyramidal cells, control their final laminar allocation of GABAergic interneurons (López-Bendito et al., 2008).

The disruption in the laminar distribution in the F2F3-DKO cortex resembles the phenotypes seen in the *Cxcr4* and *Cxcr7* mutants (Abe et al., 2014; Li et al., 2008; Sanchez-Alcañiz et al., 2011; Wang et al., 2011), suggesting that FLRTs and Cxcrs could work together. Since we found that FLRTs act in a non-cell autonomous fashion, we thought that FLRTs could act as ligands for Cxcrs. This idea was tested in our laboratory by *in vitro* binding assays where Cxcr4- or Cxcr7-transfected HEK293T cells were incubated with a recombinant fusion protein containing the FLRT-ECD with alkaline phosphatase which activity can be revealed with specific colorimetric substrates provided that binding has ocurred. However, these experiments gave negative results suggesting that Cxcr receptors do not interact physically with FLRTs. Although we cannot rule out that

the signaling mechanisms triggered by FLRTs and Cxcrs cooperate at the intracellular level, this finding suggests that another receptor, different from Cxcrs, could be expressed specifically in the interneurons transiting through the SP stream in order to mediate FLRT signaling.

It is worth noting that, in the *Cxcl12*, *Crcx4* and *Cxcr7* mutants the interneurons migration through the MZ and SVZ stream is disrupted and in concordance they then redirected to the SP stream and CP (Abe et al., 2014). So far, has not been reported the importance of the Cxcl12-Cxcr4/7 signaling in maintained the integrity of the SP stream, on the contrary the interneurons has a preference to transit through the SP stream when the Cxcl12 or Cxcr4/7 are abolished. This, together with the fact that Cxcl12 is less abundant in this stream, suggest that others mechanisms regulate the preferential interneurons migration through the SP stream. Thus, migrating interneurons may respond to other cues regulating their confining to this stream and we suggest that FLRTs play a prominent role in this specific mechanism.

5.5.2 FLRTs and Unc5 receptors

FLRTs proteins have recently been shown to bind in *trans* to Unc5 receptors (Karaulanov et al., 2009; Söllner and Wright, 2009). FLRT2 and FLRT3 act as repulsive guidance molecules for Unc5 receptor-expressing neurons. The relative binding affinities between FLRTs and Unc5 were previously measured by cell-based binding assays (Karaulanov et al., 2009; Yamagishi et al., 2011). The results revealed a specific and high-affinity interactions between FLRT2 and Unc5D (and to a lesser extent Unc5B) and between FLRT3 and Unc5B receptors (Yamagishi et al., 2011). Recently, more accurate experiments using surface plasmon resonance (SPR) confirmed the previous data but also revealed a possible FLRT3 and Unc5D interaction, although the affinity is lesser compared with

Unc5B (Seiradake et al., 2014). FLRT ECDs are shed by neurons *in vivo* raising the possibility that they may act as diffusible ligands for Unc5 receptors on opposing cells (Yamagishi et al., 2011). Indeed, the proteolytic shed FLRT2-ECD has been shown to control the pyramidal neuron migration within the developing cortex by inducing a repulsive signal through Unc5D receptors expressed by the SVZ cells (Seiradake et al., 2014; Yamagishi et al., 2011). The role of Unc5 receptors in interneuron migration is not clear. It is known that Netrin-1control the migration of GABAergic interneurons and their consequent positioning in the developing cerebral cortex (Stanco et al., 2009). Although this effect is mediated by α3β1-integrin, it cannot be discard the involvement of Unc5 receptors. Also, it has been observed that the regulation of precise Unc5B levels by the transcription factor Sip1 represents a way of sorting the different MGE cells, which changes their direction of migration without influencing their differentiation into cortical interneurons. The genetic ablation of Sip1 induces an increase of Unc5B expression in interneurons leading to a dramatic reduction of cortical interneurons (Van den Berghe et al., 2013).

All these data, suggest that the candidate receptor through which FLRT2 and FLRT3 are mediating their repulsive effect could be Unc5. To test this hypothesis, first it will be necessary to verify that definitely the intermneurons express Unc5 receptors. Moreover, to determine whether these receptors are involved in the observed phenotype the Unc5B and Unc5D DKO could be analyzed subsequently.

5.5.3 Robo-FLRT interaction

Although the cellular processes involved in cell migration and axon guidance are fundamentally different, similar molecules may be involved. Robo1 is indeed involved in both cell migration, and axon growth and guidance events (Andrews et al., 2006). Very recently our laboratory has shown that FLRT3 is a novel co-

receptor for Robo1 in rTCAs and that this interaction is required for a proper topographic projection of these axons into the developing cortex (Leyva-Díaz et al., 2014). Therefore, it is important to consider the possibility that FLRT3-Robo1 signaling could be involved in the regulation of interneuron migration.

Considering that both Robo1 and Robo2 are expressed in cortical interneurons during corticogenesis and also in the MZ, SP, as well as in the tangential migratory routes travelled by interneurons, and specially within the IZ/SVZ (W. Andrews et al., 2008), it could be suggested that these receptors may play a role during interneuron migration. *Robo1* KO mice, for instance, showed abnormalities in the formation of the corticothalamic and thalamocortical pathfinding and in the migration of interneurons to the neocortex from the ventral forebrain resulting in an increased number of cells that persisted to adulthood due to an increase of proliferation (Andrews et al., 2006, W. Andrews et al., 2008). However, the analysis of *Robo1* and *Robo2* Kos animals showed no change in the positions of the streams of migrating interneurons (W. Andrews et al., 2008). Moreover, since the phenotype of the F2F3-DKO does not show differences in the number of intracortical interneurons, in any case what appears is a decrease at E16.5, this suggests that FLRTs do not affect proliferation of interneurons as Robo1 does. Altogether, these data suggest that the *cis* interaction between the Robo1 and FLRT3 does not seem to be the primary mechanism that explain FLRTs function during interneuron tangential migration.

5.5.4 Adhesion

Another important aspect that should be considered is the role of FLRTs in cell adhesion. FLRTs proteins can interact physically, and specifically FLRT3 was implicated in the cell homotypic cell sorting through its LRR domain that was suggested to be important for cell adhesion during development of *Xenopus*

embryos (Karaulanov et al., 2006). Recently, a structural study demonstrated the FLRT-mediated adhesion function during neuronal development where FLRT3-FLRT3 homophilic interactions are implicated in the tangential dispersion of migrating pyramidal neurons during cortex development (Seiradake et al., 2014).

Since the FLRT-FLRT homophilic binding affinity is weak, FLRTs are ideal candidates for providing the finely tuned adhesive cell-cell traction required for cell migration. In contrast to, repulsive FLRT-Unc5 interaction is a low-affinity adhesive binding and also is mediated through a distinct binding surface on the FLRT LRR domain. This parallel signaling of membrane-associated (adhesion) vs. soluble FLRT ectodomains (repulstion) *in vivo* controls the delicate balance of adhesion/repulsion necessary for the adhesive properties of migrating cells. Thus, it cannot be rejected the possibility that the interneurons progress through the SP stream by using this dual mechanism. In fact, very recently, stripe assay experiments made in our group revealed that both FLRT2 and FLRT3 ECDs act as a repellent cues for MGE-derived interneurons (data no shown); however, the repulsion induced by FLRT3 was much less evident than in the case of FLRT2. This could be explained because these interneurons coexpress FLRT3 and Unc5, and FLRT3-FLRT3-mediated adhesion could counteract and attenuate FLRT3-Unc5-mediated repulsion.

5.5.5 Working model

In summary, the results of this study so far demonstrate that FLRT2 and FLRT3 are important in controlling the cortical tangential migration of interneurons. Specifically they cooperate in the maintenance of the interneurons confinement through the SP stream, by a non-cell autonomous compensatory mechanism.

Previous results have revealed that FLRTs are powerful and versatile guidance factors with structurally encoded repulsive and adhesive surfaces that allow them

to act as bimodal guidance systems for homophilic adhesion or heterophilic repulsion (Seiradake et al., 2014; Yamagishi et al., 2011). For instance, the shed ECDs of FLRT2 and FLRT3 act as soluble repulsive cues for Unc5-positive neurons, in both, the soma and the growth cone of cortical neurons (Yamagishi et al., 2011). *In vivo*, *FLRT2* ablation induces a premature migration of Unc5D^{+} SVZ cells toward the CP, consistent with FLRT2 acting as a repulsive cue for Unc5D^{+} cells (Yamagishi et al., 2011). On the other hand, FLRT3 has been also shown to be involved in pyramidal neuron adhesion and the spatial arrangement of these neurons in the tangential axis. However, in contrast to the FLRT-Unc5 repulsive effect, this adhesive function is based on a FLRT3 homotypic interaction (Seiradake et al., 2014). While until now, these FLRTs functions have been described in relation with the projection neurons, nothing related to this bi-functional effect of FLRTs has been reported for the case of interneurons. Very recent *in vitro* results from our laboratory showed by stripe assays that both FLRT2 and FLRT3 ECDs act as repellent cues for MGE-derived interneurons (data no shown).

In an effort to integrate all of this information with the experimental results obtained so far in this thesis, we propose the following model: FLRT2 and FLRT3 cooperate in the control of interneuron migration through the SP by a non-cell autonomous mechanism, via their interaction with the Unc5 receptors. First, is important to consider that there is a functional compensation between FLRT2 and FLRT3 proteins in the observed phenotype which correlates with the fact that FLRT2 and FLRT3 are indeed co-expressed only in the SP zone, where interneurons show the clearer defect in migration. In this model it is considered that interneurons migrating through the SP express Unc5 receptors. In normal conditions the Unc5-expressing interneurons are confined to navigate through the SP stream due to an inhibitory function coming from the IZ region which,

according to our data, is triggered by the shed form of FLRT2 ECD (that accumulates in the IZ) and the FLRT3 ECD present in the IZ, although it cannot be dismissed that the non-soluble FLRT3 present at the membrane of projection neurons may also be involved. In this model, both FLRT2 and FLRT3 present in the IZ act cooperatively as repulsive cues to keep the interneurons in the SP stream (Figure 50A). In addition, it cannot be excluded that the FLRT3-FLRT3 adhesion mechanism operates in this system (Figure 50A). In this model, when FLRT2 is removed the SP interneurons follow their normal trajectory because the repulsive action of FLRT3 present in the IZ (Figure 50B). It is remarkable that the interneurons do not redirect into the CP, despite the lack of FLRT2. This can be explained because the FLRT2 present in the CP, even if it could be repulsive for interneurons *in vitro* or from the IZ, is probably masked by another molecule (Figure 50A). This is consistent with the well stablished idea in the field that the CP does not contain a repulsive activity for MGE-derived interneurons and that, instead, these routes of tangential interneuron migration contain powerful attractive activities (Cxcl12 for instance) that contribute to maintain these cells away from the CP (López-Bendito et al., 2008). Furthermore, it is important to consider that the CP invasion by GABAergic neurons is temporal, tightly controlled, process and probably more of one mechanism exists to ensure the right moment for the CP invasion.

It is interesting that in *Cxcl12* mutants, migrating interneurons in the cortex have the tendency to move and migrate through the SP stream suggesting that another attractive molecule, apart from Cxcl12, is mediating the stereotyped transit through the SP stream (Abe et al., 2014). Together with the fact that in the *FLRT2/FLRT3* DKO only the SP stream seems to be affected, suggests that this stream has different molecular properties from the other two streams. In this sense, FLRT3 for instance could trigger an adhesive/attractive signal to direct the

interneurons through the SP stream. Thus, opposite signals might need to be integrated in order to control the migration of these SP interneurons which may combine the FLRT3-triggered adhesion and the FLRT3-triggered repulsion depending on its binding partner (FLRT3 and Unc5, respectively). When *FLRT3* is removed, the interneurons do not redistribute to the IZ which, in this case, is governing the well documented repulsive action of the soluble ectodomain of FLRT2 shed from the CP as was described for the radial migrating neurons (Yamagishi et al., 2011) (Figure 50C). Finally, when is removed both *FLRT2* and *FLRT3*, the IZ is deprived of all repulsive action, and the interneurons redistributed to the permissive IZ. At this point, it cannot be ruled out whether other mechanisms are involved, where other receptors or attractive molecules in the SP stream, apart from Cxcl12, might be involved and explain the questions emerged from these results.

5.6 Rnd3 in tangential interneuron migration

Significant progress has been made in identifying individual molecules and mechanism that regulate neuronal migration, specifically which guidance cues and cell-cell adhesion, transcriptional, and post-transcriptional mechanisms modulate migration. However, the modes of coordination between these mechanisms and how they converge on the cytoskeleton to drive neuronal navigation from their site of birth to their target locations in the cerebral cortex remain to be fully elucidated (Evsyukova et al., 2014). As major regulators of the cytoskeleton, the family of small Rho GTPases has been shown to play essential functions in cerebral cortex development. The Rho GTPases have been implicated in the regulation of neurogenesis, neuronal differentiation and migration regulating cytoskeletal dynamics, cell shape and migration (Hall and Lalli, 2010; Heasman and Ridley, 2008).

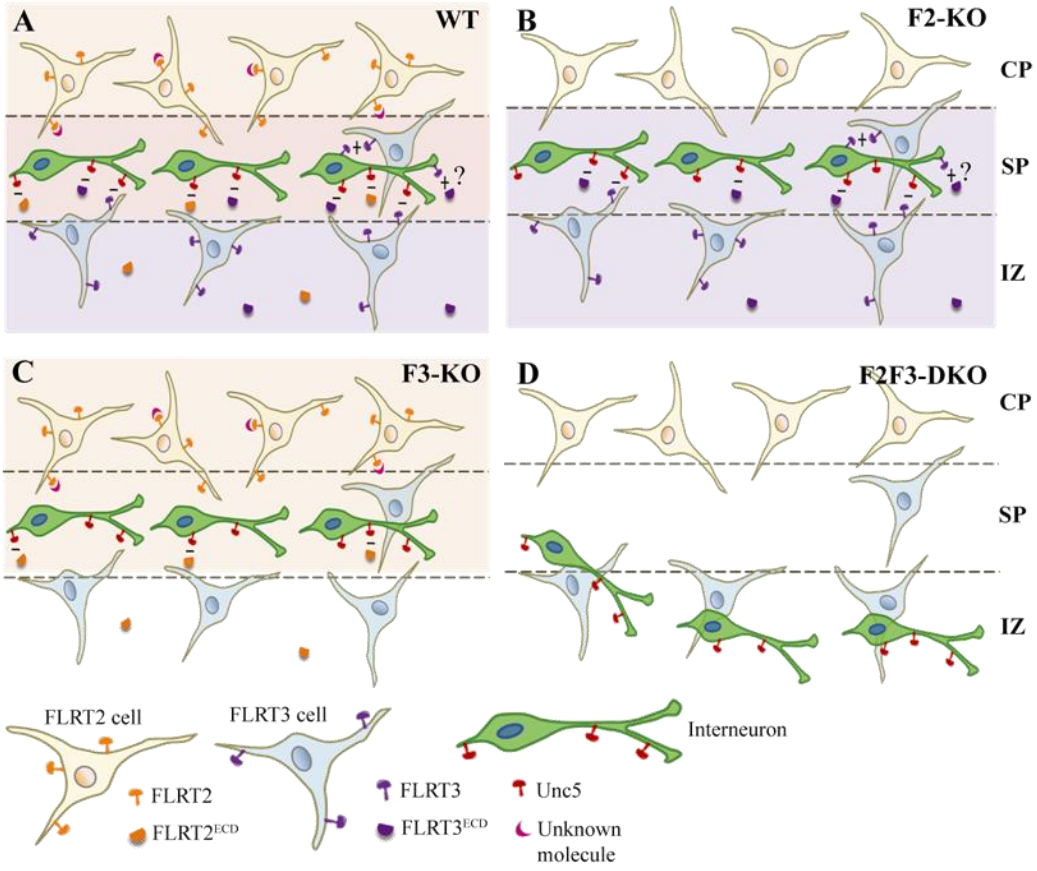

Figure 50. FLRT2 and FLRT3 cooperate in the control of tangential migration of interneuron through the SP stream. (A) In normal mice, FLRT2 protein (light pink) is expressed in the CP while FLRT3 (light blue) is expressed in the IZ, both are co-expressed in the SP. Migrating interneurons through the SP stream avoid entering the IZ due to the combination of the inhibitory action (negative signs) of FLRT2ECD (shed from the CP cells), FLRT3 bound to the membrane of migrating pyramidal cells and FLRT3ECD (present in the IZ). We postulate that this repulsion is produced by the interaction between FLRTs and the Unc5 receptors expressed in the interneurons. In addition, in the transit through the SP stream it could be that FLRT3-mediated adhesion mechanism also plays a role (positive signs and question marks in the panel). The inhibitory function of the membrane-bound FLRT2 in the CP is masked by an unknown molecule. (B) In the FLRT2-KO mice the interneurons are inhibited by the FLRT3 and FLRT3ECD present in the IZ. (C) In the FLRT3-KO mice the interneurons are inhibited by the FLRT2ECD present in the IZ. (D) In the FLRT2 and FLRT3-DKO the interneurons redistributed to the IZ which is devoid of these two important repulsive signals. Abbreviations: CP, cortical plate; IZ, intermediate zone; KO, knockout; DKO, double knockout.

Although the role of Rnd proteins in cortical neuron migration has been thoroughly investigated only in the last few years (Azzarelli et al., 2014; Heng et al., 2008; Nakamura et al., 2006; Pacary et al., 2011), it is becoming evident that Rnd proteins play important roles in cell migration during mammalian cortical development (Azzarelli et al., 2015a). Whereas the cellular and molecular functions of Rnd proteins have been thoroughly described n cortical projection neuron development, very little is known about their role in tangentially migrating cortical interneurons. This would be a fertile territory for future research. Nonetheless, the critical function of Rnd proteins in the control of neuronal migration has been further highlighted by a recent study showing the requirement of Rnd3 for the tangential migration of newborn olfactory neurons from the SVZ to the OB in the postnatal brain (Ballester-Lurbe et al., 2014).

Specifically, the Rnd functions in radial migration of projection neurons have been recently summarized (Azzarelli et al., 2015a). Respects to this, recently *in vivo* studies have shown that silencing Rnd3 in the embryonic cerebral cortex interferes with the interkinetic nuclear migration of RG stem cells, disrupts their apical attachment and modifies the orientation of their cleavage plane (Pacary et al., 2013). Also, Rnd2 and Rnd3, have crucial roles in different phases of cortical neuron migration through inhibition of RhoA signalling in different subcellular locations. Rnd2 controls the transition from the multipolar to the bipolar stage and the extension of the leading process while Rnd3 regulates locomotion (Heng et al., 2008; Pacary et al., 2011). Since Rnd proteins do not undergo the classical GTPase cycle, gene expression, protein post-transcriptional modifications and subcellular localization are predominant mechanisms that control Rnd activity (Chardin, 2006). In relation with last aspect, it has been recently shown that Rnd3 binds to a member of the Plexin family of axon guidance receptors, Plexin B2 (Azzarelli et al., 2014). The Semaphorin receptor, Plexin B2, has been implicated

in various aspects of cortical development, although its specific contribution to cortical neuron migration has been difficult to address because of earlier defects in Plexin B2 mutant cortices (Hirschberg et al., 2010). Rnd3 and Plexin B2 functionally interact in migrating neurons at the plasma membrane, and this interaction controls RhoA activity and cortical neuron migration *in vivo* (Azzarelli et al., 2014). Therefore, Rnd proteins finely orchestrate the levels of RhoA activity in migrating neurons, by directing its inactivation to specific subcellular compartments. This suggests that Rnd3 could intercat with other proteins at the plasma membrane, like FLRT3, to control the migratory behaviour of cortical neurons. In fact, FLRT3 was also identified as a target gene of Nodal signalling, inhibiting cadherin adhesion in *Xenopus* early development through interaction with the Rho family GTPase Rnd1 (Ogata et al., 2007). Moreover, Unc5B/FLRT3 complex regulates cell adhesion through the Rnd1 (Chen et al., 2009; Karaulanov et al, 2009; Ogata et al, 2007); however, all of these evidences have been tested during early *Xenopus* development but its role in neural development is unknown.

FLRTs function as homophilic CAMs (Karaulanov et al., 2006; Maretto et al., 2008; Müller et al., 2011), and very recently have been described the FLRT3-FLRT3 adhesive function in the neuronal migration context. As the small GTPase Rnd1 was shown to bind to FLRT3 and to mediate its effect on cell adhesion (Ogata et al., 2007), it is possible that other members of this family of Rho GTPases, such as Rnd3, could be potential downstream effectors of FLRT proteins. On the other hand and since Rnd1 acts downstream of Unc5B to mediate its cell deadhesion activity, it could also be that Rnd3 proteins interact with Unc5 and exert some of its effects in the nervous system, especially when Unc5 bind to FLRTs in *trans* (Yamagishi et al., 2011). Considering all these data, it is possible that during neuronal migration Rnd proteins are involved in the downstream pathways activated by FLRT homotypic interaction to control adhesion as well as

in the repulsive signaling triggered by Unc5s when activated by FLRTs. In the present work, we studied the possible functional interaction between FLRT3 and Rnd3, and also the pattern of tangential migrating interneurons in the developing brain of *Rnd3* mutant embryos.

When comparing expression pattern of *Rnd3* and *FLRT3* at E15.5, they are both expressed in thalamus, hippocampus and in the cerebral cortex, indicating that both proteins might be functionally related controlling the neuronal migration that, along with axon guidance are forming the complex neuronal circuitry. First, was assessed the physical interaction between these two proteins by *in vitro* assays. The co-immunoprecipitation assay revealed that Rnd3 interact specifically with the ICD domain of FLRT3 but does not interact with either FLRT2 or FLRT1. Moreover, Rnd3 and FLRT3 co-localize in transfected HeLa cells and the co-localization is increases upon stimulation of FLRT3 with specific antibodies, suggesting that the interaction can be regulated. These results, suggest that at least these two proteins can interact *in vitro*, and evidence that they may potentially function together *in vivo.*

Next, we analyzed the distribution pattern of tangentially migrating interneurons in the developing cortex of *Rnd3* mutant brains at E14.5. Surprisingly, it was found that there is a significant decrease in the number of CB^{+} interneurons reaching the dorsal cortex from the basal telencephalon and a shifted laminar distribution in the mutant cortices respect to controls; although is necessary to analyze more animals to corroborate these observations. These findings highlight the necessity of further studies that focus on the importance of Rnd3 in tangential migration of interneurons during development. Furthermore, during the study of these mutant brains, we observed an absence of TCAs crossing through the striatum, a phenotype that is currently being analyzed in our laboratory and that

confirms the important role of Rnd3 in neuron migration and axon guidance. Interestingly, the absence of FLRT3 produces abnormal pathfinding of TCAs *in vivo* (Leyva-Díaz et al., 2014). Thus, considering that both proteins can interact and that they also are co-expressed in the thalamus it is probably that FLRT3 and Rnd3 could be cooperate in the TCA projections.

Both neuronal migration and axon guidance are frequently studied as independent processes; however their coordination is required for establishing functional brain circuitry. Over the past few years, we have learned a great deal about the integrationof both mechanisms in some aspects of neural development, in particular in relation to the role of intermediate targets in axon guidance (Marín, 2010). Earlier studies have suggested that axons can provide a substratum for non-radial neuronal migration in the developing CNS including the cerebral cortex (Gray et al., 1990; Rakic, 1985). In the case of the tangentially migrating GABAergic interneurons derived from the MGE, this substratum has been suggested to be the developing axons of the corticofugal fiber system (Parnavelas, 2000). These cells use the corticofugal system as a scaffold to reach their positions indifferent layers of the cortex. According to this, it has been demonstrated that the adhesion molecule TAG-1, expressed by corticofugal axons, provides a substrate for the migrating neurons, lending support to the notion of common cues for axon guidance and neuronal migration (Denaxa et al., 2001). However, on the other hand, there were some other published results that reveals that the population of tangentially migrating cells within the ventral telencephalon are essential for TCAs navigation through this part of the developing brain, a process apparently mediated by Neuregulin-1/ErbB4 short- and long-range signaling (López-Bendito et al., 2006). More in detail, TCAs projection depends on the tangential migration of apopulation of the LGE-derived interneurons, which create permissive corridor forTCAs navigation in an otherwise inhibitory

MGE environment (López-Bendito et al., 2006; Molnar et al., 2012). Also, MGE- and CGE-derived GABAergic neurons use Sema3A- and ephrin-signaling pathways, as well as cell-adhesion mechanisms, to control callosal axon navigation (Niquille et al., 2009, 2013).

Therefore, having found a tangential migration and TCA navigation defects simultaneously in the *Rnd3* KOs, it is tempting to suggest that the analysis of these mutant embryos may clarify some crucial questions about the mechanism governing these two processes closely related. Also, FLRT3 functions have been shown in the radial migration together with Unc5B (Yamagishi et al., 2011), in TCA pathfinding together with Robo1 (Leyva-Díaz et al., 2014) and in tangential migration of interneurons, as it has been shown in the present work. These observations encourage us to keep investigating the possible relation between Rnd3 and FLRT3 both *in vitro* and *in vivo* and analyze the consequence to eliminate both Rnd3 and FLRT3 by a mouse model.

CHAPTER 6: CONCLUSIONS AND OUTLOOK

6. CONCLUSIONS AND FUTURE OUTLOOK

1.) FLRT2 and FLRT3 are expressed in the E14.5 mouse cerebral cortex, specifically in the CP and IZ, respectively; overlapping their expression in the SP.

2.) The CB^+ interneurons disperse tangentially through the three mainly streams (MZ, SP and IZ/SVZ), following routes that mainly avoid the CP and IZ, where FLRT expression is enriched.

3.) The single disruption of *FLRT3* by the nervous specific Cre lines, *Nestin-Cre* or *Sox1-Cre*, does not affect the tangential migration of CB^+ interneurons at E14.5.

4.) The single disruption of *FLRT2* by the nervous specific Cre line, *Sox1-Cre*, does not affect the tangential migration of CB^+ interneurons at E14.5.

5.) The simultaneous disruption of both *FLRT2* and *FLRT3* with the nervous system specific Cre line, *Sox1-Cre*, leads to a phenotype that affects the intracortical distribution of CB^+ interneurons with a decrease in the number of CB^+ cells through the SP stream and an increase of CB^+ cells in the IZ, at E14.5.

6.) The tangential progression of CB^+ interneurons along the SP stream is decreased in the F2F3 DKO at E14.5.

7.) The total number of CB^+ interneurons migrating into the CP at E16.5 in the double *FLRT2* and *FLRT3* KOs is reduced.

8.) Morphology and laminar pyramidal-cell structure of the cortex of the double *FLRT2* and *FLRT3* KO brains is largely unafected.

9.) Deletion of both *FLRT2* and *FLRT3*, with the palial (pyramidal neurons) specific Cre line, *Emx1-Cre*, shows similar intracortical migration phenotypes as the whole nervous system deletion of the two genes.

10.) *FLRT2* and *FLRT3* deficient interneurons migrate normally *in vitro* compared to controls.

11.) Rnd3 is expressed in the cerebral cortex (CP, VZ) and in the basal telencephalon (VZ, SVZ), thalamus and hippocampus of the developing mouse brain.

12.) Expression pattern of Rnd3 overlaps with that of FLRT3 in several regions including, the cerebral cortex, thalamus and hippocampus.

13.) Rnd3 interact physically and specifically with FLRT3 *in vitro*, in heterologous cells. This interaction takes place in *cis* and requires the intracellular domain of FLRT3.

14.) The *Rnd3* $^{gt/gt}$ mutant mice have a decrease in the total number of CB^{+} interneurons reaching the dorsal cortex.

15.) Prelimiar results indicate that the distribution of tangentially CB^{+} interneurons migrating within the cortex is disrupted at E14.5.

6.1 Future outlook

Although lot of questions remains to be elucidated the current study demonstrate that there is a wide range of molecular cues and complex mechanism controlling the interneurons journey during cerebral cortex development. Importantly, points to that the attractive mechanism is not exclusively guiding the interneurons, instead here it is shown that inhibitory mechanisms may also contribute to ensure the proper allocation of the neurons in the cerebral cortex. Arriving here with these findings it is important to note that, nevertheless, further experiments are necessaries to elucidate the molecular signaling pathways triggered by FLRTs that regulates interneuron migration and distribution within the developmental cortex. Indeed, in our laboratory we are already performing *in vitro* studies as for example the stripe assay to demonstrate the tentative proposal that FLRTs are chemorepulsives cues. The preliminary results show that in fact, FLRTs act as inhibitory molecules for MGE-derived interneurons *in vitro.* Besides this, it would be interesting to study the morphology of the affected interneurons in the F2F3-DKO cortices and see how the lack of FLRTs modifies some morphological parameters of the leading process. This could be easily studied (even by live imaging) by by doing a conditional deletion of FLRTs in the MGE (*Nkx2.1-Cre* line, for instance) together with an IRES-GFP if such line would be available or crossing with a lox-STOP-lox-GFP reporter mouse line. Thus, it could be studied the angle of leading processes, their branching morphology, if they are polarized along the dorsal tangential dimension or are non-continuous with their cell bodies, suggesting changes in the orientation respect to the cortical plane of section as in the Myers and collaborators study (Myers et al., 2014). Also, the speed of these affected interneurons could also be analyzed in these mice. In addition to these experiments, the analysis of the Unc5B/Unc5D-DKO, would be important to address the hypothesis if these candidate receptors are involved in this system. Also, the analysis of the distribution of interneuron subtypes in the F2F3-DKO

postnatal cortices will provide a more complete understanding of the molecular mechanism and the consequences behind the phenotype found.

Another important aspect which necessarily must be considered is the balance between the repulsion and cell adhesion in cortical development by FLRTs proteins (Seiradake et al., 2014). Recently has been described that the adhesive FLRT3-FLRT3 interaction in *trans* is required for the spatial arrangement of pyramidal neurons in the tangential axis. This separation of adhesive and repulsive functionalities allows FLRTs to regulate the behavior of migrating pyramidal neuronsin distinct ways; FLRT2 repels Unc5D^{+} neurons and thereby controls their radial migration, while FLRT3-FLRT3 homophilic interactions regulate their tangential distribution. We speculate that these cell-cell communication mechanisms operating during radial and tangential patterns of migration of pyramidal neurons, suggest that also interneurons integrate these adhesive and repulsive effects. First, we have seen that cortical interneurons express certain levels of FLRTs proteins. If we can confirm this expression then, it would be interesting to check whether FLRT3 is involved in the adhesive properties of migrating interneurons and thereby participates in the delicate balance of adhesion/repulsion necessary for cell migration (Cooper, 2013). Since, the adhesive FLRT interaction reduces the repulsive response triggered by FLRT-Unc5 interaction in a combinatorial way; it could be studied whether the repulsive effect ofFLRTs on MGE-derived interneurons in the stripe assay is enhanced in interneurons lacking FLRT3.

The possibility that FLRTs regulate adhesion in migrating interneurons open the possibility that they could trigger, in a cell-autonomous manner, some intracellular signaling pathways in these cells. It would be therefore interesting to analyze gene expression in the GFP labeled cortical interneurons and compare gene expression using microarrays of FACS purified cells. In addition, to assess

the non-cell autonomous role of these two proteins, it will perform the crossing between the FLRTs conditional KOs mice and *Nkx2-1-Cre* line in order to remove *FLRT2* and *FLRT3* from the MGE-derived interneurons destined to the cortex.

CHAPTER 7: PUBLICATIONS AND MEETINGS

7. PUBLICATIONS AND MEETINGS

Spinet, C., Gonzalo, H., Fleets, C., Menal, M.J., Elea, J., 2015. Oxidative Stress and Neurodegenerative Diseases: A Neurotrophic Approach. *Current Drug Targets.* 16, 20-30.

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CHAPTER 8: REFERENCES

8. REFERENCES

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