Universitat de Lleida

TESI DOCTORAL

ROLE OF CYTOPLASMIC CYCLIN D1 IN RADIAL MIGRATION OF CORTICAL NEURONS DURING BRAIN DEVELOPMENT

Daniel Rocandio Durán

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida

Programa de Doctorat en Salut

Director
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IRBLleida
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Memòria de tesi doctoral presentada per Daniel Rocandio Durán per optar al grau de Doctor per la Universitat de Lleida (Programa de Doctorat en Salut).

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

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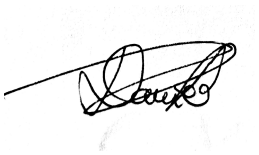
Doctorand



Daniel Rocandio Durán

Director de tesi

Joaquim Egea Navarro

A mis padres,

A Esther,

Os quiero

"Toda persona puede ser, si se lo propone, escultor de su propio cerebro"

Santiago Ramón y Cajal

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INDEX

INDEX

ABBREVIATIONS

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CDK	Cyclin-Dependent Kinase
CKIs	Cyclin-dependent Kinase Inhibitors
CP	Cortical Plate
CSF-1	Colony Stimulating Factor-1
CycD1	Cyclin D1
DCX	Doublecortin
ECM	Extracellular Matrix
EdU	5-Ethynyl-2′-deoxyuridine
FAs	Focal Adhesions
FGFR	Fibroblast Growth Factor Receptor
G1	GAP 1
GFP	Green Fluorescent Protein
GTP	Guanosine-5’-triphosphate
IP	Intermediate Progenitor
IZ	Intermediate Zone
Lis1	Lisencepahly 1
MAPs	Microtubule-Associated Proteins
MZ	Marginal Zone
PAK1	Serine/threonine-proteinkinase
PCR	Polymerase Chain Reaction
PP	Preplate
pRb	Retinoblastoma tumor suppressor protein
Rho	Ras Homologous
RGC	Radial Glial Cell
SP	Subplate
SVZ	Subventricular Zone
VZ	Ventricular Zone
WT	Wild-Type

ABSTRACT

ABSTRACT

Cyclin D1 may interact with CDK4 or CDK6 to form an active complex in the cell nucleus promoting the step from G1 to S phase of cell cycle. Recently, it has discovered that cancer cells overexpress Cyclin D1 to the tumor growth, but also it has seen that Cyclin D1 can exit from the nucleus to increase the metastasis capacity due to the recently discovered function of adhesion and migration observed also in the cytoplasm of keratinocytes, fibroblasts and macrophages. In this context, our group study molecular mechanisms that may mediate in the murine embryonic neurogenesis, such neuronal migration and neuroprogenitors adhesion to the extracellular matrix and other nervous system cells. By this way, our hypothesis is related with the possible cytoplasmic expression of Cyclin D1 in the embryonic nervous system to participate in the neuronal migration. Through immunofluorescence, we have seen that Cyclin D1 is expressed in the cytoplasm of the RGCs processes and in the basement membrane of telencephalon. This expression occurs parallel to the neurogenetic ventro-dorsal gradient. In addition, it was performed the intraventricular injection and *in utero* electroporation assay to overexpress Cyclin D1 and its CDK4/6 negative dominant. Furthermore, CycD1$^{-/-}$embryos and postnatal mice were analyzed. Our results suggest that Cyclin D1 may controls radial migration through the activity regulation of cytoskeleton of migrating neurons in different phases and localizations. This participation would be essential for the correct layer V formation in the adult cerebral cortex.

RESUMEN

Ciclina D1 puede interaccionar con CDK4 o CDK6 para formar un complejo activo en el núcleo celular que promueve el paso de la fase G1 a S en el ciclo celular. Recientemente, se ha descubierto que células cancerígenas sobreexpresan Ciclina D1 para el crecimiento tumoral, pero también se ha visto que Ciclina D1 puede salir del núcleo para incrementar la capacidad metastásica gracias a la reciente función descubierta de adhesión y migración observada también en el citoplasma de queratinocitos, fibroblastos y macrófagos. En este contexto, nuestro grupo estudia mecanismos moleculares que pueden intervenir en la neurogénesis embrionaria murina, como por ejemplo la migración neuronal y la adhesión de neuroprogenitores a la matriz extracelular y otras células del sistema nervioso. De este modo, nuestra hipótesis está relacionada con la posible expresión citoplasmática de Ciclina D1 en el sistema nervioso embrionario para participar en la migración neuronal. A través de immunofluorescencia, hemos visto que Ciclina D1 se expresa citoplasmáticamente en los procesos de las células de glía radial y en la membrana basal del telencéfalo. Esta expresión ocurre paralela al gradiente ventrodorsal neurogenético. También se realizó la inyección intraventricular y la electroporación *in utero* para sobreexpresar Ciclina D1 y su dominante negativo de CDK4/6. Además también se analizaron embriones y ratones postnatales $CycD1^{-/-}$. Nuestros resultados sugieren que Ciclina D1 puede controlar la migración radial gracias a la regulación de la actividad del citoesqueleto y la adhesión neuronal en diferentes fases y localizaciones. Esta participación sería esencial para la correcta formación de la capa V de la corteza cerebral adulta.

RESUM

Ciclina D1 pot interaccionar amb CDK4 o CDK6 per formar un complex actiu en el nucli cel·lular que promou el pas de la fase G1 a S en el cicle cel·lular.

Recentment, s'ha descobert que cèl·lules cancerígenes sobreexpressen Ciclina D1 per al creixement tumoral, però també s'ha vist que Ciclina D1 pot sortir del nucli per incrementar la capacitat metastàsica gràcies a la recent funció descoberta d'adhesió i migració observada també en el citoplasma de queratinòcits , fibroblasts i macròfags.

En aquest context, el nostre grup estudia mecanismes moleculars que poden intervenir en la neurogènesi embrionària murina, com ara la migració neuronal i l'adhesió de neuroprogenitors a la matriu extracel·lular i altres cèl·lules del sistema nerviós. D'aquesta manera, la nostra hipòtesi està relacionada amb la possible expressió citoplasmàtica de Ciclina D1 en el sistema nerviós embrionari per participar en la migració neuronal.

A través d'immunofluorescència, hem vist que Ciclina D1 s'expressa citoplasmàticament en els processos de les cèl·lules de glia radial i en la membrana basal del telencèfal. Aquesta expressió passa paral·lela al gradient ventrodorsal neurogenètic. També es va realitzar la injecció intraventricular i l'electroporació in utero per sobreexpressar Ciclina D1 i la seva dominant negativa de CDK4 / 6. Es van analitzar embrions i ratolins post-natals CycD1 - / -. Els nostres resultats suggereixen que Ciclina D1 pot controlar la migració radial gràcies a la regulació de l'activitat del citoesquelet i l'adhesió neuronal en diferents fases i localitzacions. Aquesta participació seria essencial per a la correcta formació de la capa V de l'escorça cerebral adulta.

INTRODUCTION

1. INTRODUCTION

Cyclins are a broad family of approximately 29 proteins in humans, collected in three major groups and 16 subfamilies: Cyclin B group (A, B, D, E, F, G, J, I and O); Cyclin Y group; and Cyclin C group (C, H, K, L and T) (Ma et al. 2013). The structural characteristic of these proteins is the presence of the "cyclin box", a nearly 100 amino acid residues domain that forms a stack of five α-helices. The majority of cyclins have two cyclin boxes, one amino-terminal box for bind and activate cyclin-dependent kinases (CDKs), a conserved family of catalytic proteins, and a carboxy-terminal region domain that allows its threonine phosphorylation for the regulation of cyclins proteolysis (Malumbres 2014).

Many of cyclins participate in the regulation of the passage from one cell cycle phase to another. They control and respond to a variety of external and internal cues to ensure the proper regulation of cell cycle. The activity of specific cyclin-CDK complexes rises and falls as the cell progresses through the cell cycle, and these oscillations lead directly to cyclical changes in phosphorylation of intracellular proteins that initiate or regulated the majority of events of the cell cycle (Alberts et al. 2002; Pines 1995). Therefore, we could name cyclins as the regulators that decide the cell cycle synchronization, while CDKs are the effectors that turn on the machinery of every cell cycle state. This kinase activity is also regulated by a pool of CDK inhibitors (CKIs), which under unfavorable conditions interrupt the cyclin-CDK complex activity (Lim and Kaldis 2013;Morgan 2007).

Although, CDK1 appears to be the only essential CDK for the control of the mammalian cell cycle (Santamaría et al. 2007), CDKs and cyclins reveal their absolute requirements in some specific tissues and cellular processes: CDK6 and Cyclin A2 in erythropoiesis and more generally in the haematopoietic lineage, Cyclin D3 for lymphocyte development and T cell leukemia (Sicinska et al. 2003), CDK4 in the pancreas, and CDK2 in meiosis (Bendris, Lemmers, and Blanchard 2015).

The passage from G1 to S phase in cell cycle is controlled, in the first period, by D-type, and, at the end of G1 through most S phase by E-type cyclins (Roberts 1999). The

complexes formed with their corresponding CDKs phosphorylate the retinoblastoma tumor suppressor protein (pRb), releasing the E2F transcription factor which trigger the necessary pathway for cell cycle progression (Bendris, Lemmers, and Blanchard 2015).

1.1. D-type cyclins

In mammalian cells have been enumerated three D-cyclins (Cyclin D1 (CycD1), Cyclin D2, and Cyclin D3) (Kiyokawa et al. 1992; Xiong et al. 1991). The three proteins are encoded by separate genes located on different chromosomes, but show significant amino acid similarity and are closely related in protein domain structure (Inaba et al. 1992; Xiong et al. 1991).

The expression levels of D-cyclins are inducted largely by the extracellular environment by the presence of external mitogens, and their levels decline when mitogens are removed or when anti-mitogens are added (Matsushime et al. 1994). Therefore, the activation of their corresponding CDKs (CDK4 and CDK6) to progress through cell cycle (Bates et al. 1994; Meyerson and Harlow 1994; Tam et al. 1994) depend of the extracellular environment that link the mitogenic pathways to the formation of cyclin D-CDK4/6 complex (Malumbres et al. 2009). This complex phosphorylates their numerous target substrates, not only pRb required for the transition from G1 to S phase of cell cycle, but also pRb-like proteins involved in centrosome duplication, mitochondrial function, DNA damage response, cell growth, cell adhesion and motility, and cytoskeletal modeling (Malumbres et al. 2009).

In addition, D-type cyclins interact with diverse chromatin-modifying enzymes and transcription factors to regulate different pool of genes to play non-catalytic roles involved in proliferation, differentiation, migration, DNA damage response, and, importantly for cell cycle progression, sequestration of CKIs (Musgrove et al. 2011).

D-type cyclins are regarded as developmental regulators, since during mouse embryogenesis, are expressed in a dynamic and highly expression pattern in different tissues, often in mutually exclusive cell types, suggesting that a unique promoter

exist in the three genes (Ciemerych et al. 2002; Kozar et al. 2004). Within the early embryo, cyclins D1 and D2 transcripts are first detected before differentiation of progenitors of the epiblast. In the other hand, Cyclin D3 transcript is just detectable in extraembryonic tissues of epiblast and trophoblast. In many developing tissue, Cyclin D1 and Cyclin D2 show specific expression levels in opposite localizations. For instance, during hindbrain development, Cyclin D1 and Cyclin D2 display opposite, showing a highly specific expression pattern (Wianny et al. 1998). In the developing skin, keratinocytes express Cyclin D1, and Cyclin D2 is absent, while the opposite happens in developing hair follicles. Within the developing nervous system, cyclins D1 and D2 are expressed in distinct proliferating compartments. This specific, often mutually exclusive pattern of D-cyclin expression is also preserved in several organs of the adult animals (Ravnik, Rhee, and Wolgemuth 1995; Robker and Richards 1998).

Although the activation of CDK4/6 by all three D-type cyclins leads to consider redundant functions, reports in mice expressing only one single D-type cyclin show focused abnormalities in particular tissues depending de protein expressed (Ciemerych et al. 2002), suggesting that individual D- type cyclins are required for specific roles in proliferating and differentiating cell populations during embryo development.

1.1.1. *Ccnd1*

The mouse *Ccnd1* gene (13,4kb) encodes the CycD1 protein. In recent years it has learnt about other cell cycle non-related functions of CycD1 (Fu et al. 2004; Pestell 2013). Nuclear CycD1 forms physical associations with a wide range of transcription factors, co-activators and co-repressors that influence histone acetylation (Fu et al. 2004) and chromatin remodeling proteins (C. Wang et al. 2004), controlling cellular metabolism, adipogenesis (Lamb and Ewen 2003; C. Wang et al. 2004), differentiation (Hulit et al. 2004), nuclear hormone receptor responses (McMahon et al. 1999; Reutens et al. 2001) and growth (Ma et al. 2013), inhibition of mitochondrial metabolism (C. Wang et al. 2004; Sakamaki et al. 2006) and other functions related with the nuclear

localization of CycD1 (Fig. 1) (Fu et al. 2004; Pestell 2013). The CycD1-CDK4/6 complex also is related with roles in centrosome duplication, mitochondrial function, cell growth, cell adhesion and motility, and cytoskeletal modeling (Fig. 1).

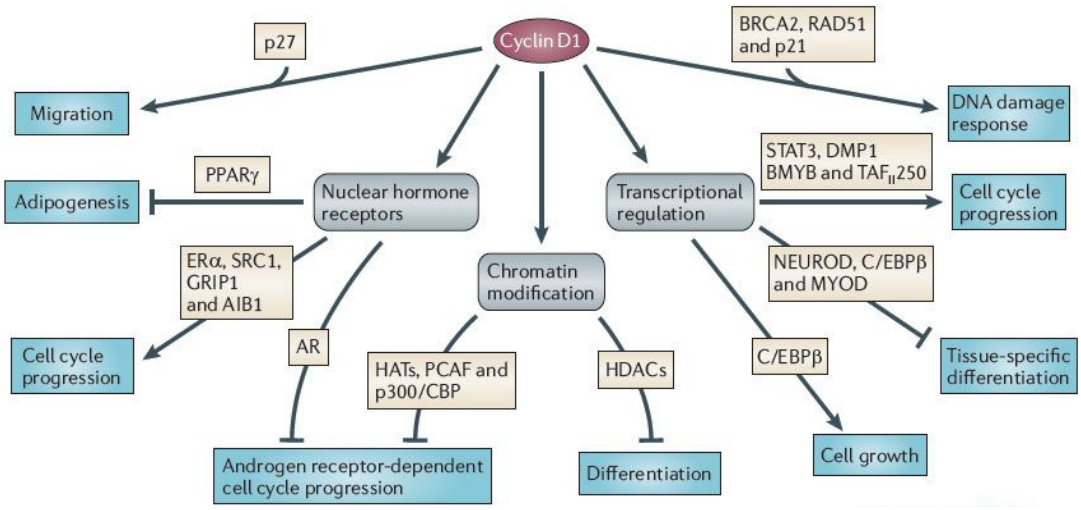

Image 1. Cyclin D1 (CycD1) functions besides cell cycle.
Schematic representation of CycD1 interactions with different molecules in diverse tissues. Depending the interaction CycD1 controls different processes (Musgrove E, 2011).

1.1.2. *Ccnd1* deficient mice phenotype

Anatomic analysis of mice lacking *Ccnd1* revealed a significant degree of embryonic lethality and underdeveloped when compared with their heterozygous and wild-type littermates (Fantl et al. 1995; Sicinski et al. 1995). Furthermore, there was observed a misalignment of the incisor teeth, leading to their excessive growth (Fantl et al. 1995; Sicinski et al. 1995).

The histological study showed a striking reduction in thickness and organization of the retinal layers, due to a severe reduced ability of mutant retinal cell precursors to proliferate during embryonic development (Fantl et al. 1995; Sicinski et al. 1995). In addition, *Ccnd1*$^{-/-}$ females displayed poor lobuloalveolar development and very little secretory activity (Fantl et al. 1995; Sicinski et al. 1995). These mentioned defects could be expected knowing the cell cycle function of CycD1 but attending to the abnormal limb reflex phenotype observed in knock-out mice (Fantl et al. 1995; Sicinski

et al. 1995), we could suggest an irregularity in the functioning of the nervous system, since in several neurologic mutants the clasping of the limbs has been reported (Urbánek et al. 1994). Remarkably, all the tissues analyzed developed normally in Cyclin D2 or Cyclin D3 deficient animals, revealing a unique requirement for CycD1 in vivo in selected tissues (Sicinska et al. 2003; Sicinski et al. 1995).

1.1.3. *Ccnd1* in cancer

Ccnd1 is also known as an oncogene, since its and/or CDK4/6 expression dysregulation can lead to prolonged CycD1-CDK4/6 activation, giving cancer cells the power to enter the cell cycle continuously or shortening their cell cycle increasing the proliferation activity (Choi and Anders 2014). Moreover, may triggers aberrant cellular growth and tumorigenesis (Weinstat-Saslow et al. 1995), enhancing angiogenesis and resistance to apoptosis (Tashiro, Tsuchiya, and Imoto 2007), originating a variety of human cancer, including mantle cell lymphoma (Jares, Colomer, and Campo 2007), non-small cell lung cancer (Jin et al. 2001), and the majority of breast carcinomas (Barnes and Gillett 1998), head, neck (Bartkova et al. 1995), and esophagus (Shamma et al. 2000).

The *Ccnd1* overexpression in tumor cells may occur as a result of copy number alterations, or more rarely, by mutation and chromosomal translocations, gene amplification or the disruption of trafficking, protein stabilization or proteolysis mechanisms (Haverty et al. 2008), or as a consequence of an aberrant transcriptional signaling downstream of oncogenes such as ERBB2 (Lee and Muller 2010). Clinical studies have revealed that transgenic mice overexpressing *Ccnd1* in the mammary gland develop breast carcinoma (T. C. Wang et al. 1994), whereas mice lacking *Ccnd1* are resistant to different oncogene-induced tumorigenesis (Robles et al. 1998; Yu, Geng, and Sicinski 2001).

In vitro fibroblasts experiments demonstrated that overexpression of *Ccnd1* increased the expression of fibroblast growth factor receptor-1 (FGFR-1) and fibroblast growth factor receptor-2 (FGFR-2), sensitizing the cells to growth stimulation by bFGF, molecule produced by stromal cells enhancing adjacent tumor progression (Tashiro, Tsuchiya, and Imoto 2007).

1.1.4. Cytoplasmic CycD1 controls cell detachment and motility

Recent studies in fibroblasts, keratinocytes and epithelial cell lines (Fernández et al. 2011; Fernández-Hernández et al. 2013) show the interaction of CycD1 in cytoplasmic foci with Ral A, Ral B and Sec6, small GTPases that regulate exocyst assembly and participate in the control of cell attachment and migration by integrin recycling or/and by targeting the exocyst to focal adhesion complexes (Bodemann and White 2008; Rosse et al. 2006).

Other analysis with *Ccnd1*-deficient primary bone macrophages revealed altered cell morphology, increased adhesion, and decreased motility and chemotaxis toward Colony Stimulating Factor-1 (CSF-1). In addition, the cells were constitutively well spread and attached, yielding a flattened, circular morphology with reduced membrane ruffles. This phenotype has been attributed to the nuclear role of CycD1 as a transcriptional regulator of genes controlling cell adherence and migration. However, the attachment of macrophages was mediated via the increment of numbers of circumferentially arrayed focal complexes rich tyrosine-phosphorylated paxilin (Neumeister et al. 2003), described as a structural and regulatory component of focal adhesions (FAs) (Glenney 1989; Turner, Glenney, and Burridge 1990), the macromolecular assemblies through which the cytoskeleton connects to the extracellular matrix. Furthermore, it has discovered the interaction and phosphorylation of paxilin by CycD1/CDK4 complex in membrane ruffles of normal fibroblasts and tumor cells (Fusté et al. 2016).

In addition, reports with MCL cell lines and primary tumor cells have been described the correlation of subcellular distribution of CycD1 with the natural physiology of cancer cells. Tumor cells proliferation may be controlled by nuclear CycD1, whereas tumor cells invasiveness may be controlled by the cytoplasmic fraction of the protein (Body et al. 2017). This function is dependent on CDK4-associated kinase activity and independent of pRB. Recently, it has been demonstrated that there exists an association between the levels of CycD1–CDK4 activity and invasiveness and metastasis development in MDA-MB-231 breast cancer cell lines (Zhong et al. 2010). Previously, CycD1 overexpression had already been correlated with tumor metastasis (Drobnjak et al. 2000).

These data suggest that cytoplasmic CycD1 overexpression in cancer cells could contribute to the invasiveness and/or metastatic phenotype, independently of effects on proliferation, by regulating cellular adhesiveness and motility through the collaboration with Rho GTPases, and, in the same manner, CycD1 may control cell migration mechanisms in normal physiology context.

1.2. Migration in the developing cerebral cortex

Nervous system is the organ through which animals perceive, interpret, and respond to the world around them. They consist of specialized, electrically active cells connected together in networks resulted from a precisely regulated process that is critical for the development of brain architecture. Neurons that constitute the cerebral cortex must migrate long distances from their place of birth, and through several anatomical boundaries, to reach their final position within the correct cortical layer, following complex routes, changing direction at landmarks along the way (Cooper 2013).

Recent investigations have yielded new insights into the mechanisms that regulate migration and have pointed to migration abnormalities in several naturally occurring genetic defects in humans (Gleeson and Walsh 2000; Lambert de Rouvroit and Goffinet 2001).

In the developing brain, neurons arise from the proliferative epithelium that covers the ventricular space throughout the neural tube, an area named the ventricular zone (VZ). Two modes of migration have been identified: radial and tangential (Hatten 1999; Marín and Rubenstein 2003) (Fig. 2).

In tangential migration, GABAergic inhibitory interneurons, which arise from the two proliferating cell masses of the ventral telencephalon named lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE), migrate into cortex parallel to the surface of the brain along axons or other neurons and often transgress regional boundaries (Nadarajah et al. 2002). Radial migration, the principal mode of migration of glutamatergic excitatory projection neurons (or pyramidal neurons), is

characterized because neuroprogenitors originated in the VZ of cerebral cortex move orthogonal to the surface of the brain oriented to radial glia fibers that span the entire depth of the parenchyma (Ayala, Shu, and Tsai 2007; Marín and Rubenstein 2003; Marin et al. 2010).

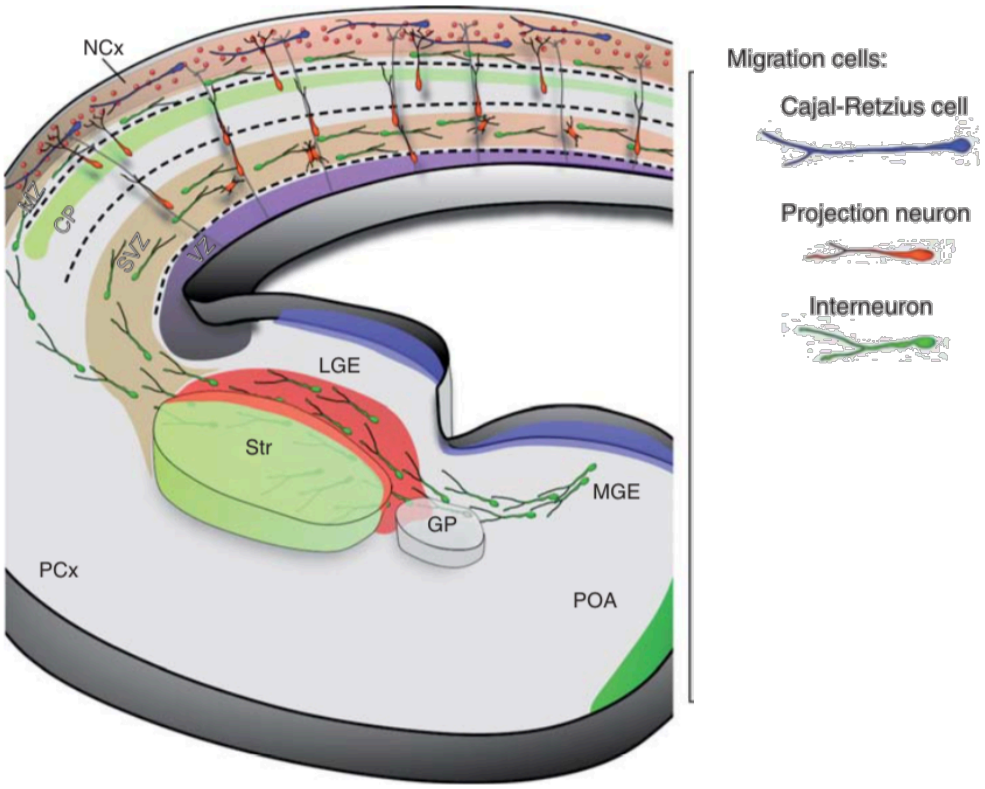

Image 2. Schematic representation of the two modes of neuron migration along telencephalon. The schema shows a coronal slice of the telencephalon, in which the cortex is populated by two types of neurons. Interneurons or GABAergic neurons, originated in the ventral telencephalon, perform tangential migration through subpalio. In the other hand, glutamatergic projection neurons origin in the ventricular zone of the cortex and migrate radially to upper layer of the cortex. (Marín O, 2010).

1.2.1. Neurogenesis in the cerebral cortex

Pyramidal cortical neurons arise from the undifferentiated neuroepithelial progenitor cells in the proliferative pseudostratified epithelium at the surface of the embryonic cerebral ventricles, establishing the ventricular zone (VZ) (Rakic 1990). Prior to the onset of neurogenesis, radial glial cells (RGCs), bipolar cells with the cell body in the VZ and radial fibers that extend to the pial surface, undergo symmetric cell division to produce two daughter cells that adopt the progenitor fate, expanding their population

(Ayala, Shu, and Tsai 2007; Bystron, Blakemore, and Rakic 2008; Götz and Huttner 2005; Koizumi et al. 2006; A. Kriegstein, Noctor, and Martínez-Cerdeño 2006). As neurogenesis begins, the majority of RGCs in the VZ divide asymmetrically with two modes of cell division, with different results (Koizumi et al. 2006; Noctor et al. 2001; Noctor et al. 2004; Noctor, Martínez-Cerdeño, and Kriegstein 2008). The neurogenic division produces a self-renewing RGC and a daughter neuron, and the progenitor division results in a self-renewing RGC and an intermediate progenitor (IP) (A. Kriegstein, Noctor, and Martínez-Cerdeño 2006; Noctor et al. 2004; Noctor, Martínez-Cerdeño, and Kriegstein 2008).

The IPs are multipolar cells, which migrate away from the ventricular surface to undergo symmetrical division in the other proliferative layer of the developing cortex so-called subventricular zone (SVZ). In the SVZ, symmetric divisions allow the increase of IPCs population, until they divide symmetrically to produce two neurons, playing a critical role in cortical development by expanding the neuronal population (A. Kriegstein, Noctor, and Martínez-Cerdeño 2006; Martínez-Cerdeño, Noctor, and Kriegstein 2006; Noctor et al. 2004; Noctor, Martínez-Cerdeño, and Kriegstein 2008).

The first cohort of postmitotic neurons leaves the germinal VZ to constitute the transient primordial plexiform layer or preplate (PP) (E11) (Caviness, Takahashi, and Nowakowski 1995). The subsequent wave of neuronal migration splits the PP into two layers: the more superficial marginal zone (MZ), which consists of the Cajal-Retzius cells born in the first wave of migration; and the deeper subplate (SP), which is constituted by the rest of the primordial cells (Ayala, Shu, and Tsai 2007; Ghashghaei, Lai, and Anton 2007; Kwan, Sestan, and Anton 2012; Marín and Rubenstein 2003; Marin et al. 2010).

Successive waves of migrating neurons arrive to occupy progressively layers II-VI of cerebral cortex in an inside-out pattern. Neurons generated earlier reside in deeper layers, whereas later-born neurons migrate past existing layers to form superficial layers, forming the cortical plate (CP) (Angevine and Sidman 1961; Rakic 1990). Between CP and the SVZ appears the intermediate zone (IZ), a layer that will eventually contain the afferent and efferent axons of the cortex (white matter).

1.2.1.1. Somal translocation and glia-dependent locomotion

Neurons destined to migrate radially adopt two separate modes of movement: earliest born neurons use somal translocation during the early stages of corticogenesis to form the PP, when the cerebral wall is still relatively thin and distances that neurons need to migrate are relatively short; by contrast, glia-dependent locomotion is required to guide CP neurons along more convoluted (and longer) paths in complex cortices during the later stages of CP formation, when the cerebral wall is considerably thicker and need the RGCs support to reach to their place (A. R. Kriegstein and Noctor 2004; Marín and Rubenstein 2003; Nadarajah and Parnavelas 2002; Nadarajah et al. 2001).

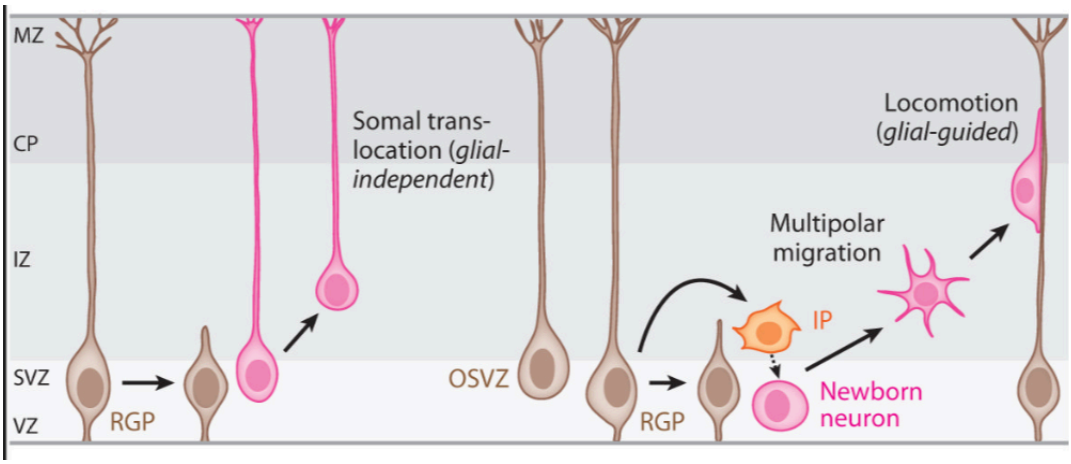

Image 3. Schematic representation of the two modes of radial migration.
The left side of the schema shows somal translocation mode used by early-born neurons which attach with their leading process to basement membrane. In the right side, late-born neurons migrate to SVZ to differentiate to intermediate progenitor cells, which divide and differentiate. Then, they attach to RGCs which us as scaffold to migrate to CP. (Evsyukova I, 2013).

Nevertheless, these two modes are not cell-type specific, because many locomoting cells switch to somal translocation when the leading process reaches the pial surface at the final stage of their migration (Nadarajah et al. 2001). In both movement modes, the migrating neurons are characterized by the important role of their basal process so-called leading process, with structures that are similar to the growth cones of migrating axons, and as such are thought to play an important role in sensing the surrounding microenvironment and guide neurons (Rakic 1990; Yee et al. 1999) (Fig. 3).

Some studies report that somal translocation is an older mode of movement in the evolution of the cerebral cortex, because PP and early CP neurons that are generated at the onset of corticogenesis are phylogenetically older, whereas later generated cells are a more recent evolutionary addition ((Marin-Padilla 1978; Goffinet 1983).

Somal translocation

The cells that undergo somal translocation typically have a long, branched radially oriented basal process attached to the pial surface, and a short, transient trailing process which is actually the axon, growing from the rear (T Miyata et al. 2001; Nadarajah et al. 2002; Tabata and Nakajima 2003; A. R. Kriegstein and Noctor 2004; Noctor et al. 2004; Sakakibara et al. 2014). After terminal division, translocating cells extends their leading process radially from the VZ and remain attached to the pial surface, while soma moves upward by nucleokinesis continuously (60 μm/h), without significant pausing (Nadarajah et al. 2001).

During the nucleokinesis of migrating neurons, a cytoplasmic swelling form 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 et al. 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 by mechanisms that involve dyneins associated with the microtubule network, resulting in a shorter leading process and a longer trailing process (Rivas and Hatten 1995; Solecki et al. 2004; Tsai and Gleeson 2005).

In addition, actomyosin contraction in the rear of the cell contributes to drive the nucleus in the same direction (Bellion et al. 2005; Schaar and McConnell 2005). These two steps are repeated producing the typical saltatory movement of migrating neurons. This mode of migration does not depend on radial glial guides, but attachment of the leading process to the intact pial basement membrane is likely essential.

Glia-dependent locomotion

Neurons that adopt glia-dependent locomotion possess a short, unbranched, motile leading and trailing process that extend and retract rapidly, resulting in forward movements of the entire cell, interrupted by stationary phases. (Ayala, Shu, and Tsai 2007; Marín and Rubenstein 2003; Marin et al. 2010; Nadarajah and Parnavelas 2002; Tabata and Nakajima 2003; Noctor et al. 2004; Sakakibara and Hatanaka 2015).

This process is entwined around radial glia process, which function as guides that direct migrating neurons from their birthplace in the VZ to their final destination in the CP. Thus, RGCs not only give rise to neurons but also provide a scaffold for locomoting neurons. The leading process may help create a passage between the radial glia fibers and surrounding differentiating neurons, while the base of the leading process close to the cell body, provides adhesion sites for moving the nucleus.

Neurons undergoing locomotion follow three synchronized steps to move (Ayala, Shu, and Tsai 2007; López-Bendito et al. 2006). First, the cell extends its leading process that explores the immediate environment for attractive or repulsive cues. Second, the nucleokinesis, which typically occurs in a saltatory pattern, resulting in slower average speed (35 μm/h). In the final step, the migrating neuron retracts its trailing process, which leads to the net movement of the cell. The subsequent remodeling of the leading process will initiate a new migratory cycle, which will be repeated until the neuron reaches its final destination.

Neurons migrating by locomotion switch to somal translocation during the final stages of their migration, right after their leading process makes contact with the pial surface and the nucleus migrates smoothly up the leading process (T Miyata et al. 2001; Takaki Miyata and Ogawa 2007; Nadarajah and Parnavelas 2002; Nadarajah et al. 2001).

1.2.2. Molecular mechanisms of radial migration

Although somal translocation and glia-guided locomotion are regulated by distinct cellular mechanisms, these modes of movement might share some signaling cascades and proteins, although with differing temporal patterns. Several neuronal and glial receptor systems have been implicated in the adhesion and signaling between migrating neurons and their substrate, which may be radial glial fibers or the pial surface.

Extracellular guidance cues are interpreted through receptors that relay signals to a network of intracellular signaling pathways, ultimately converging onto the cytoskeleton. Consequently, it is essential to view neuronal migration as a concerted mechanical decision made by a large interactive signaling network. Both microtubule and actin networks are believed to operate synergistically to mediate migration.

Whereas the extension of the leading process is mediated by the polymerization and reorganization of actin microfilaments, microtubules are important for nucleokinesis. The morphological changes at the early phase of migration require the proper regulation of microtubule dynamics and actin cytoskeletal reorganization and polymerization.

RHO GTPases

Small GTPases regulate various cellular events, such as cell adhesion, migration, proliferation and signal transduction. Among five subfamilies (Ras/Rap/Ral, Rho, Rab, Arf/Sar and Ran), Rho family small GTPases are mainly involved in cytoskeletal regulation, whereas Rab family proteins regulate membrane trafficking. It is widely accepted that three major Rho family members, RhoA, Rac1 and Cdc42, are central regulators for cell adhesion and cytoskeleton reorganization that occurs in the morphological changes of migrating neurons in the developing cerebral cortex (Nobes and Hall 1995).

During radial migration, inhibitory pathways decreases RhoA levels, promoting the movement and leading process growth through the disassembly of focal adhesions, detachment from extracellular matrix (ECM) adhesions via integrin-mediated pathway

and actin stress fiber formation. Instead, RhoA activity is up-regulated in the rear of the neuron tail retraction via promoting myosin-dependent contractility (Kaibuchi, Kuroda, and Amano 1999; Lauffenburger and Horwitz 1996; Nobes and Hall 1995). By contrast, Rac1 and Cdc42 regulate several aspects of neuronal migration, axon formation and neuroprogenitor proliferation and survival (Cappello et al. 2006; S. Kawauchi et al. 2005; Kholmanskikh et al. 2006; Konno et al. 2005) (Fig. 4).

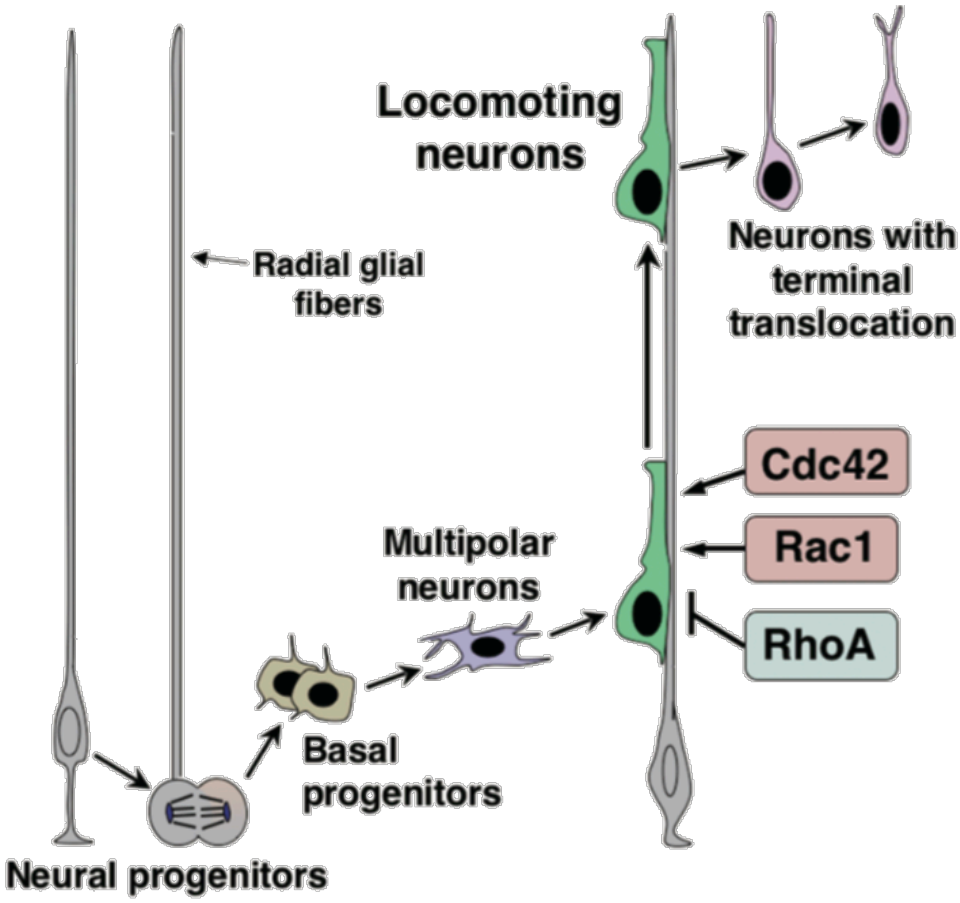

Figure 4. Small GTPases regulate specific phases of glial-guided locomotion migration. In this mode of radial migration neural progenitors use radial glial cells (RGCs) as scaffold. The leading process of migrating neurons round the RGCs processes to move. In this context, Cdc42 and Rac1, but not RhoA, promote microtubule dynamics. (Kawauchi T, 2011).

MAPs

Classical microtubule-associated proteins (MAPs), such as MAP1B, MAP2, and Tau, were among the first identified microtubule regulators (Dehmelt and Halpain 2004).

Phosphorylation of MAP1B leads to an increase of its association with microtubules, whereas phosphorylation of MAP2/Tau generally results in their dissociation from microtubules (Zhang and Dong 2012). Thus, MAP1B, MAP2, and Tau may act synergistically to facilitate neuronal migration.

Furthermore, two nonclassical MAPs, Lisencepahly 1 (Lis1) and doublecortin (DCX), are characterized microtubule and centrosome-associated proteins which provide stability and play complementary roles in the swelling formation and the centrosome association with the nucleus during nucleokinesis (Koizumi et al. 2006; Moores et al. 2004), and the stabilization of actin cytoskeleton of leading process microtubules (Nasrallah et al. 2006). DCX is required for the microtubule stability of leading process branches, while Lis1 promotes leading process branching to enhance neuron guidance (Kappeler et al. 2006).

Several reports suggest that DCX can interact with Lis1 and probably function in a similar pathway to regulate microtubule dynamics during neural development (Caspi et al., 2000). In addition, Lis 1 is associated with the downregulation of RhoA levels and upregulation of Rac1 and Cdc42 activity, suggesting the participation of Lis1 in the tail retraction mediated by RhoA (Kholmanskikh et al. 2003; Kholmanskikh et al. 2006).

$p27^{kip1}$ and CDK5

Similar to its role in other cells, $p27^{kip1}$ controls the G1 length of neural progenitors and cell cycle exit through the inhibition of CDK-cyclin activity (Mitsuhashi et al. 2001; Tarui et al. 2005). In addition, $p27^{kip1}$ promotes the extension of the leading process and subsequent neuronal migration by suppressing RhoA activity (T. Kawauchi et al. 2006). Therefore, $p27^{kip1}$ has dual functions in neural progenitors and migrating neurons, and provides a molecular link between the cell cycle exit and migration start.

Nevertheless, $p27^{kip1}$ is phosphorylated and stabilized by CDK5, a serine/threonine cyclin-dependent kinase, regulating the levels of F-actins and modulating the extension of the leading process in the migrating neurons (Dhavan and Tsai 2001; T. Kawauchi et

al. 2006). Moreover, CDK5 phosphorylates wide range of key cytoskeleton structural proteins such as intermediate and heavy chains of neurofilaments, MAPs, including MAP1b, Tau, Ndel1 and DCX. Furthermore, with the collaboration of $p27^{kip1}$, CDK5 controls other actin regulatory protein as serine/threonine-protein kinase 1 (PAK1) (Kato and Maeda 1999; T. Kawauchi et al. 2006; Paglini et al. 1990).

Adhesion mechanisms in radial migration

During both modes of neuron movement in developing cortex is required a coordinated interaction between migrating neurons and other surrounding cells or the extracellular matrix (ECM), which provides a correct guiding migration. In addition, leading process need a tight anchorage to radial glia in the case of glia-dependent locomotion, or to pial surface in the case of somal translocation. These continuous interactions are mediated by several adhesion proteins which cooperatively modulate the radial migration of cortical neurons (Graus-Porta et al. 2001).

Adhesion proteins in glial-dependent locomotion

In the glial-dependent locomotion movement there are complex morphological changes of the postmitotic neurons which begin a long-distance journey along radial glial fibers. This migration requires the trafficking of transmembrane receptors proteins, which provides a ligand for neuronal adhesion to the apposed radial glial fiber, having an essential role for postmitotic neurons exit from the VZ (Fox and Walsh 1999). Among the most important transmembrane receptors, there are connexins that form gap junctions (Dere and Zlomuzica 2012), cadherins (regulated by the small GTPase Rap1), which establishes adherents junction-like structures (Franco et al. 2011), astrotactins and neuregulins which interact with FLN1, and several integrins (Adams et al. 2002; Anton et al. 1997; Edmondson et al. 2018; Fishell and Hatten 1991; Stitt and Hatten 1990).

Adhesion in somal translocation

When CPNs undergo somal translocation, the tip of the leading process requires a safe mechanism to be fixed into the pial surface and provide anchorage. This molecular adhesion requires transmembrane proteins including N-cadherin or ß1 integrin (Franco et al. 2011; Sekine et al. 2012).

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Cerebral cortex development is characterized by precisely regulated and coordinated migration of newly originated neurons from diverse proliferative regions to their final position, to assembly in a functional circuit glutamatergic projection neurons and GABAergic interneurons from dorsal and ventral telencephalon, respectively. Several studies have analyzed the molecular mechanisms that control the radial migration of neuroprogenitor cells in the cortex suggesting a precise integration of several receptors, ligands and other extracellular cues. However, the detailed mechanisms that control each step during radial migration are largely unknown.

In this context, cytoplasmic CycD1 may be an important candidate to participate in the molecular framework of radial migration. Firstly, it is reported the specific expression of CycD1 in the nervous system during mouse neurogenesis (Freeman, Estus, and Johnson 1994; Lange, Huttner, and Calegari 2009; Salles et al. 2007; Sicinski et al. 1995).

In the other hand, Sumrejkanchanakij P (Sumrejkanchanakij, Eto, and Ikeda 2006), reported the cytoplasmic localization of CycD1 in postmiotic neurons *in vitro*, as a nuclear export mechanism of the cell to stop cell cycle and avoid apoptosis. According to our hypothesis, this nuclear CycD1 export should also be necessary for the promotion of neuron differentiation, an essential fact occurred in the radial migration initiation.

Secondly, several studies have shown that nuclear or cytoplasmic CycD1 collaborates by different ways to the motility of many kind of cells, such keratinocytes, metastatic cells, fibroblasts or macrophages (Bodemann and White 2008; Body et al. 2017; Drobnjak et al. 2000; Fernández et al. 2011; Fusté et al. 2016; Li et al. 2006; Neumeister et al. 2003; Rosse et al. 2006; Zhong et al. 2010). In addition, it has been attributed to CycD1 an adhesion regulatory role in several studies (Fernández-Hernández et al. 2013; Fusté et al. 2016), which contribute to their motility capacity.

Finally, CycD1 interact with many molecules known for their involvement in the intracellular pathways that governs cell attachment and motility. For instance, Fusté N demonstrated that cytoplasmic CycD1-CDK4 complex activated Rac1 pathway through paxilin interaction, controlling cell adhesion, migration and metastasis (Fusté N, 2016). Other reports also propose that CycD1 promotes cellular migration by the activation of $p27^{Kip1}$, inhibiting RhoA, which controls microtubules and cytoskeleton dynamics for migration (Li et al. 2006; Ridley 2015).

Besides controlling migration, our collaborators Dr Eloi Garí and Dra Neus Pedraza (Cell Cycle Group) have performed *in vitro* experiments using transfected cortical $CycD1^{-/-}$ and $CycD1^{+/+}$ neurons from E15.5 mice embryos, which overexpress CycD1 in the cytoplasm (unpublished data). They observed that $CycD1^{-/-}$ neurons did develop short neurites compared with $CycD1^{+/+}$ neurons, and the CycD1 transfection to $CycD1^{-/-}$ neurons rescued the wild-type phenotype. In the other hand, the overexpression of cytoplasmic CycD1 in $CycD1^{+/+}$ neurons improve the neuritogenesis respect to control neurons.

2.2 Objectives

Considering all this background we hypothesize that cytoplasmic expression of CycD1 may be a relevant molecular element during dorsal telencephalon development, in particular during radial migration or newly born cortical neurons.

The specific objectives are:

1. **Analyze the expression of CycD1 protein during neurogenesis in the developing telencephalon of wild-type embryos.**

    a. Determine the subcellular localization of CycD1 in the different neuronal cells, specially focus in the cytoplasm expression and its distribution along the cell.

    b. Verify the specificity of used CycD1 antibody in CycD1$^{-/-}$ embryos.

    c. Determine the expression pattern of CycD1 along developing telencephalon to stablish a defined gradient, to relate it with the proliferation or the neurogenesis activity pattern studied in the mice embryos brain.

    d. Compare the expression pattern between nuclear and cytoplasmic CycD1 in the developing telencephalon.

    e. Reveal the presence in the developing telencephalon of proteins which interact with CycD1 in other tissues according previous studies.

    f. Discover same CycD1 expression pattern of proteins known for their neurogenesis function in the developing telencephalon.

2. **Examine cell cycle activity in CycD1$^{-/-}$ embryos to discard proliferation defects in the observed phenotypes during their analysis.**

3. **Analyze the development of telencephalon from CycD1 genetic manipulated embryos through intraventricular DNA injection and *in utero* electroporation assay.**

    a. Overexpress cytoplasmic CycD1 in E13.5 wild-type embryos and analyze electroporated neurons during different phases of developing and postnatal telencephalon.

    b. Study the development and postnatal telencephalon of wild-type embryos electroporated at E13.5 with a negative dominant of cytoplasmic CycD1-CDK4/6.

4. **Analyze the telencephalon development of CycD1$^{-/-}$ embryos and postnatal mice.**

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 Mice

Animal care was performed in accordance with the Guidelines of University of Lleida for Animal Experimentation in accordance with Catalan, Spanish and European Union regulations (Decret 214/1997, Real Decreto 53/2013 and Directive 63/2010). Animals were housed in the animal house of the University of Lleida with 12:12h light/dark cycle and food/water available ad libitum. All efforts were made to minimize the number of animals used and their suffering.

Ccnd1 deficient mice was previously designed by Sicinski P (1995) in this manner. Mouse genomic fragment encoding Ccnd1 was isolated and assembled from five exons that are spread over a genomic distance of more than 7 kb. A gene targeting construct was prepared by deleting a restriction fragment containing the coding portion of exon I as well as exons II and III and replacing it with a cassette expressing the neo gene. These exons were chosen for deletion because they encode the so-called cyclin box, a stretch of over 100 amino acid residues that is conserved among the cyclins of all eukaryotes and is believed to be essential for cyclin function (Xiong and Beach, 1991). Following electroporation, embryonic stem cell clones were selected using the positive-negative selection method (Mansour et al., 1988) expanded, and screened for homologous recombination events by Southern blotting analysis of their DNA. The stem cells which were found to be heterozygous at the CycDl locus were expanded and injected into mouse blastocysts, giving rise to CycD1 heterozygotes. Heterozygotes were bred to produce cyclin $Dl^{-/-}$ mice, which were identified by Southern blotting and polymerase chain reaction (PCR) amplification of tail DNA. The absence of CycDl protein in mutant mice was confirmed by Western immunoblot analysis of embryonal fibroblast lysates. Transgenic and wild-type mice were maintained in C57/bl6 and CD1 background, respectively.

For embryos dissection, the day on which the vaginal plug was found was considered as embryonic (E) day 0.5. The date of birth was considered as postnatal (P) day 0.

Female mouse was sacrificed by cervical translocation and its abdomen was opened in order to remove the uterus from the abdominal cavity. Uterus was placed in PBS and embryos were taken out using forceps.

3.2 DNA extraction and purification by proteinase K

Tails of embryo and postnatal mice were collected and warmed at 54º C in a 200 µl solution of genotyping buffer (1 M Tris.Cl pH8.0, 500 mM EDTA pH8.0, 10% SDS, 5 M NaCl) and proteinase K (20 mg/ml) during 20´ at 950 rpm into a Thermo-Shaker (Eppendorf). Dissolved tails were centrifuged 5´ at 10000 rpm to collect the supernatant and warmed at 65ºC to inactivate the proteinase K. Afterward, DNA was washed with ethanol and isopropyl alcohol and finally precipitated and suspended in H_2O.

3.3 PCR

PCR mix (24 µl) for 1 µl of DNA embryo sample contained: Taq Buffer 10x (HM); 25 mM $MgCl_2$; 100 mM dNTPs (Biotools); 100 µM/primer (Forward (CTCCGTCTTGAGCATGGCTC), Reverse (CTAGTGAGACGTGCTACTTC) and Common (TAGCAGAGAGCTACAGACTTCG)) (Integrated DNA Technologies); 7,5 units of Taq Polymerase (Biotools) and distilled water to the required volume. The PCR program used in the Thermal Cycler (T100 Bio-Rad) was: 94°C 3' + 38 x (94°C 30'' + 62°C 45'' + 72°C 1') + 72°C 2' + 4°C ∞. The amplified product of DNA was loaded and run (300 V; 400 A; 25 minutes) in a 1,5% Agarose (NBS Biologicals) gel with TAE 1X (Tris base (Sigma-Aldrich), acetic acid (Scharlau) and EDTA (Sigma-Aldrich). Gel was bathed in EtBr solution during 10´ and revealed under the UV light (Fig. 1).

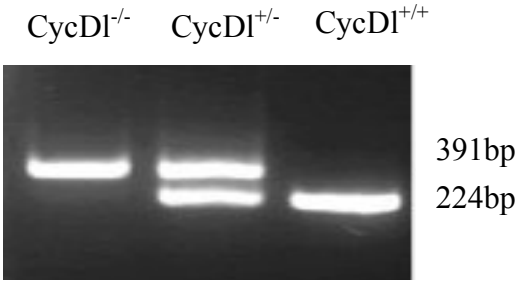

Figure 1. Representative image of electrophoresis of PCR products from CycD1 genotyping assay. Three possibilities of genotype are indicated in the top of the image, and the base pair of each allele in the right side.

3.4 Plasmid design for intraventricular injection and *in utero* electroporation

Myc-tagged *Ccnd1*^{CAAX} and Ccnd1$^{K112\text{-}CAAX}$ were inserted into pCAGIG vector (constructed by Takahiko Matsuda) (Addgene). CAG promoter is efficient to introduce genes into mammalian cells. Furthermore, all these plasmids have the gene encoding the enhanced green fluorescent protein (Pmx-IRES-GFP (Nosaka et al., 1999)) to facilitate the detection of transfected cells. *Ccnd1*$^{K112\text{-}CAAX}$ encodes a mutated CycD1 protein in the Lysine 112, the amino acid that is crucial for the interaction with CDK4/6. This construct is used as dominant negative of the CDK4/6 dependent functions. The CAAX motif is used to target proteins to the endomembrane in order to study the non-nuclear function of CycD1. As control plasmid, it was used pCAGIG vector and a plasmid containing the gene coding for CycD1 without the CAAX motif, (CycD1N), thus, transfected protein was maintained into the nucleus. Vectors were inserted using the cloning protocol, digesting with specific restriction enzymes and then realizing the ligation.

Chemical-competent bacteria *E. coli* were transformed with the designed plasmids, placing them at 42oC for 30´´ (thermal shock) and growing the transformed bacteria in LB/ampicillin medium during 24h. Finally, DNA was extracted from bacteria using Plasmid Maxi Kit (Qiagen) protocol.

3.5 Intraventricular injection and *in utero* electroporation

E13.5 pregnant CD1 wild-type mice were deeply anesthetized with isoflurane (IsoFlo, Zoetis) and administrated during whole operation. In order to relax uterus muscles, 0,1 ml of the β_2 agonist Ritodrine (Sigma (R0758)) (13.9 mg/ml) was administrated intraperitoneally, and 0,1 ml of buprenorphine (Buprex (100 mg/ml)) subcutaneously as an analgesic. The abdomen was sterilized with 70% ethanol and shaved. A 2 cm laparotomy section was made, and the uterine horns were carefully exposed and lubricated with NaCl 0,9% at 37ºC. Approximately, 2 to 4 microliters of purified plasmid DNA dissolved in PBS (1 µg/µl) was injected in the lateral ventricles of each

embryo using a glass capillary (World Precision Instruments) sharpened previously by Puller P-97 (Sutter Instrument). Fast Green (Sigma-Aldrich) solution was added (0,025 %) in order to monitor the injection in the lateral ventricles. Platinum electrodes (CUY701P20L, Nepagene) were placed across the head locating positive pole around the neocortex where DNA should go, in order to enhance the permeability of the cell membrane and allow the entrance of DNA. Five 30 mV electric pulses of 50 ms with intervals of 950 ms were charged by an electroporator (ECM830, BTX). After the uterine horns were placed back into the abdominal cavity to allow the embryos to continue their development until the required age, abdomen wall and skin were sutured with surgical suture (Aragò). During whole operation embryos were manipulated with ring forceps (Fine Science Tools) (Fig. 2).

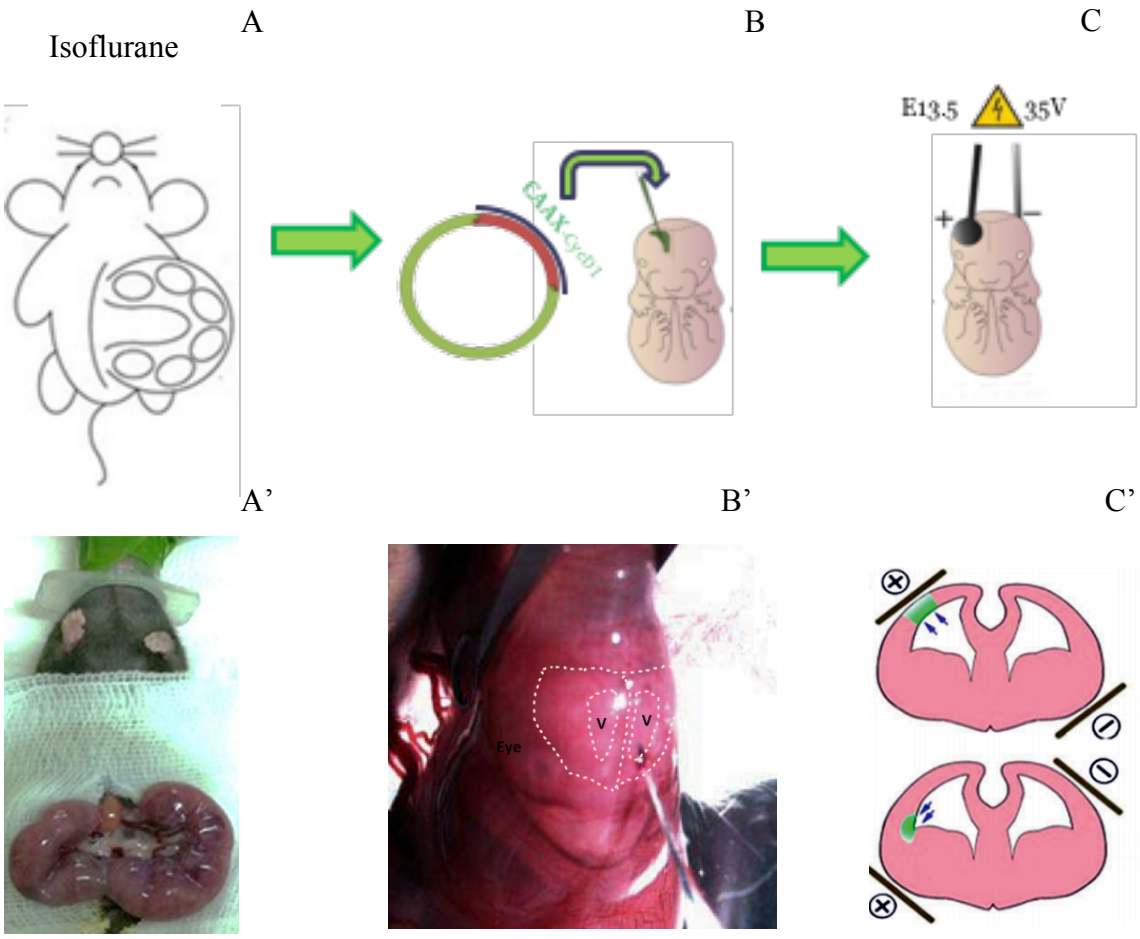

Figure 2. Intraventricular injection and in utero electroporation assay. **A, A'**: E13.5 embryo extraction from anesthesized pregnant female by isoflurane. **B**: Intraventricular injection of 3 µl of DNA in a lateral ventricle (V in **B'**) of each embryo. **C**: Electroporation through five 35V electric pulses of 50 ms with polarized paddles in both sides of the embryo brain. **C'**: Poles orientation to manage transfected neurons to desired region.

3.6 Immunofluorescence

Embryonic brains were fixed for 4 h in 4% PFA in PBS. Postnatal mice were anesthetized with Rompun (Xylacine 0.01 mg/g, Bayer)/Imalgene (Ketamine 0.1 mg/g, Merial) and perfused transcardially with PBS followed by 4% PFA in PBS. Adult brains were dissected and fixed overnight in 4% PFA in PBS. After fixation, brains were washed with PBS + 50mM NH_4Cl to quench the aldehyde group of PFA. Subsequently, brains were cryoprotected in 30% sucrose (Scharlau) in PBS O/N, embedded in cryoprotective Tissue-Freezing Medium (General Data) and stored at -80°C. Serial 20 µm coronal sections were made in cryostat (Leica CM3000), and collected in Superfrost Plus™ slides (Thermo Fisher). The slides were washed with PBS to take off impurities, permeabilized and blocked with 5% donkey serum in 0,1% Triton X-100 (Sigma-Aldrich) in PBS for 1 h at room temperature. Subsequently, the slides were incubated with primary antibodies diluted in blocking buffer for 12-24 h at 4°C. After washing, sections were triple stained with DAPI, Alexa Fluor 488 and Cy3, diluted in blocking buffer (2 h at room temperature).

3.7 EdU Labeling

E14.5 pregnant mice were injected intraperitoneally with 100 µg/g body weight of EdU (ThermoFisherC10337) in a solution of 10 mg/ml with PBS. Pregnant mice were sacrificed 1h later and the embryos were processed as explained above (Immunofluorescence chapter). For immunostaining with anti-EdU antibody, sections were washed with PBS and permeabilized and blocked with 5% goat serum in 0,1% Triton X-100 in PBS for 1 h at room temperature. Subsequently, the slides were incubated during 30 minutes with Click-iT reaction cocktail (Click-iT reaction buffer, CuSO4, Alexa Fluor azide, Reaction buffer additive) protected from the light. After washing, DAPI was added to sections during 2 h at room temperature for nucleus staining.

3.8 Immunofluorescence microscopy

Samples were mounted with antifading mounting medium Fluoromount-G (Southern Biotech), covered with a cover slip (Menzel-Gläser) and visualized with an upright fluorescence microscope (Olympus BX51) or with a confocal laser scanning biological microscope FV1000, FLUOVIEW (Olympus). Images were acquired with the Olympus DP30BW camera. Images were brightness and contrast adjusted with Adobe Photoshop. Cell quantification was executed with the cell counter tool of ImageJ. Adobe Photoshop CS3 was used to merge images and impose false colors by assigning images into green, red and blue channels accordingly to show co-localization of expression.

3.9 Antibodies

Primary	**Reference company**	**Working dilution**	**Antigen retrieval**	**Species**
α-GFP	Abcam	1/300	No	Goat
α-Cyclin-D1	Dako	1/300	No	Rabbit
α-Tbr2	Abcam	1/300	No	Rabbit
α-PH3	Sigma-Aldrich	1/100	No	Rat
α-Sox2	Abcam (ab97959)	1/300	No	Rabbit
α-Tbr1	Abcam (ab31940)	1/300	No	Rabbit
α-Ctip2	Abcam (ab31940)	1/300	No	Rat
α-Cux1	Proteintech	1/200	No	Rabbit
α-Pax6	Hybridoma Bank	1/300	No	Mouse
α-Nestin	Abcam (ab6142)	1/100	Si	Mouse

Secondary	**Reference company**	**Working dilution**	**Species**
Alexa Flour 488	Jackson ImmunoResearch	1/300	Donkey – anti goat, rabbit, rat or mouse
Cy3	Jackson ImmunoResearch	1/300	Donkey – anti goat, rabbit, rat or mouse

3.10 Statistical analysis

Two tailed unpaired Student's *t* tests, calculated in GraphPad program, were used to determine statistical differences between groups. The *p* value in each experiment is indicated and significance was considered when $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***). Error bars were calculated using the standard deviation of the mean (SD).

RESULTS

4. RESULTS

4.1 Cytoplasmic Expression of Cyclin D1 in the developing neocortex

In light of the mentioned role of CycD1 to regulate adhesiveness and motility of diverse type of cells, we have examined and characterized the expression profile and the specific cellular localization of CycD1 in the developing mouse brain by immunohistochemistry. This analysis will help to elucidate the function of this protein in the developing telencephalon.

We first analyzed the presence of CycD1 in coronal slices of E14.5 mouse embryos, when active neurogenesis occurs. As we expected, nuclear expression of CycD1 was detected in the periventricular zone through developing telencephalon and thalamus (Fig. 1A, 1B). We observed that nuclear CycD1 was practically limited to the VZ/SVZ where neural stem cells and progenitor cells reside, and colocalized almost exclusively with Pax6 (Fig. 1C).

But surprisingly, CycD1 staining was also observed in a specific cytoplasmic pattern, forming processes from VZ to the basal surface, in particular in the ventral telencephalon and thalamus (Fig. 1D). These CycD1-expressing processes co-localized with Nestin, a marker of neural progenitor cells, suggesting that the majority of observed cytoplasmic CycD1 processes belong to RGCs (Fig 1E). This cytoplasmic expression was heterogeneous through the RGC process with higher expression in middle regions and distal end and low expression closer to the soma in the VZ (Fig 1B). Interestingly, CycD1 positive staining accumulated at the tip of the process forming intermittently “buttons” adjacent to the BM along the entire cortex (Fig. 1F). These buttons represent the endfoot of the RGC processes, the structure that keep these cells anchored to the BM. Remarkably, as was mentioned, the last part of the RGCs is not as stained as the middle part, but just where the RGCs reach into the BM, the CycD1 expression increases considerably.

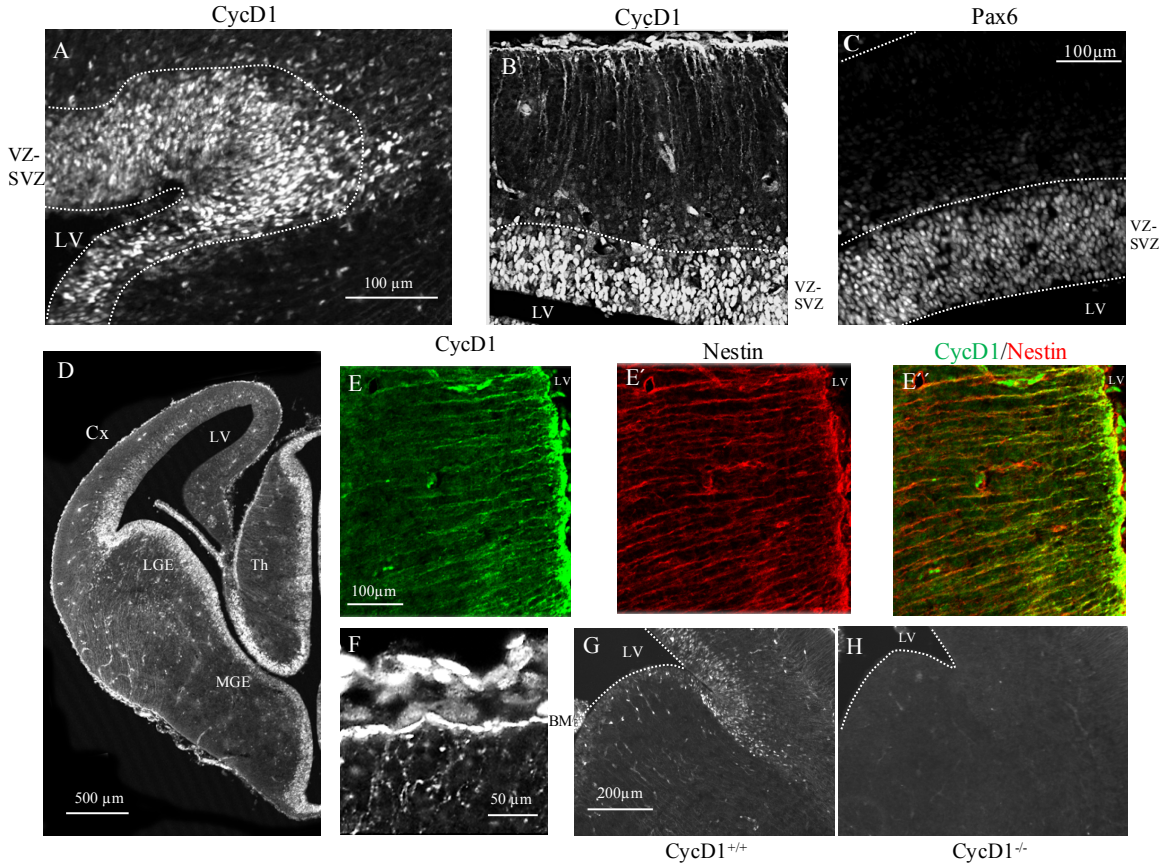

Figure 1. Specific cytoplasmic CycD1 expression in the RGCs and BM along telencephalon and thalamus.
A: Nuclear expression of CycD1 in the neuroprogenitor cells of the VZ and SVZ region. VZ-SVZ was limited by a discontinuous line. **B**: Representative immunofluorescence of the cortex labeled with CycD1. Nuclear localization was observed in the VZ-SVZ region, and cytoplasmic CycD1 in the RGCs processes which reach to BM, also positive for CycD1 (indicate with arrowheads). **C**: Neuroprogenitors expressing Pax6 in the same zone than CycD1 positive nuclei along telencephalon. **D**: Whole CycD1 expression in E14.5 coronal slice of telencephalon and thalamus (only one hemisphere is shown). **E**: Cytoplasmic expression of CycD1 in thalamus. **E´**: Nestin positive RGCs processes in the thalamus. **E``**: RGCs processes of thalamus labeled with Nestin (red) and CycD1 (green) antibody. **F**: Cytoplasmic expression of CycD1 in the RGCs endfoot and the adyacent BM. In the top of the image there are CycD1 positive nuclei of endothelial cells (indicate with arrows, arrowheads, asterisks, etc ... all these structures). **G**: Specific CycD1 expression in $CycD1^{+/+}$ telencephalon. **H**: Negative CycD1 expression in $CycD1^{-/-}$ telencephalon. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **MGE**: Medial Ganglionic Eminence; **LGE**: Lateral Ganglionic Eminence; **BM**: Basement membrane; **LV**: Lateral Ventricle; **Th**; Thalamus; **Cx**: Cortex.

To verify the specificity of the staining observed with the antibody we used against mouse CycD1, the same immunofluorescence was performed in coronal slices of E14.5 CycD1-deficient mice. Any signal was detected along the telencephalon and thalamus (Fig. 1H), just a weak expression into BM much less intense than the observed in wild-type embryos (Fig. 1G). Having thus validated the CycD1 antibody specificity, we decided to use it to perform a spatiotemporal analysis of expression of CycD1 during

mouse neurogenesis period (E12.5-E16.5), to unveil an evidence of why CycD1 is localized in this specific cytoplasmic pattern.

Analysis of integrin β1 expression in the telencephalon development was performed to relate the preview adhesion role of cytoplasmic CycD1 observed in other tissues (Fernández RMH, 2013; Fusté N, 2016; Neumeister P, 2003). Integrin β1 was localized in the cytoplasm of whole CycD1 positive RGCs processes along the telencephalon. In addition, integrin β1 was expressed in the BM colocalizing with CycD1 (Fig. 2).

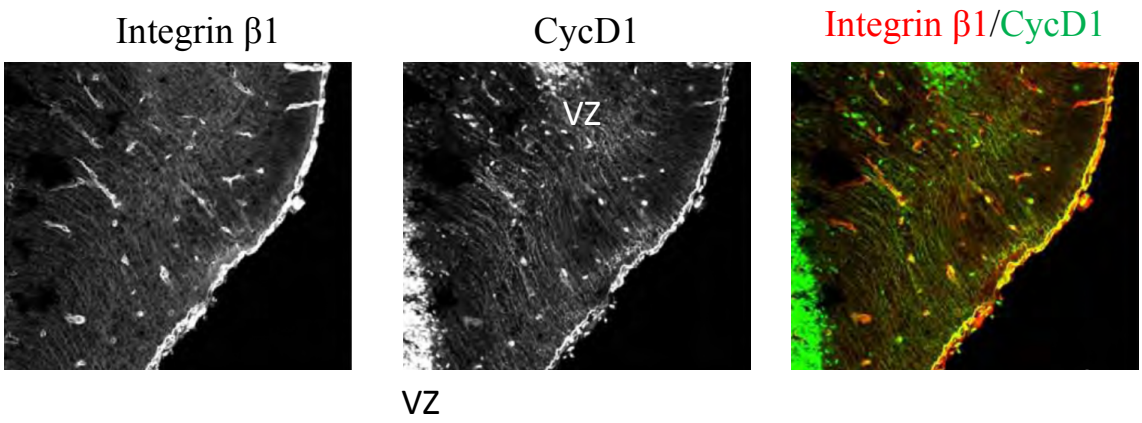

Figure 2. Colocalization of integrin β1 and cytoplasmic CycD1 observed in the development of telencephalon. RGCs processes and BM show double staining of both proteins, in a similar pattern. **VZ**: Ventricular zone.

Spatiotemporal analysis of CycD1 expression through neurogenesis

Neurogenesis period starts when NSCs switch their identity and turn into RGCs. Into the mouse telencephalon, the first RGCs are originated at E10.5, however, due to our interesting in cytoplasmic localization, we have started the analysis when the RGCs process formation occurs, thus, at E12.5. Subsequently, the end of neurogenesis is considered when the majority of postmitotic cells have reached to CP and begin the axogenesis and gliogenesis at E17.5 in the telencephalon, thus, the last age analyzed was E16.5.

E12.5

At this period, nuclear CycD1 expression was predominantly restricted to NPCs localized in the VZ and SVZ of MGE, in the most ventral telencephalon region, while

more dorsal regions in LGE and cortex showed a weak nuclear signal in VZ and SVZ (Fig. 3A). Remarkably, there was not a progressive decrease in the staining intensity into the boundary between MGE and LGE, but this expression difference occurred suddenly. Nevertheless, cytoplasmic expression of CycD1 was observed in long processes exclusively from RGCs, which their soma was located into VZ and SVZ of MGE, while dorsal regions did not show this cytoplasmic staining. The CycD1 positive processes reached to the BM of piriform cortex region (Fig. 3B), which, significantly, showed much more intense CycD1 expression than the BM that belonged to more dorsal regions of telencephalon, which could be unspecific signal.

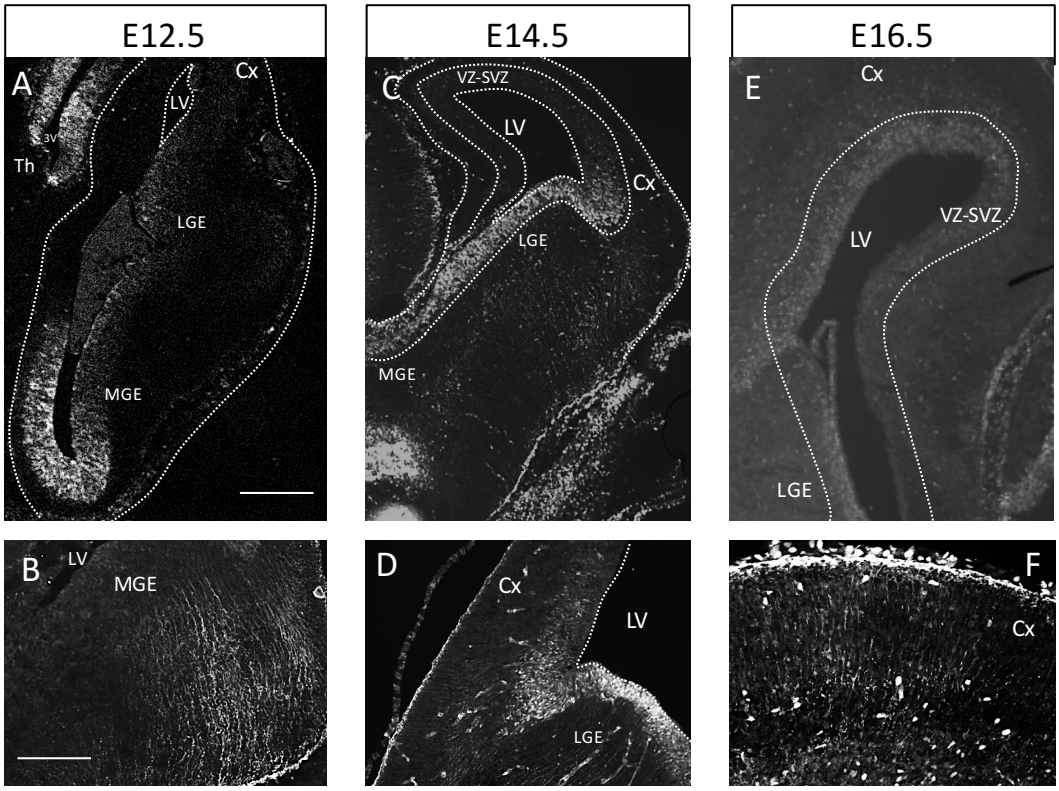

Figure 3. CycD1 expression follows the ventral-dorsal neurogenetic gradient during neurogenesis period.
A, B: Representative immunofluorescence of E12.5 telencephalon coronal slice labeled with CycD1. Nuclear localization of CycD1 in the ventral MGE **(A)** and RGCs processes from MGE along the ventral telencephalon **(B)**, without expression in upper parts. **C, D**: Representative immunofluorescence of E14.5 telencephalon coronal slice labeled with CycD1. CycD1 positive cells and processes in the middle part of telencephalon at E14.5, without dorsal expression **(C)**, where finishes in the LGE/cortex boundary **(D). E, F**: Representative immunofluorescence of E16.5 telencephalon coronal slice labeled with CycD1. Stained nuclei in the VZ-SVZ region of the cortex at E16.5 **(E)** and RGCs processes in the cortex with some positive nuclei in the CP **(F)**. Scale bar A, C, E: 500 μm. Scale bar B, D, F: 200 μm. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **MGE**: Medial Ganglionic Eminence; **LGE**: Lateral Ganglionic Eminence; **LV**: Lateral Ventricle; **Th**; Thalamus; **Cx**: Cortex.

E14.5

Approximately in the middle stages of neurogenesis, nuclear expression of CycD1, with similar strength, progressed dorsally to NPCs which are localized into VZ and SVZ of LGE, while the ventral nuclear expression detected at E12.5 was maintained (Fig. 3C). At E14.5, the ventral CycD1 positive RGCs processes detected at E12.5 were not observed at this stage, although positive CycD1 cytoplasmic expression was observed in processes from RGCs settled into the VZ and SVZ of the LGE, occupying the dorsal region of subpaleo. These CycD1 positive processes reached to the BM of the middle part of the telencephalon in the ventral-dorsal axis, some to the BM corresponding to the subpaleo, and others reached to the ventral portion of the BM cortex, crossing pallio-subpallio boundary (Fig. 3D). Cortical VZ and SVZ showed similar nuclear CycD1 signal as observed at E12.5, but there was not any CycD1 positive RGC process in the cortex.

E16.5

During the last phases of neurogenesis, when CP is almost formed and populated by differentiating neurons and the last migrating neurons are reaching this region most of the CycD1 expression was observed along neocortex (Fig. 3E). Cortical VZ and SVZ at the most dorsal regions showed a nuclear CycD1 expression not seen before in this region at previous analyzed stages, but not as intense as VZ and SVZ of ventral regions at E12.5 and E14.5. This nuclear intensity decrement may be because of at this neurogenesis period proliferative activity and amount of VZ and SVZ populating cells is in decline. Similarly, cytoplasmic CycD1 positive processes of RGCs and BM were observed exclusively in the cortex, also less intense than the stained processes at previous analyzed stages. In addition, there were fewer CycD1 positive nuclei distributed through IZ and CP (Fig. 3F).

Regarding the CycD1 expression in the thalamus during neurogenesis, also is localized in the nuclei of NPCs of periventricular zone of third ventricle, and in the processes of RGCs which reach to the basal side of thalamus (Fig. 1B). In this brain area, no neurogenetic gradient was observed during the neurogenesis period studied (E12.5-E16.5).

As we know, described ventral-dorsal expression gradient of CycD1 follows the ventral-dorsal neurogenetic gradient that occurs during telencephalon development (Bayer SA, 1991). To approach the reason of this specific CycD1 gradient, we performed a parallel expression pattern analysis of the proliferative nuclear marker PH3 (Fig. 5) during neurogenesis through mouse telencephalon.

CycD1 is well known for its role in the regulation of cell cycle and it was kind of surprising to observe that not all active dividing progenitors, especially those located in the dorsal cortex, expressed low levels of CyclinD1 compared to more ventral progenitors. Intriguingly, the ventral (high)-dorsal (low) expression gradient of CycD1 follows the ventral-dorsal neurogenetic gradient that occurs during telencephalon development (Bayer SA, 1991). We investigated more in detail this observation and performed a parallel expression pattern analysis with other proliferative nuclear markers such as PH3 (Fig. 4) during neurogenesis through mouse telencephalon.

The expression observed did not follow any spatiotemporal gradient as observed in CycD1 expression analysis, suggesting the existence of a non-related cell cycle function of CycD1 in the neurogenesis machinery of telencephalon.

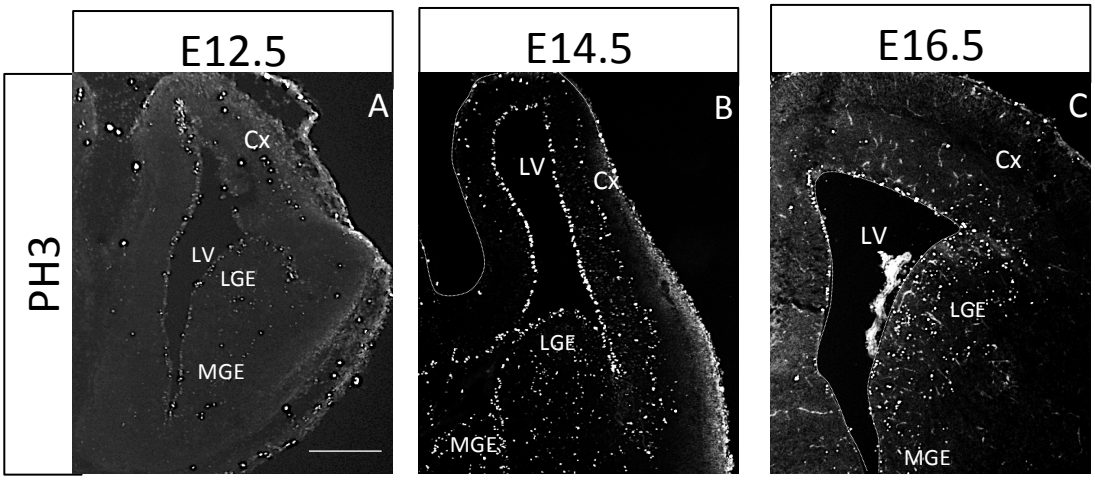

Figure 4. Lack of neurogenetic gradient in proliferative markers during neurogenesis.
Representative immunofluorescence of coronal slice of telencephalon labeled with PH3 at E12.5 **(A)**, E14.5 **(B)** and E16.5 **(C)** staining the same regions along this neurogenesis period, confirming that proliferative gradient is not related with CycD1 expression gradient. Scale bar: 500 µm. **MGE**: Medial Ganglionic Eminence; **LGE**: Lateral Ganglionic Eminence; **LV**: Lateral Ventricle; **Cx**: Cortex.

Lacking proliferation defects in CycD1 knock-out embryos

To support this idea and check the relevance of CycD1 in the cell cycle progression of telencephalon NPCs during neurogenesis, proliferation activity of CycD1-deficient embryos was analyzed by diverse methods. Immunofluorescence against PH3 protein was performed to study mitosis activity, which there was no significative differences comparing CycD1-deficient embryo with wild-type (Fig. 5A). 5-ethynyl-2′-deoxyuridine (EdU) incorporates in the DNA of divided cells since its injection. EdU was injected in E16.5 pregnant mice and sacrificed just 1 hour later, not allowing to the cells which have incorporate EdU differentiate and migrate to upper layers. No significative differences were observed in the number of EdU positive cells between both genotypes studied (Fig. 5B). These results suggest that there is not CycD1 dependence for cell cycle progression of NPCs, at least during neurogenesis in the embryo telencephalon, as it occurs in many developing tissues where the three D-type cyclins are largely exchangeable (Ciemerych MA, 2002).

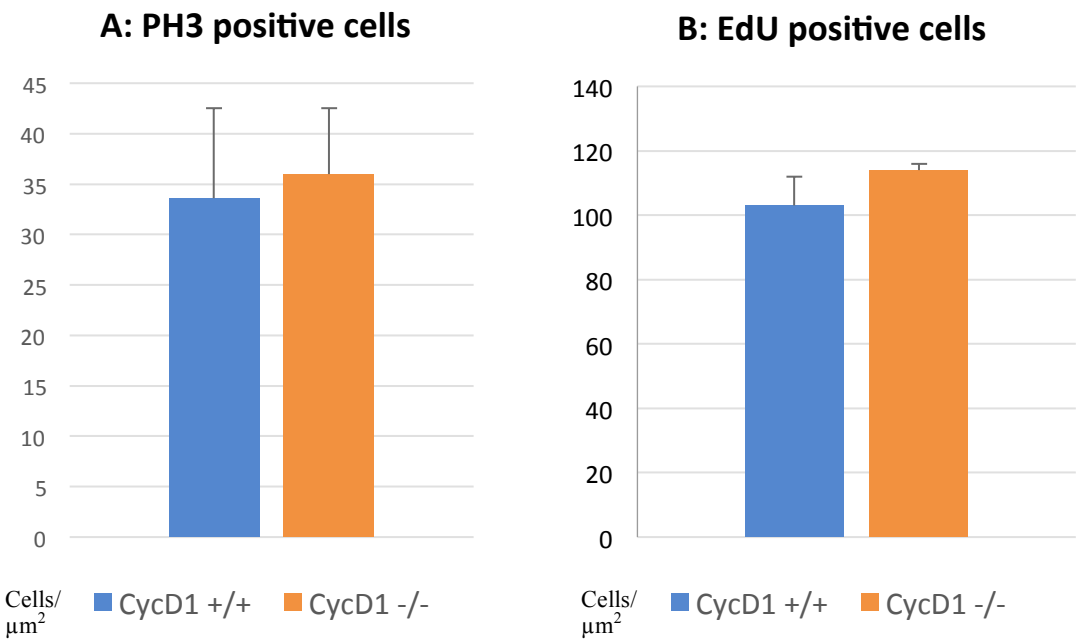

Figure 5: Lack of CycD1 do not trigger proliferative defects in the telencephalon during neurogenesis. Quantification graphic of PH3 positive cells **(A)** and EdU positive cells **(B)** in the cortex of E14.5 CycD1$^{+/+}$ and CycD1$^{-/-}$ embryos. The selected area for the quantification was the same region and with the same µm^2 in each analysis. EdU injection was 1 hour before sacrifice the embryos to their analysis. Values represent mean ± SD (n=3 experiments). P (A) = 0.7764 . P (B) = 0.1077.

Because CycD1 is expressed in the cytoplasm of RGCs and considering the described spatiotemporal neurogenetic gradient, we wondered whether CycD1 may contribute during any phase of neurogenesis through its participation in the control of migration and adhesion as previously suggested in other cell types (Body S, 2017; Fernández RMH, 2011; Fernández RMH, 2013; Fusté N, 2016; Neumeister P, 2003).

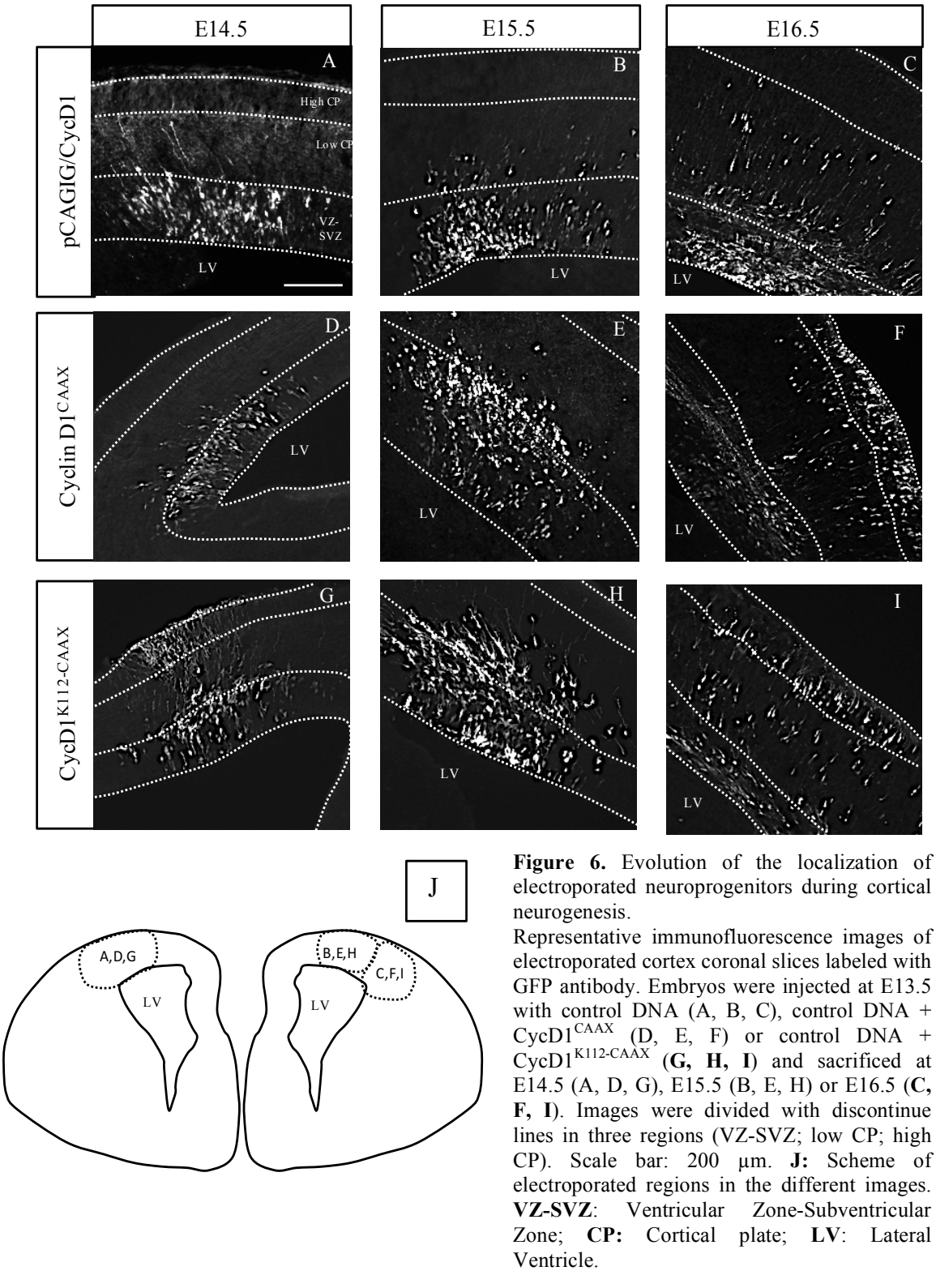

Figure 6. Evolution of the localization of electroporated neuroprogenitors during cortical neurogenesis.
Representative immunofluorescence images of electroporated cortex coronal slices labeled with GFP antibody. Embryos were injected at E13.5 with control DNA (A, B, C), control DNA + $CycD1^{CAAX}$ (D, E, F) or control DNA + $CycD1^{K112\text{-}CAAX}$ (**G, H, I**) and sacrificed at E14.5 (A, D, G), E15.5 (B, E, H) or E16.5 (**C, F, I**). Images were divided with discontinue lines in three regions (VZ-SVZ; low CP; high CP). Scale bar: 200 µm. **J:** Scheme of electroporated regions in the different images. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **CP:** Cortical plate; **LV**: Lateral Ventricle.

To unravel this potential function of CycD1, intraventricular injection and *in utero* electroporation assay was performed in order to overexpress different constructs harboring several CyclinD1 mutations that will allow to ascertain the role of cytoplasmic CycD1 in neuron development. We targeted progenitors cells in neocortex of *CD1* E13.5 embryos, injecting in their lateral ventricle different constructs in the pCAGIG plasmid: GFP (control), CycD1 (control), $CycD1^{CAAX}$ or $CycD1^{K112\text{-}CAAX}$, which is unable to produce active kinases complexes due to the lack of the lysine 112 residue, which is required for the attachment between the cycD1-CDK4/6 complex. Thus, the use of both mutant plasmids ($CycD1^{CAAX}$ and $CycD1^{K112\text{-}CAAX}$) will allow determine if the observed results are related or not with the cycD1-CDK4/6 complex functions. CAAX motif was incorporated to mutant plasmids to target codified proteins to the plasma membrane, in order to study just the possible cytoplasmic function of CycD1 in electroporated neuroprogenitor cells, thus, the results obtained in this assay will be attributed to the observed cytoplasmic expression of CycD1 observed during neurogenesis.

4.2 CycD1 controls neuron migration

Neocortical neural stem cells were electroporated at E13.5 and their localization along the neocortex was analyzed at E14.5, E15.5 and E16.5 to study the neuron migration during neurogenesis. Developing neocortex was divided in three zones (VZ-SVZ; low CP/IZ; high CP/MZ), which the boundaries between them were highly plain watching GFP positive cells.

At first sight, cortices electroporated with control construct showed the majority of GFP positive progenitor cells in VZ-SVZ area at E14.5 and E15.5, and some invaded the CP at E16.5 (Fig. 6A, 6B, 6C). In the case of cortices electroporated with $CycD1^{CAAX}$ and $CycD1^{K112\text{-}CAAX}$ plasmids, at E14.5 also GFP positive cells were maintained into VZ-SVZ area (Fig. 6D, 6G), but at E15.5 some of GFP positive cells invaded the low CP (Fig. 6E, 6H), and most of them were in the high CP at E16.5 (Fig. 6F, 6I).

In order to quantify this altered neuron distribution of modified electroporated cells, images of injected neocortex were divided in the three zones explained above (VZ-SVZ, low CP and high CP), and the percentage of GFP positive cells was quantified in each zone from multiple comparable sections (≈6), obtained at least from n = 3 embryos per construct.

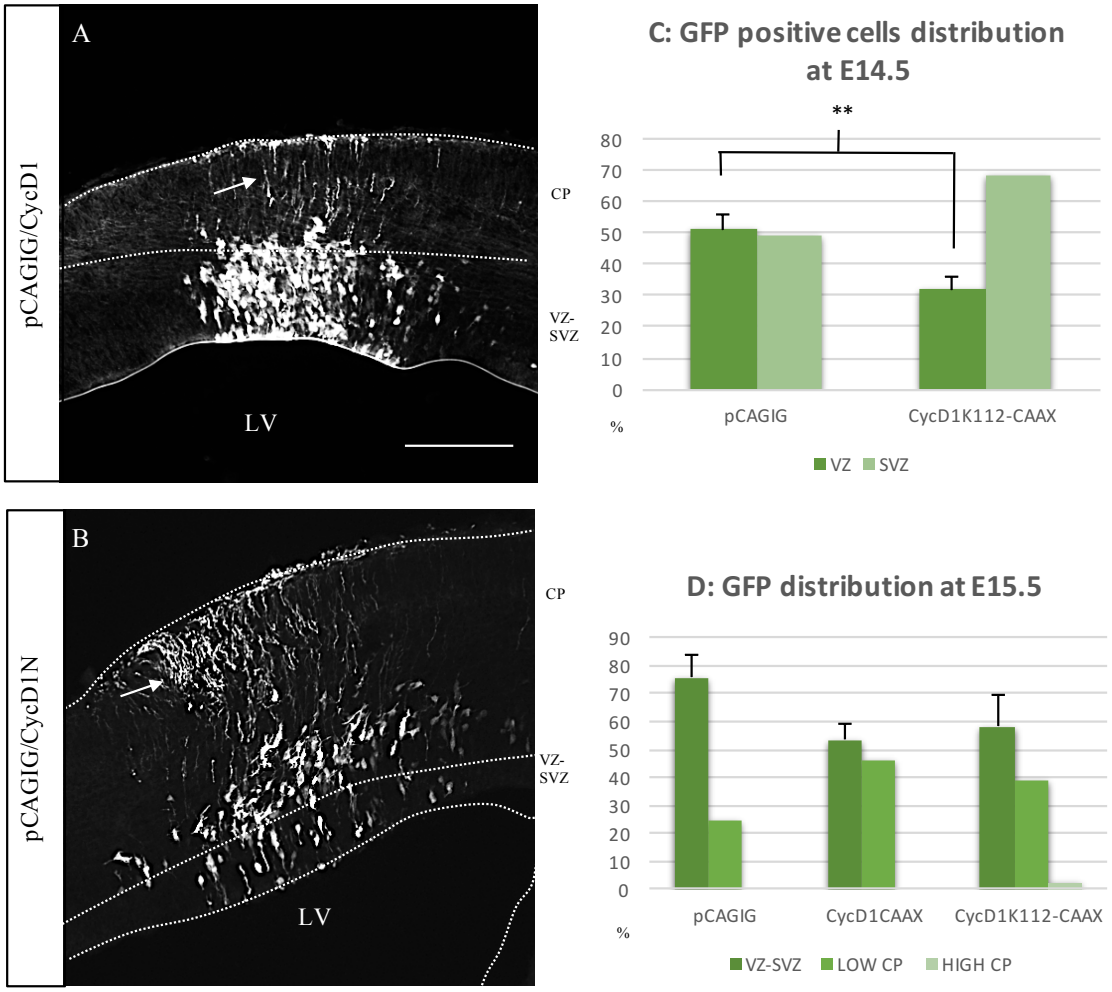

Figure 7. Increase density of CycD1$^{K112\text{-}CAAX}$electroporated cells in the SVZ.
A, B: Representative immunofluorescence images of coronal cortex slices electroporated at E13.5 and sacrificed at E14.5, labeled with GFP antibody. Discontinue line was drawn to identify the VZ-SVZ boundary. Arrow heads indicate the leading process thrown by electroporated cells. Scale bar: 200 µm. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **CP:** Cortical Plate; **LV**: Lateral Ventricle.
C: Quantification graphic of E14.5 electroporated cells located in VZ and SVZ between control DNA and CycD1$^{K112\text{-}CAAX}$ plasmid. Data are mean percentage ± SD (n=3 experiments). **P = 0.0051.
D: Quantification graphic of E15.5 electroporated cells located in VZ and SVZ between control DNA, CycD1^{CAAX} and CycD1$^{K112\text{-}CAAX}$ plasmid. Values represent mean percentage ± SD (n=3 experiments). P = 0.0578.

One day after *in utero* electroporation, at E14.5, GFP positive cells did not leave VZ-SVZ sector. Nonetheless, there was a remarkable difference inside this area. In control

samples, electroporated cells were scattering homogenously, with the 51,06% in the VZ and the 48,94% in the SVZ (Fig. 7A). Instead, in CycD1$^{K112\text{-}CAAX}$ samples there was a significant heterogeneity with the 31,71% of GFP positive cells in the VZ and the 68,29 in the SVZ (Fig. 7B)(CycD1^{CAAX} was not measured).

At E15.5, some cells reached to low CP but none the high CP. In control samples, 75,62% of GFP positive cells were maintained in the VZ-SVZ sector, and just the 24,38% were in the low CP. In mutant samples, the distribution was approximately similar between them. 53,56% of CycD1^{CAAX}-expressing cells were in VZ-SVZ region and 46,44% in the low CP. In like manner, 58,36% of CycD1$^{K112\text{-}CAAX}$-expressing cells were in VZ-SVZ section and the 39,10% in the low CP. Anecdotally, there were 2,54% of cells in the high CP of CycD1$^{K112\text{-}CAAX}$ –electroporated brain (Fig. 7D).

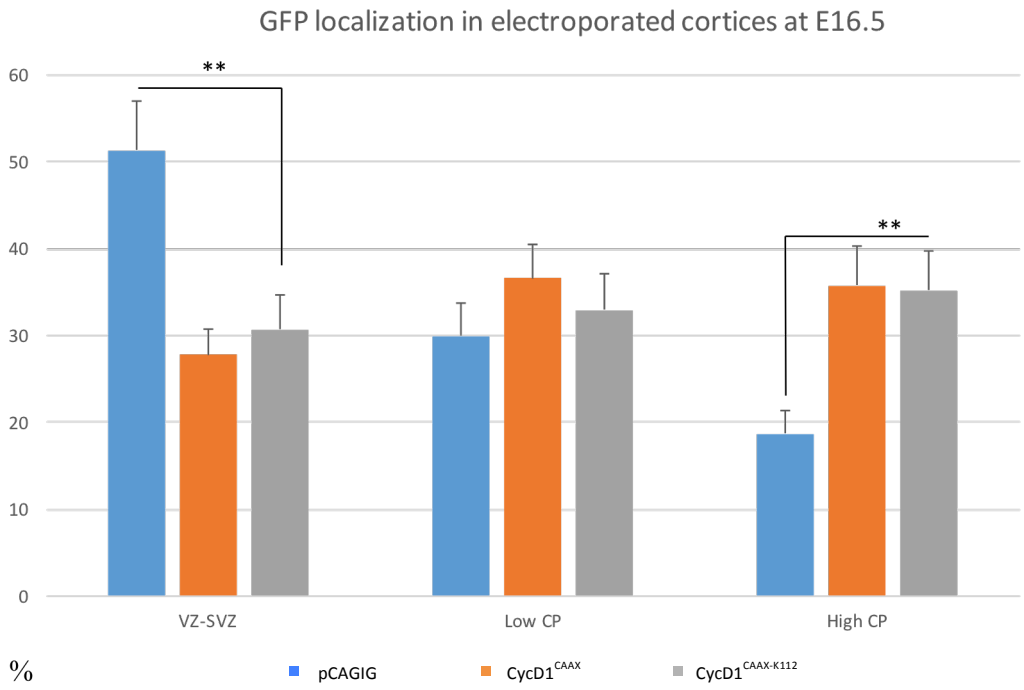

Figure 8: Early migration of transfected cells which overexpress CycD1.
Quantification graphic of E13.5 electroporated cells in embryos sacrificed at E16.5. Cortex was divided in three zones (VZ-SVZ, low CP, High CP) to analyse the placement of control, CycD1^{CAAX} and CycD1$^{K112\text{-}CAAX}$ -expressing neurons. Values represent mean percentage ± SD (n=3 experiments). **P (VZ-SVZ) = 0.0054; **P (High CP) = 0.0072.

As illustrated above (Fig. 6C, 6F, 6I), at E16.5 approximately half of control GFP positive cells (51,29%) maintained their localization in the VZ-SVZ subdivision,

29,97% in the low CP and 18,74% in the high CP. But the percentage of CycD1$^{K112-CAAX}$ and CycD1^{CAAX}-expressing neurons was even higher in high CP than VZ-SVZ area. In CycD1^{CAAX} samples just 27,77% of cells were in VZ-SVZ sector, the 36,60% in the low CP and the 35,80% in the high CP. In like manner, CycD1$^{K112-CAAX}$ samples showed similar percentages having 30,73% of cells in VZ-SVZ subdivision, 32,94% in the low CP and 35,24% in the high CP (Fig. 8).

Migration of Tbr2 positive cells electroporated with CycD1$^{K112-CAAX}$ is affected

In order to know the identity of mutant cells that displayed the upper localization in the brains electroporated with CycD1$^{K112-CAAX}$ and CycD1^{CAAX}, coronal sections were stained against different cortical layer markers of developing neocortex to find the colocalization with the GFP positive cells which are placed in the low and high CP at E16.5. In the neocortex, Ctip2 protein is restricted in developing CP, nevertheless, any scattered CycD1$^{K112-CAAX}$ and CycD1^{CAAX} expressing cells did not colocalized with Ctip2.

Surprisingly, almost the total amount of CycD1$^{K112-CAAX}$-expressing neurons located in the CP at E16.5 colocalized with Tbr2, a SVZ marker (Fig. 9C). In addition, CycD1$^{K112-CAAX}$-electroporated cells placed in the VZ-SVZ, did not express Tbr2. In the other hand, control-expressing neurons which colocalized with Tbr2 were restricted in the VZ-SVZ region, and the control cells which were in the low and high CP did not express Tbr2 (Fig. 9A). Thus, Tbr2 positive cells were placed normally in the SVZ, but in the electroporated samples with CycD1$^{K112-CAAX}$, whole transfected Tbr2 positive cells were scattered in the CP. In the case of CycD1^{CAAX} samples, there were some transfected scattered Tbr2 positive cell in the low CP and very few in the high CP, and, in like manner as CycD1$^{K112-CAAX}$ samples, there was not colocalization with Tbr2 in the VZ-SVZ region (Fig. 9B).

This anomalous localization of electroporated Tbr2 positive cells was quantified dividing neocortex in the three compartments explained in previous analysis performed,

comparing this time four constructs, adding a plasmid with the Ccnd1 insert without CAAX, as control of the cytoplasmic function carried by the CAAX motif in CycD1^{CAAX} and CycD1$^{K112\text{-}CAAX}$ results. The percentage of the quantification in both control samples (pCAGIG and CycD1) is pointed as pCAGIG/CycD1, in this order (Fig. 9D). Solely very few of control electroporated cells (7,07%; 1,78%) colocalized with Tbr2, placed practically all of them (96,01%; 98,72%) in VZ-SVZ region (Fig. 9A).

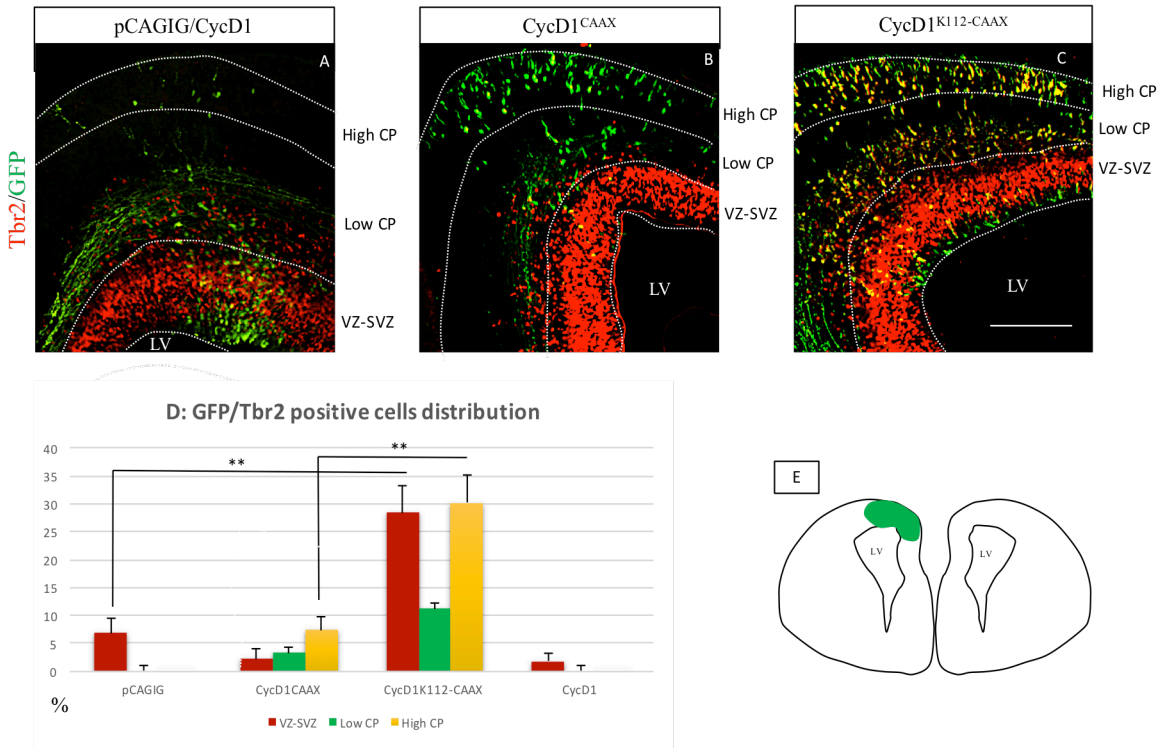

Figure 9. CycD1$^{K112\text{-}CAAX}$ Tbr2 positive neurons perform early migration to high CP.
Representative immunofluorescence of coronal cortices slices electroporated at E13.5 with control DNA (**A**), CycD1$^{K112\text{-}CAAX}$ (**B**) or CycD1$^{K112\text{-}CAAX}$ (**C**) and sacrificed at E16.5. Images were divided with discontinue lines in three regions (VZ-SVZ; low CP; high CP). Slices were labeled with Tbr2 (red) and GFP (green), thus, yellow cells observed in CycD1$^{K112\text{-}CAAX}$ slices are electroporated IPCs. Scale bar: 200 µm. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **LV**: Lateral Ventricle; **CP:** Cortical Plate. **D:** Quantification graphic of the colocalization between Tbr2 and GFP observed in electroporated slices. Values represent mean percentage ± SD (n=3 experiments). **P (VZ-SVZ) = 0.0024; *P (High CP) = 0.0018. **E**: Scheme of electroporated region in the electroporated brains.

In like manner as control samples, CycD1^{CAAX}-expressing cells minimally colocalized with Tbr2 (12,87%), but in this case just some of them (17,09%) were in VZ-SVZ area, while the majority (82,91%) were in CP (31,22% low CP; 68,78% high CP). This result may be considered as normal, due to the higher quantity of CycD1^{CAAX}-expressing cells

placed in the CP compared with control samples. As observed in Fig. 8B, the number of CycD1^{CAAX}-expressing cells which colocalized with Tbr2 in CP is very slight, given that just a few of CP electroporated cells (14,78%) colocalized with Tbr2.

Differently, whole CycD1$^{K112\text{-}CAAX}$ neocortex showed a great percentage of GFP/Tbr2 colocalization (69,86%), even in VZ/SVZ sector was overmuch superior than the CycD1^{CAAX} VZ/SVZ (40,71%), but the majority was located in the CP sector (59,29%), mainly in the high CP (26,96% in low CP; 73,04% in high CP). A fact to keep in mind is the practically inexistence of no electroporated Tbr2 positive cells in upper layers from SVZ (Fig. 8C). This considerable colocalization of GFP/Tbr2 in the CP of CycD1$^{K112\text{-}CAAX}$ samples, translates in a massive spread of Tbr2 positive cells in the CP produced by the action of CycD1$^{K112\text{-}CAAX}$.

In this context, quantification and distribution analysis of the whole number of Tbr2 positive cells (also SVZ) of electroporated neocortex was performed, dividing their allocation in the three regions mentioned above (VZ-SVZ; low CP; high CP) (Fig. 10D). In control samples (Fig. 10A), there were anecdotic Tbr2 positive cells in the low CP (0,18%; 0,56%). In CycD1^{CAAX} neocortex (Fig. 10B), some scattered Tbr2 positive cells was observed in low CP (4,83%) and in high CP (3,93%). Finally, notable number of scattered Tbr2 positive cells was observed in CycD1$^{K112\text{-}CAAX}$ low CP (5,90%) and a remarkable number in high CP (10,72%), keeping in mind the elevated quantity of Tbr2 positive cells placed in the SVZ normally (Fig. 10C).

As we know, some neural stem cells leave VZ and acquire a multipolar morphology in the SVZ becoming IPCs, continuing the cell division. When they migrate to upper layers to assembly into the CP, IPCs switch off Tbr2 signal and loss the capacity of division. In this context, immunofluorescence against PH3 was performed to study the cell cycle activity of Tbr2 positive cells placed in CP expressing CycD1$^{K112\text{-}CAAX}$, to determine if they still were IPCs or just they did not switch off Tbr2 signal.

As observed in the Fig. 11, there was not colocalization between PH3 and Tbr2 in the electroporated cell of the CP. This result suggest that CycD1 may control Tbr2 switch off migrating IPCs, therefore, scattered CycD1$^{K112\text{-}CAAX}$-Tbr2 positive cells should not be IPCs because of their loss of cell cycle activity.

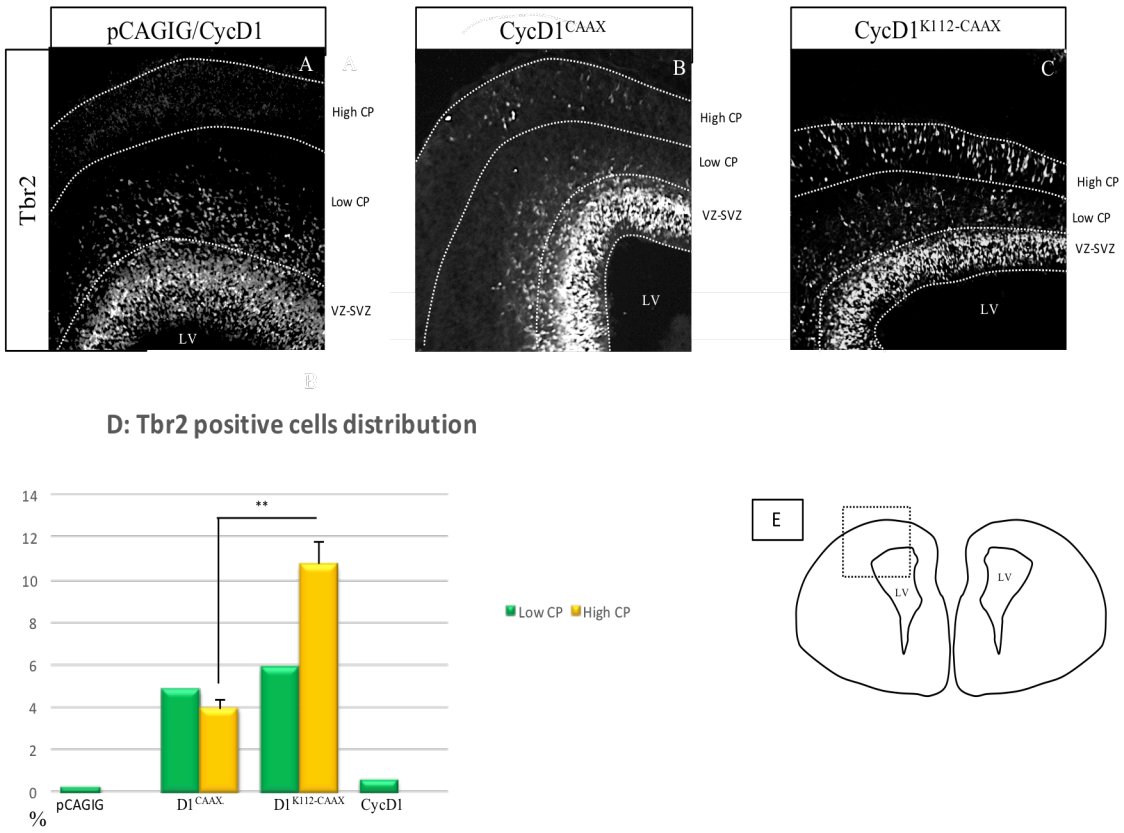

Figure 10. Tbr2 positive cells are extremely scattered in CycD1$^{K112\text{-}CAAX}$ injected brains.
Representative immunofluorescence of E13.5 electroporated coronal slices with control DNA (**A**), CycD1$^{K112\text{-}CAAX}$ (**B**) or CycD1$^{K112\text{-}CAAX}$ (**C**) and sacrificed at E16.5. Images were divided with discontinue lines in three regions (VZ-SVZ; low CP; high CP). Slices were labeled with Tbr2. Scale bar: 200 µm. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **LV**: Lateral Ventricle; **CP:** Cortical Plate. **D:** Quantification graphic of the colocalization of Tbr2 positive cells. Values represent mean percentage ± SD (n=3 experiments). **P = 0.0098. **E**: Scheme of the captured region of electroporated brains.

CycD1 controls the morphology and the attachment during translocation of radial migration

Migrating bipolar IPCs cells extend their leading processes and anchor to the BM or to the extracellular matrix to migrate into the CP by somal translocation. The soma moves upward in a spring-like manner by rapidly shortening the leading process. During somal translocation, neurons shorten their leading processes to move their cell bodies to their final positions.

For this reason, morphology of electroporated neurons was analyzed to determine the mechanism of radial migration occurring in the different electroporated neocortex with the three studied plasmids during the three ages studied (E14.5, E15.5 and E16.5). At

E14.5 it was not observed any significant difference between the morphology of control and CycD1$^{K112\text{-}CAAX}$ –expressing cells. Both neocortex showed similar proportion of multipolar GFP positive cells in the VZ/SVZ area, and some bipolar cells in the SVZ with processes reaching to the BM, but there were not significant differences neither the processes number nor length.

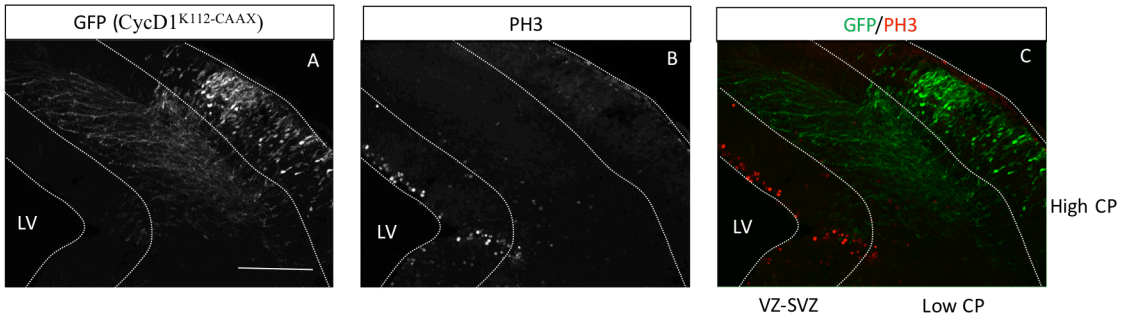

Figure 11. Transfected CycD1$^{K112\text{-}CAAX}$-Tbr2 positive cells do not express PH3 protein in the CP.
A, B, C: Representative immunofluorescence of E13.5 electroporated cortices slices of E16.5 embryos. **A:** Transfected cells labeled with GFP antibody in the high CP. **B:** PH3 positive cells located in the VZ of electroporated cortex. **C:** Double-stained slice labeled with GFP (green) and PH3 (red). Scale bar: 200 µm. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **LV**: Lateral Ventricle; **CP:** Cortical Plate.

At E15.5, as mentioned above, some GFP positive cells were in the low CP. Significative differences were observed analyzing the processes which throw these electroporated cells from low CP to high CP. In control samples, which the percentage of electroporated cells in the low CP is minor than the other studied conditions, it was observed some processes which did not reach to BM (Fig. 12A). Instead, in CycD1$^{K112\text{-}CAAX}$ samples, processes of low CP GFP positive cells showed numerous long processes which reached to BM (Fig. 12C). Remarkably, in CycD1^{CAAX} samples, it was not observed any process from the low CP electroporated cells (Fig. 12B). On the other hand, the morphology analysis of GFP positive cells did not report any significative difference in the proportion between multipolar and bipolar cells neither in the VZ/SVZ nor in the low CP.

In the E16.5 electroporated cortices was observed three types of morphology or condition of GFP positive cells in the neocortex: multipolar, bipolar and, just in the high CP zone, bipolar cells anchored to basement membrane (Fig. 13).

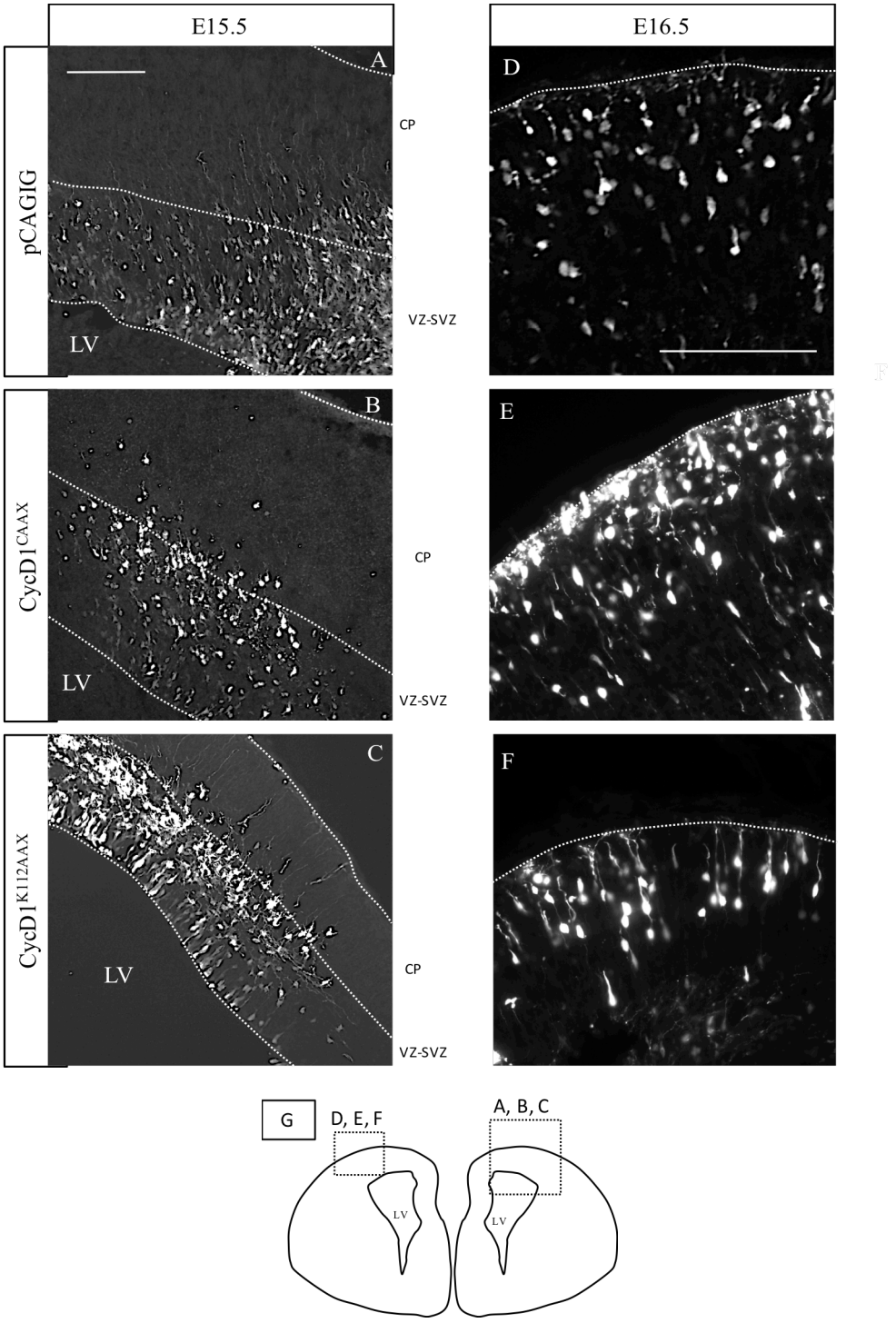

Figure 12. Leading process length depends of CycD1-CDK4/6 expression. **A, B, C:** Representative immunofluorescence images of E15.5 electroporated neocortex slices stained with GFP antibody. **A**: pCAGIG positive processes from electroporated cells (GFP positive) placed in the low CP, although these processes did not reach to BM. **B**: Transfected cells placed in the low CP did not throw processes to the CP of electroporated neocortex by $CycD1^{CAAX}$. **C**: Long and numerous processes in the neocortex electroporated by $CycD1^{K112\text{-}CAAX}$. The majority of these processes reached to BM. Scale bar: 200 µm.
D, E, F: Representative immunofluorescence images of electroporated cells in E16.5 high CP stained with GFP antibody. **D**: The little percentage of control electroporated cells which reach to high CP develop the leading process to anchor to BM. **E**: $CycD1^{CAAX}$ electroporated cells in the high CP with a great percentage of multipolar cells close to BM with a short leading process or without it. **F**: $CycD1^{K112\text{-}CAAX}$ electroporated cells relatively distant from BM but anchor to it through a long leading process. Arrow heads indicate leading process. Scale bar: 200 µm. **G**: Scheme of the captured region of electroporated brains. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **LV**: Lateral Ventricle; **CP:** Cortical Plate.

In the VZ-SVZ and low CP there were not significant differences, although there was a little increment in the percentage of bipolar CycD1$^{K112\text{-}CAAX}$ –expressing neurons (72,89%) in the low CP region compared with control and CycD1^{CAAX}–expressing neurons (52,92% and 42,19%, respectively) (Fig. 13). Into high CP, the percentage of multipolar GFP positive cells increased in the brains electroporated with CycD1^{CAAX} (63,33%), while the percentage of the brains electroporated with the control and CycD1$^{K112\text{-}CAAX}$ was significantly minor (24,7% and 17,18%, respectively). There was no significant difference in the proportion of bipolar GFP positive cells, but there was a great increment of the percentage of bipolar cells which were anchored to the basement membrane in the brains electroporated with CycD1$^{K112\text{-}CAAX}$ (53,11%) (Fig.11C), instead, in the brains electroporated with the control plasmid this percentage was a bit smaller (38,47%)(Fig. 12A) and quite decreased in CycD1^{CAAX} samples (17,46%) (Fig. 12B).

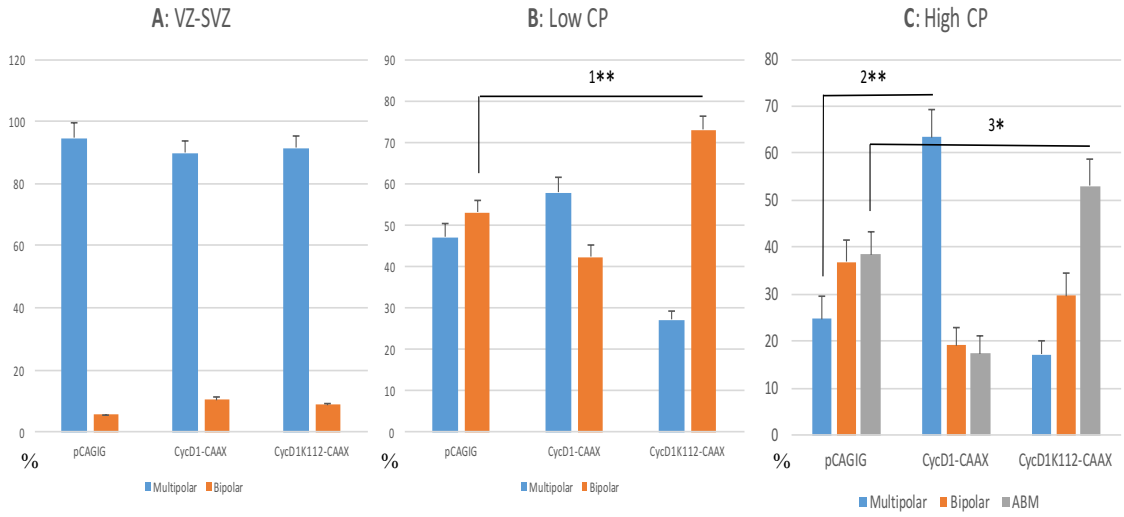

Figure 13. CycD1-CDK4/6 participates in the leading process formation.
Quantification analysis of transfected cells morphology along whole cortex. E13.5 embryos brain were electroporated and sacrificed at E16.5. Images were divided with discontinue lines in three regions (**A**: VZ-SVZ; **B**: low CP; **C**: high CP). Bipolar morphology was determined when a polarized leading process was observed. Values represent mean percentage ± SD (n=3 experiments). ^{1}**P = 0.0054; ^{2}**P = 0.001; ^{3}**P = 0.0286.

Not only the number of cells anchored to BM was increased, but observing the length of the leading process to reach to BM, there was significant longer in CycD1$^{K112\text{-}CAAX}$–expressing cells than control and CycD1^{CAAX}–expressing cells (Fig. 12D, E, F). The length of leading processes from the cells anchored to BM was measured and the average of the length was calculated. As it is described in Fig. 14A, the average length

of the leading process of anchored electroporated cells expressing CycD1$^{K112\text{-}CAAX}$ was 38,49 micrometers, while the control cells had an average leading process of 27,83 micrometers, and the CycD1^{CAAX}-leading process measured an average of 24,62 micrometers.

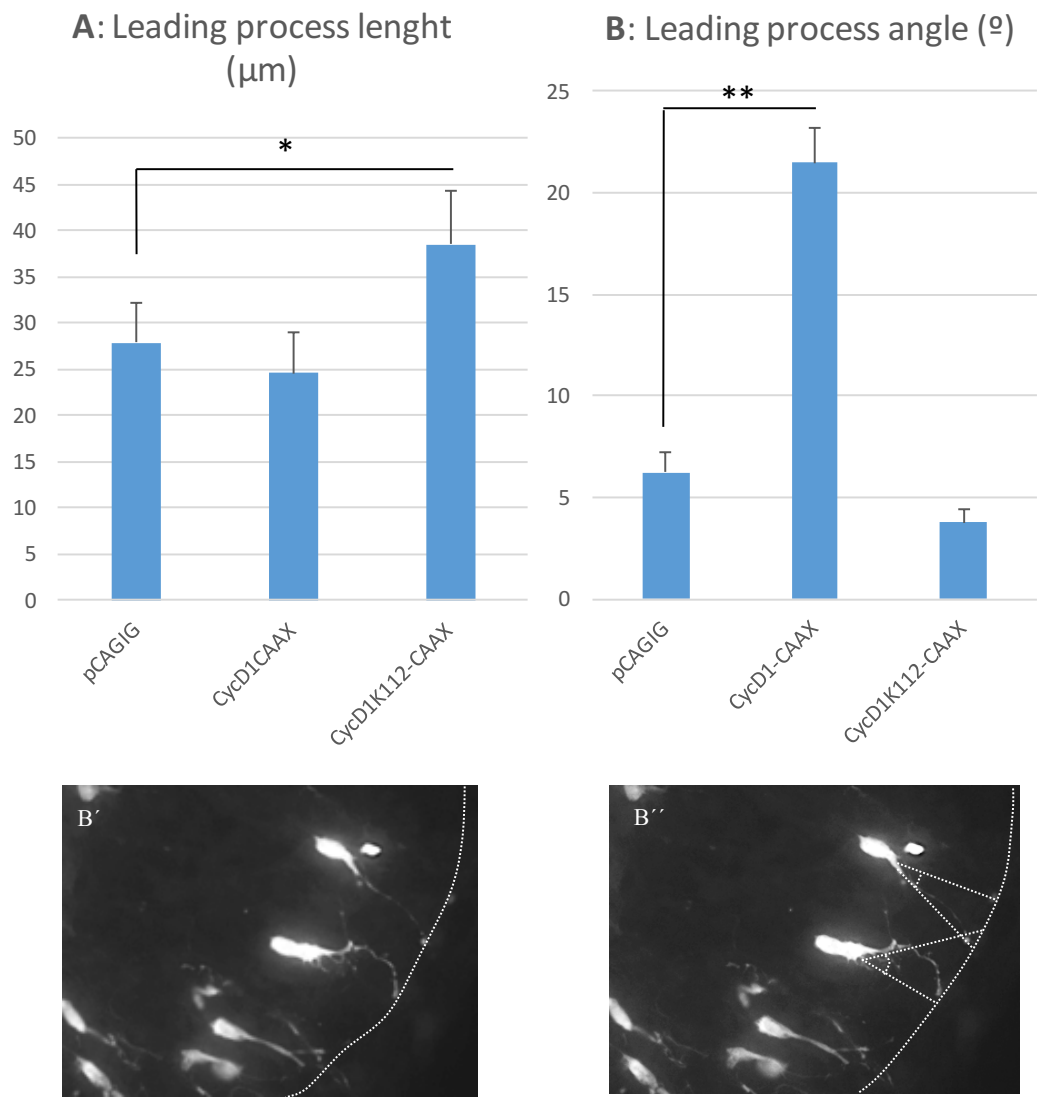

Figure 14. CycD1-CDK4/6 controls the polarization and the elongation of the leading process.
A: Quantification of the leading process length (µm) of transfected cells along E16.5 CP. Leading process length was measured since the closest part from the soma to the tip of the leading process. It was measured in pixels and converted to µm. Values represent mean ± SD (n=3 experiments). *P = 0.0302.
B: Analysis graphic of the angle formed between BM perpendicular and the leading process orientation of transfected cells. As shown in the images B´ and B´´, the alignment of the leading process was determined taking into account the closest portion to the soma of the leading process Scale bar: 50 µm. Values represent mean ± SD (n=3 experiments). *P = 0.002.

The analysis of the leading process of electroporated cells in the high CP makes us observe other significant difference between studied plasmid conditions. The angle which formed the leading process outset with the BM perpendicular, which should be approximately 0°, the CycD1^{CAAX}-leading process used to bend in most cases. In order to define this observation, the mentioned angle was measured and the average was calculated (Fig. 14C, D). As described in the Fig 14B, the average angle formed in control and CycD1$^{K112\text{-}CAAX}$ samples is quite close to be totally perpendicular to BM (6,25° and 3,74°, respectively). Contrarily, in CycD1^{CAAX} samples the angle was highly superior (21,34°).

4.3 Tbr2 positive cells are scattered in the neocortex of CycD1 knock-out embryos

To get further in the knowledge of cytoplasmic CycD1 function in the neocortex development, immunofluorescence analysis of E16.5 CycD1 knock-out (CycD1$^{-/-}$) embryos was performed. Diverse neocortex markers were stained comparing with wild-type (CycD1$^{+/+}$) embryos, including Tbr2, Ctip2, Nestin and Pax6.

Just with the Tbr2 analysis, as *in utero* electroporation assay, significant differences were observed. As illustrated in Fig. 15B, a considerable quantity of Tbr2 positive cells were scattered in the closest upper layers of the CycD1$^{-/-}$ SVZ, while into CycD1$^{+/+}$neocortex the whole of Tbr2 positive cells were restricted to SVZ. To improve our knowledge about this E16.5 CycD1$^{-/-}$ phenotype, Tbr2 expression analysis was performed at previous (E14.5) and subsequent (E18.5) neurogenesis phases to observe its development. At E14.5, when the IPCs migration to the CP has not yet happened, no significative differences were observed between CycD1$^{+/+}$ and CycD1$^{-/-}$ embryos. In the other hand, at E18.5 occurred similar phenotype as at E16.5, as observed in the Fig. 15C. This observed phenotype is quite clear, but less aggressive than Tbr2 positive cells phenotype observed in electroporated brains. Consequently, quantification method was more accurately dividing a fragment of neocortex in 10 equal bins from the apical to the basal side, and Tbr2 positive cells number was measured in each bin (Fig. 15A).

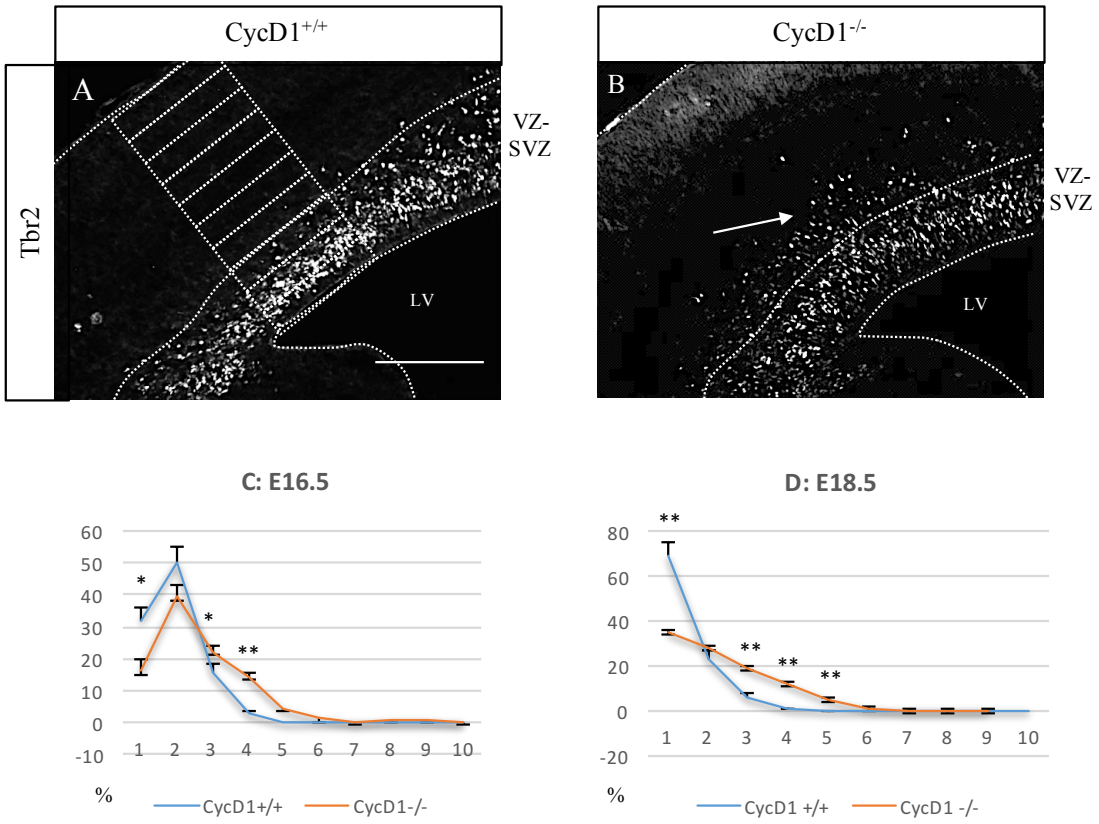

Figure 15. Tbr2 positive cell are scattered inCycD1$^{-/-}$ cortices at final stages of neurogenesis. Representative immunofluorescence images of CycD1$^{+/+}$ **(A)** and CycD1$^{-/-}$ **(B)** coronal slices labeled against Tbr2.The VZ-SVZ of E16.5 and E18.5 images was determined to observe scattered Tbr2 positive cells (arrowhead). Scale bar: 200 µm. **VZ-SVZ**: Ventricular Zone-Subventricular Zone; **LV**: Lateral Ventricle. **C, D**: Quantification analysis of Tbr2 positive cells throughout CycD1$^{+/+}$ and CycD1$^{-/-}$ neocortex at E16.5 and E18.5. Analyzed images were divided in 10 bins to enhance the accuracy of the quantification, being bin 1 the closest to LV. Values represent mean percentage (n=3); **C**: *P = 0.0119; *P = 0.0352; **P = 0.0020. **D**: **P = 0014; **P = 0.0059; **P = 0.0010; **P = 0.0018.

At E16.5/E18.5, as described in the Fig 15C/15D, the majority of Tbr2 positive cells (81,30%/92,56%) from CycD1$^{+/+}$ neocortex was in the first two bins (corresponded to roughly VZ and half SVZ). Instead, a little more than half of CycD1$^{-/-}$ Tbr2 positive cells (55,5%/63,63%) were in these bins. In the other hand, almost the rest of CycD1$^{+/+}$ Tbr2 positive cells (18,5%/7,3%) were in the three following bins (corresponded to the other half SVZ and IZ), while a significant higher percentage of CycD1$^{-/-}$ Tbr2 positive cells (41%/35,23%) were in these 3, 4 and 5 bins. Regarding the other bins corresponded to CP, there were a bit more percentage of Tbr2 positive cells in all CycD1$^{-/-}$ bins, but not enough to be significant due to the little number of cells in upper layers in both samples.

In analyzed neocortex images there were approximately similar number of Tbr2 positive cells between both genotypes studied. This data discards a quantification or immunofluorescence method error. In addition, as demonstrated in previous points, the phenotype observed was not due to proliferation differences caused by the lack of CycD1.

4.4 Layer 5 disorganization in new born mice cortices electroporated with CycD1$^{K112\text{-}CAAX}$

To examine the evolution of electroporated Tbr2 positive cells in the neocortex development, which may trigger an adult defect, postnatal analysis was performed in CycD1$^{K112\text{-}CAAX}$ electroporated mice. For reasons related with the *in utero* electroporation technique, just P1 mice were available for the analysis.

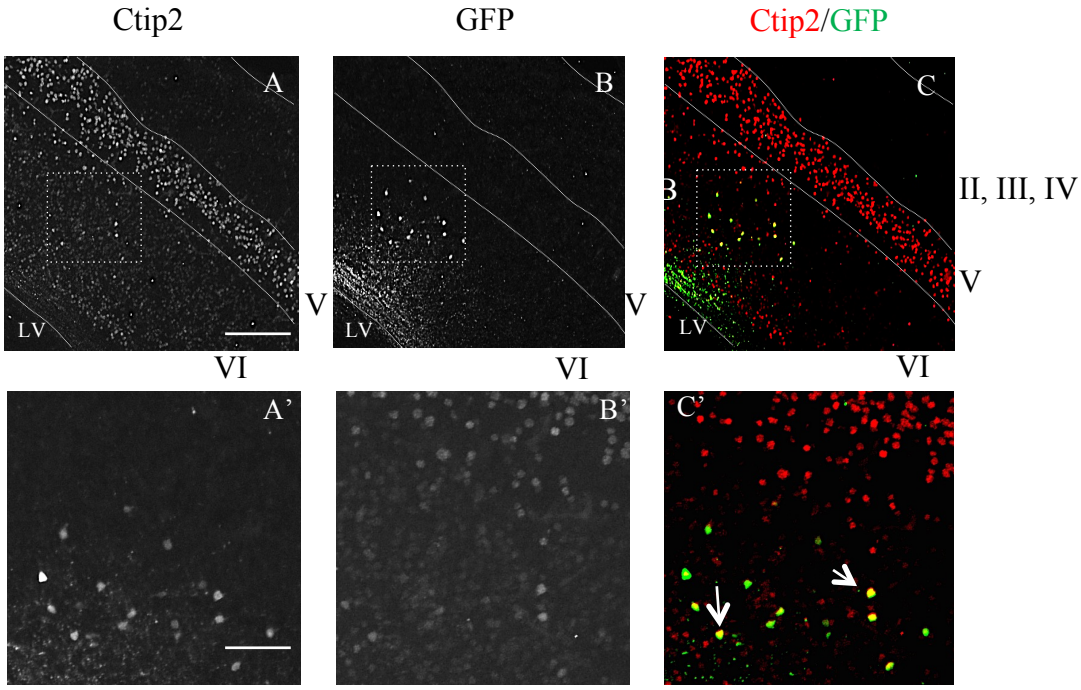

Figure 16. Transfected CycD1$^{K112\text{-}CAAX}$ Ctip2 positive cells are scattered from layer V in electroporated postnatal mice. **A, B, C**: Representative immunofluoresence of E13.5 electroporated cortices slices of P5 mice. **A**: Some Ctip2 positive cells located in the layer VI of electroporated cortex. **B**: Transfected cells labeled with GFP antibody in the layer VI. **C**: Double-stained slice labeled with GFP (green) and Ctip2 (red). The only Ctip2 positive cells located in layer VI are CycD1$^{K112\text{-}CAAX}$ expressing neurons. Scale bar: 200 µm. Square of discontinued lines indicate A', B' and C' images limits. **A', B', C'**: Amplified images of the scattered CycD1$^{K112\text{-}CAAX}$ electroporated Ctip2 positive cells located in the layer VI pointed with arrows. Scale bar: 100 µm.

In these mice, Tbr2 did not express along P1 neocortex, thus neocortex analysis was performed with different layer markers (Cux1, Ctip2 and Tbr1) to determine the identity of the GFP positive cells. Any of electroporated cells were positive for Tbr1 nor Cux1, but some of them colocalized with Ctip2 around the layer V (Fig. 16). Close zones from the electroporated focus were taken as control samples, and Ctip2 positive cells were analyzed in both regions. In control neocortex, Ctip2 positive cells were classified in two groups; high Ctip2 cells were placed in the layer V, and low Ctip2 cells were dispersed between the layer V and the layer VI, as shown in the right part of the Fig. 16. Instead, in the CycD1$^{K112\text{-}CAAX}$ electroporated region, GFP positive cells colocalized under the layer V with high Ctip2 cells, which were the only ones that were not in their corresponded layer. Furthermore, layer V in the electroporated region was significantly more disorganized and wider than the next control area, even without being CycD1$^{K112\text{-}CAAX}$-expressing cells.

Cyclin D1 deficient postnatal phenotype

CycD1 deficient mice have a life expectancy of 10-15 days. Using different cortical layer markers like Cux1 (layer 2, 3, 4), Ctip2 (layer V) and Tbr1 (layer VI), we wanted to analyze the cortical structure of postnatal knockout mice of P8-P10 compared with wild-type to know if the embryonic phenotype observed entails a posterior defect in the cortical architecture.

The analysis of Cux1 and Tbr1 did not reveal any significant defect in the localization of the positive cells, but the Ctip2 expression in knock-out cortices was slightly different compared with the layer V of wild-type (WT) cortices (Fig. 17A, 17B).

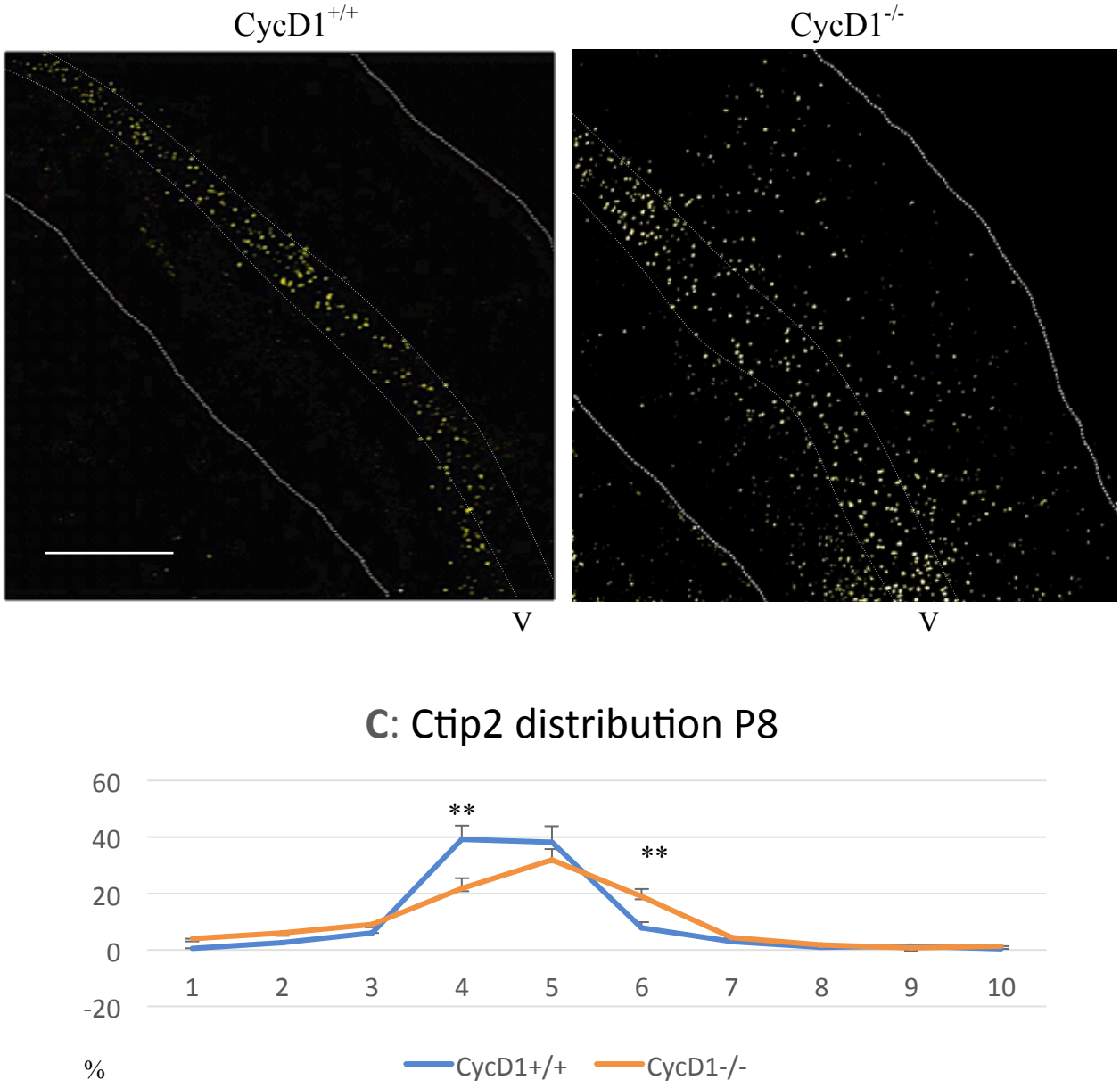

Figure 17. Ctip2 positive cells are disorganized in the P8 cortex CycD1$^{-/-}$ mice.
Representative immunofluorescence images of CycD1$^{+/+}$ **(A)** and CycD1$^{-/-}$ **(B)** coronal slices labeled against Ctip2. Layer V was marked to observe scattered Ctip2 positive cells. Scale bar: 200 µm. **LV** Lateral Ventricle. **C**: Quantification analysis of Ctip2 positive cells throughout CycD1$^{+/+}$ and CycD1$^{-/-}$ neocortex at P8. Analyzed images were divided in 10 bins to enhance the accuracy of the quantification being bin 1 the closest to LV. Values represent mean percentage (n=3); **P = 0.0079; *P = 0.0044.

DISCUSSION

5. DISCUSSION

In recent years, novel cytoplasmic CycD1 functions have been discovered in many tissues related with integrative mechanisms of adhesion between cell-cell or cell-ECM interaction, and of motility enhancement by cytoskeleton regulation during migration of several cell types.

In the present thesis we have made two important observations about the expression of CycD1 in the developing telencephalon. First we have revealed that CycD1 expression follows a graded pattern (from high in ventro-lateral regions to low in dorsal-medial areas) different from other proliferation markers. Second, we have observed a specific cytoplasmic CycD1 localization along the radial glia fiber and in important adhesion sites. We have investigated the role of CycD1 during neuron differentiation and tackle specifically its cytoplasmatic function in relation with CDK4/6 interaction in a in vivo context. For this, we performed in utero electroporation of the developing mouse brain with different CycD1 mutants.

We have demonstrated that CycD1 displays CDK4/6 dependent and independent functions. For instance lack of cytoplasmatic CycD1-CDK4/6 interaction triggers IPCs migration and morphology alterations in the developing cortex. Interestingly, some of these defects were also observed in *CycD1* knock-out embryonic brains. All together, these results suggest that the cytoplasmic CycD1 plays an important role in brain development probably by regulating the adhesion assembly of essential neuronal mechanisms and/or the motility of migrating neurons during radial migration.

5.1 Specific cytoplasmic CycD1 follows neurogenic gradient expression in the developing telencephalon

Nuclear localization of CycD1 is mainly related with cell cycle and transcription factor regulation. In recent years, several studies have pointed that CycD1 act as a multifunctional protein that is able to localize and perform specific functions in the cytoplasm of many cell types, including fibroblasts (Li et al. 2006), macrophages

(Neumeister et al. 2003), keratinocytes (Fernández-Hernández et al. 2013) and metastatic cells (Body et al. 2017; Fernández et al. 2011; Fusté et al. 2016). Initially, the cytoplasmatic expression of CycD1 was thought to be part of a mechanisms to remove CycD1 from the nucleus just to restrain cell proliferation and promote differentiation of the cells, including postmitotic neurons (Sumrejkanchanakij 2003; Sumrejkanchanakij, Eto, and Ikeda 2006). Recently, however, it has unveiled many important roles of cytoplasmic CycD1. Predominantly these CycD1 cytoplasmic functions are related with adhesion regulation in the detachment between cell to cell or cell-ECM, and locomotion during cells migration and their differentiation in many kind of cells. Through activation of a CycD1/CDK4-paxillin-Rac1 axis (Fusté et al. 2016) and the CycD1 collaboration with Ral GTPases (Fernández et al. 2011), it was shown a functional relevant mechanism operating under normal and pathological conditions to control cell detachment, migration and metastasis (Li et al. 2006; Neumeister et al. 2003; Fernández-Hernández et al. 2013).

We have expanded these observations and revealed that CycD1 cytoplasmatic expression can be also observed during brain development *in vivo*, in particular in the radial process of the RGCs of the developing telencephalon and thalamus. In addition, this cytoplasmic localization follows ventro-dorsal gradient during the neurogenesis of telencephalon (Bayer and Altman 1987).

Additionally, because of its important cell cycle roles in other localizations, cytoplasmic expression of CycD1 was compared with well-established proliferation markers analysis such PH3 and EdU during neurogenesis, and it reveals that the new-born neurons division do not follow the same gradient as cytoplasmic CycD1 observed in the telencephalon. While proliferation markers are expressed homogenously during the studied phases of telencephalon development (E12.5-E16.5), there was a clear difference in the staining intensity of cytoplasmic CycD1 between ventral and dorsal zones in the telencephalon depending the studied ages.

This unexpected expression parallel to neurogenesis and not to proliferation gradient along developing telencephalon suggests not only that cytoplasmic CycD1 do not participate in cell cycle functions, but may be important for the proper neurogenesis progress. In addition, this result is according to the CycD1$^{-/-}$ proliferation analysis which

do not reveal any mitosis defect analyzing the staining of EdU and PH3 antibodies, which labeled similar number of cells in $CycD1^{+/+}$ and $CycD1^{-/-}$ cortices. All together suggest that CycD1 is being compensated by other proteins, surely CycD2 also expressed in the telencephalon, to the accurate functioning of cell cycle in CycD1 deficient mice (Glickstein et al. 2009; Tamaru, Okada, and Nakagawa 1994).

The analysis of subcellular localization of cytoplasmic CycD1 shows that in the final section of RGCs close to BM, the CycD1 expression disappears, and appears again in the tip of the process. Furthermore, integrin ß1 colocalizes with CycD1 in the RGCs processes. These results suggest that cytoplasmic CycD1 may participate in the glial-guided locomotion during neurogenesis, probably controlling the interaction between locomoting neurons and RGCs fibers, by the regulation various membrane-bound cell adhesion molecules, such integrin ß1 or other integrins, which mediate the interaction of migrating neurons and RGCs (Adams et al. 2002; Anton et al. 1997; Edmondson et al. 2018; Fishell and Hatten 1991; Stitt and Hatten 1990).

Furthermore, as explained in the results section, cytoplasmic CycD1 was localized not only in the RGC fibers, but also close to BM in the tip of RGCs or translocating neurons leading process. This basal expression colocalizes with integrin ß1. When early-born migrating neurons undergo somal translocation, the leading process is anchored to the BM. This adhesion has been reported to require N-cadherin or ß1 integrin (Franco et al. 2011; Sekine et al. 2012).

These results may suggest that the adhesion control in the attachment of translocating cells to BM, essential for the correct migration of translocating neurons, may occurs by the CycD1-integrin ß1 pathway. In the same manner RGCs attach to BM. RGCs are the first cohort of neuronal precursor in the neurogenesis, which divide asymmetrically to origin a new-born neuron and an IPC. Some studies report that neurons can inherit the radial process during division and the daughter neuron migrates to the cortical plate through somal translocation (T Miyata et al. 2001; Nadarajah and Parnavelas 2002; Noctor et al. 2001; Tamamaki et al. 2001; Nadarajah et al. 2001). This background may suggest that CycD1 in the tip of RGCs processes plays de same role as the CycD1 in the tip of leading process of translocating neurons.

5.2 CycD1 may increase motility in late-born neurons

Using the technique of the intraventricular injection and *in utero* electroporation, we have shown that, at E16.5, transfected neuroprogenitor cells which overexpress a cytoplasmatic membrane bound CycD1 move to the CP earlier than control cells, overexpressing only EGFP. Interestingly, this effect is independent of CDK4/6 interaction, since there are not differences between $CycD1^{K112\text{-}CAAX}$ and $CycD1^{CAAX}$-expressing neurons. In addition, at E14.5, neuroprogenitor cells overexpressing $CycD1^{K112\text{-}CAAX}$ appear to collect in the SVZ, while control transfected cells were distributed along VZ-SVZ region homogenously. These results demonstrate that cytoplasmic CycD1 ($CycD1^{CAAX}$) is able to modulate the dynamics of cell locomotion of neuron progenitors *in vivo* and that this effect is mediated by effectors different of CDK4/6.

Overexpression of CycD1 has been reported in many human cancers such breast, colon, prostate and hematopoietic malignancies, and it is related with metastasis promotion since it was discovered that CycD1 has a central role in mediating invasion and migration of cancer cells by the repression of the Rho GTPases signalling (Li et al. 2006). In addition, CycD1 modulates levels of $p27^{kip1}$ protein, which is also accumulated in metastatic cells. $p27^{kip1}$ is an inhibitor of CycD1-CDKs complexes and plays a crucial role in cell cycle regulation. Recent evidence showed an important role of $p27^{kip1}$ in promoting cellular migration of fibroblasts modulating RhoA activity. Then, Fusté et al. showed that cytoplasmic CycD1 promotes cell locomotion by the regulation of two types of Rho small GTPases, RhoA and Rac1, in an opposite way (Fusté N, 2016).

In the other hand, it has reported that $CycD1^{-/-}$ fibroblasts or macrophages displayed increased cellular adherence, and defective motility (Li et al. 2006; Neumeister et al. 2003). By contrast, we suggest that overexpression of CycD1 decreases adhesion capacity of the cell to stick to ECM or other cells, increasing the migration of those cells.

In the exposed *in utero* electroporation results, we suggest that the electroporated cells overexpressing cytoplasmic CycD1 display faster radial migration than control

electroporated cells because that cytoplasmic CycD1 interacts and inhibit the Rho GTPases pathway which controls neuron locomotion (Li et al. 2006).

New-born neurons in the VZ attach to RGCs to migrate by glial-guided locomotion to CP. This mode of migration needs the adhesion of migrating neurons to RGCs which attach to the process and use them as scaffold to reach to CP (Marin et al. 2010; T Miyata et al. 2001; Takaki Miyata and Ogawa 2007; Nadarajah and Parnavelas 2002). Neumeister P reported that CycD1 deficient macrophages showed an adhesion increase in their migration related with its metastasis capacity (Neumeister P, 2003). We also suggest that overexpression of cytoplasmic CycD1 in the *in utero* electroporation assays, affects to the interaction between migrating electroporated neurons and RGCs, through the reduction of adhesion between both cells. This absence of interaction may be one of the reasons of the quick migration of overexpressing CycD1 electroporated neurons.

5.3 CycD1-CDK4/6 may control leading process dynamic in translocating neurons

Differences in the neuronal and leading process morphology were observed between $CycD1^{K112\text{-}CAAX}$ and $CycD1^{CAAX}$-expressing neurons. For example, at E15.5, the majority of $CycD1^{K112\text{-}CAAX}$-expressing cells located in the SVZ threw a leading process that reached the BM, while $CycD1^{CAAX}$ –expressing neurons located in the same region did not form any process. Furthermore, at E16.5, $CycD1^{K112\text{-}CAAX}$-transfected neurons in the high CP that are located close to the BM show significant longer leading processes than $CycD1^{CAAX}$-expressing leading process, which appear a bit unstable in regard to the perpendicular line of radial migration.

Previous reports have shown in different cell types that CycD1-CDK4/6 controls cytoskeleton organization and cell adhesion enhancing cell motility in collaboration with Ral GTPases (Fernández et al. 2011). Formation of the leading process requires the microtubule synthesis through the activation of Cdc42 and Rac1 and the inhibition of RhoA (Bourne, Sanders, and McCormick 1991; Nobes and Hall 1995; Spiering and

Hodgson 2018). It has been reported that $p27^{kip1}$ inhibit the activity of RhoA, and as explained above, also the CDK4/6 activity to promote the migration. All together suggest that CycD1-CDK4/6 play the same of RhoA, which must to be decreased by $p27^{kip1}$ to promote leading process formation and motility during neurogenesis, while other Rho GTPases such Cdc42, Rac1 and Ral interact with CycD1 (Fernández et al. 2011) to enhance the cell migration.

5.4 CycD1-CDK4/6 may regulate IPCs differentiation and the exit from the SVZ

One of our more interesting observations was that neurons located in the CP and overexpressing $CycD1^{K112\text{-}CAAX}$ (but not those overexpressing $CycD1^{CAAX}$) were expressing Tbr2, a marker for IPCs normally located in the SVZ.

IPCs are distinguished from other neuronal cells by a unique molecular profile, principally the specific expression of the Tbr2 transcription factor (also known as Eomes; NCBI Gene Eomes) (Englund et al. 2005; Gal et al. 2006; Stancik et al. 2010). IPCs are originated from RGCs that leave the VZ to accumulate in the SVZ, where they acquire a multipolar morphology and divide symmetrically to generate two IPCs, or two neurons. Subsequently, these cells leave the SVZ in order to migrate in a bipolar shape to CP by glial-guided locomotion and finally by somal translocation in the last phase of their migration (Tarabykin 2001; Zimmer et al. 2004). The start of this migration towards the CP correlates with a downregulation of Tbr2. However, in the $CycD1^{K112\text{-}CAAX}$ overexpressing neurons, Tbr2 expression is not downregulated and it is still observed in cells within the CP. This observation may suggest three possibilities.

First, Tbr2 positive cells located in the CP are still IPCs, which also divide and have multipolar morphology, but, due to the overexpression of $CycD1^{K112\text{-}CAAX}$, its adhesion control and glial-guided locomotion is somehow disturbed and do not make the "SVZ stop" and migrate directly to the CP. When $CycD1^{K112\text{-}CAAX}$ expressing IPCs should remain in the SVZ, they reach to the CP expressing still the Tbr2 marker. According with the results showed in other *in utero* electroporation assays performed (Artegiani,

Lindemann, and Calegari 2011; Lange, Huttner, and Calegari 2009), overexpression of CycD1-CDK4 may shorten G1 and delay neurogenesis, promoting the expansion of IPCs. This report makes us to consider that CycD1$^{K112\text{-}CAAX}$-Tbr2 overexpressing neurons located in the CP have delayed their neurogenesis outset and for this reason they still express Tbr2, but they have migrated to CP due to their locomotion increase. In the other hand, this fact do not explain the differences showed in the localization between the CycD1$^{K112\text{-}CAAX}$-Tbr2 and CycD1^{CAAX}-Tbr2 expressing neurons, because in the CycD1$^{K112\text{-}CAAX}$ electroporated cortices, CycD1-CDK4 functions do not develop.

The second possibility would be that CycD1-CDK4/6 controls the morphology and differentiation of IPCs cells. As explained in the previous section, CycD1 may control microtubules organization through CDK4/6 linking, and regulate leading process dynamic. The negative dominant expression of this complex (CycD1$^{K112\text{-}CAAX}$) in the IPCs could increase the microtubule synthesis activity required to form the leading process and migrate around RGCs by glial-guided locomotion in later phases than control IPCs, leaving SVZ but still expressing Tbr2 due to their improved locomotion capacity.

The third possibility would be the control of Tbr2 expression by any CycD1 pathway, due to it has reported that this protein regulates the activity of transcription factors, coactivators ad corepressors (Fu et al. 2004), but in this case any report let us to take this possibility seriously.

To select one of these possibilities, we did two inquiries. Proliferation analysis of CycD1$^{K112\text{-}CAAX}$ -Tbr2 positive cells by PH3 marker revealed that they were not proliferative active and consequently did not divide symmetrically in the CP. In addition, the majority of CycD1$^{K112\text{-}CAAX}$ -Tbr2 positive cells located in the CP show a polarized bipolar shape, with a main leading process, which contrasts with the multipolar shape of IPC neurons in the SVZ. Accordingly, both analyses support the second possibility exposed and the idea that CycD1$^{K112\text{-}CAAX}$ -Tbr2 positive cells can not be considered as IPCs because they do not have mitotic capacity nor multipolar morphology.

5.5 CycD1-CDK4/6 may control somal translocation regulating migrating neuron anchorage to basement membrane through integrin ß1 interaction

Leading process of late-born migrating neurons surrounds RGC fiber while perform glial-guided locomotion until it is close to pial surface, when extends the leading process to anchorage to BM and finishes its migration by somal translocation (Cooper 2013). Basement membrane is a layer of specialized extracelular matrix that form part of the cortex architecture, and interact with the surrounding membrane cells through integrins, growth factor interactions, and dystroglycan (Yurchenco 2011).

Integrins are transmembrane heterodimeric receptors that mediate signaling initiated by ligand binding, mainly by components of the extracellular matrix. They act in a bidirectional fashion and are modulated by the mechanical properties of the cell-ECM interface (Berrier and Yamada 2007; Takagi 2007). Integrins undergo clustering that concentrates intracellular components involved in signaling. Integrins affect actin organization through modulation of small GTPase activities and can provide firm anchorage to the cell through linkages formed with recruited cytoplasmic proteins to F-actin (Vicente-Manzanares, Choi, and Horwitz 2009). Basement membrane components, especially the laminins, interact with a number of related β1-integrins (α1β1, α2β1, α3β1, α6β1, and α7β1) (Wu and Reddy 2012).

Our results show that specific expression of CycD1 close to the basement membrane in the telencephalon parallels neurogenic gradient (Bayer S, 1987) and colocalizes with integrin β1. In addition, the marginal zone analysis of electroporated cortex revealed significative differences between $CycD1^{K112\text{-}CAAX}$ and $CycD1^{CAAX}$ in the percentage of CP transfected neurons which are attached to BM with its leading process. Regarding to electroporated cells with their soma placed close to BM, the majority of $CycD1^{K112\text{-}CAAX}$-expressing leading process were attached to BM, while respect to $CycD1^{CAAX}$-expressing neurons, their short leading process were disengaged to BM, “floating” in the MZ. Both results together make us suggest that CycD1-CDK4/6 complex may control the adhesion between BM and translocating neurons through the modulation of integrin β1 and other macromolecules of ECM. The difference between $CycD1^{K112\text{-}CAAX}$

and CycD1^{CAAX} may be triggered because the integrin β1 interaction with CycD1 is through the K112 union site, suggesting that CycD1-CDK4/6 would inhibit the BM integrin β1 adhesion function in the somal translocation.

5.6 CycD1 could enhance layer organization in postnatal cortex

Both postnatal analyses in CycD1$^{-/-}$ P8 mice and CycD1$^{K112\text{-}CAAX}$ electroporated P5 mice revealed similar phenotypes respect to layer V positive for Ctip2. Lack of CycD1 triggered the dispersion of Ctip2 positive cells regard to layer V. Unfortunately, due to technical causes, CycD1^{CAAX} *in utero* electroporation provoked the embryos abortion before their birth, and in the absence of postnatal CycD1^{CAAX} samples. For this reason, we cannot distinguish if this defect is triggered by CycD1 or the CycD1-CDK4/6 complex.

Other question we make is if scattered Tbr2 positive cells observed in CycD1$^{-/-}$ embryos and CycD1$^{K112\text{-}CAAX}$ electroporated brains originate scattered Ctip2 positive cells in postnatal cortices. Accordingly to the inside-out pattern, it has always been thought that IPCs, which migrate radially in the last phases of neurogenesis, populate CP upper layers (II, III, IV). But recent studies (Kowalczyk et al. 2009) report that IPCs contribute originating pyramidal neurons of all layers of cerebral cortex, suggesting that scattering Tbr2 positive cells may affect to the layer V development.

Pyramidal neurons from layer V integrate inputs from many sources and distribute outputs to cortical and subcotical structures. In the motor cortex, pyramidal neurons constitute the origin of the corticospinal tract and the electric signals of voluntary movements and reflexes (Salimi, Friel, and Martin 2008). Injuries in the layer V of motor cortex provide evidences that appear symptoms including spasticity, hyperactive reflexes, a loss of the ability to perform fine movements, and an extensor plantar response known as the Babinski sign. In this context, Sicinski P reported that CycD1 deficient mice behavior shows an abnormal limb reflex and the clasping of the limbs (fine movement) was defective. This signs suggest that the lack of CycD1 trigger

defects in the radial migration of IPCs, and subsequently, the disorganization of Ctip2 positive cells of layer V in postnatal mice lacking CycD1 originating neurological defects related with layer V pyramidal neurons functions.

CONCLUSIONS AND FUTURE OUTLOOK

6. CONCLUSIONS AND FUTURE OUTLOOK

6.1 Conclusions

1) Cyclin D1 is expressed in the processes of RGCs and in the basement membrane of developing telencephalon and thalamus.

2) Cytoplasmic expression of Cyclin D1 follows a ventro-dorsal gradient during telencephalon development (E12.5-E16.5) in like manner occurs the mouse brain neurogenesis.

3) Integrin ß1 expression colocalizes with the whole cytoplasmic CycD1 expression.

4) Cyclin D1 deficient embryos do not show proliferation defects, thus Cyclin D1 is not essential for the neuroprogenitors cell cycle progression in the developing telencephalon.

5) Overexpression of CDK4/6 independent cytoplasmic Cyclin D1 promotes radial migration.

6) Overexpression of CDK4/6 dependent cytoplasmic Cyclin D1 inhibits leading process formation in migrating bipolar neurons. Contrarily, overexpression of its negative dominant provokes a great increase of the leading process length.

7) Overexpression of CDK4/6 dependent cytoplasmic Cyclin D1 triggers defects in the polarity of the short leading process of migrating bipolar neurons, forming a non-perpendicular process with the basement membrane.

8) Overexpressing CDK4/6 dependent cytoplasmic Cyclin D1 neurons placed in the high cortical plate do not anchor to basement membrane even having a leading process. By contrast, the majority of neurons expressing its negative dominant are

strongly attached to basement membrane.

9) Cyclin $D1^{-/-}$ intermediate progenitor cells and Cyclin $D1^{+/+}$ intermediate progenitor cells transfected with CDK4/6 dependent cytoplasmic Cyclin D1 negative dominant differentiate early and leave from the SVZ expressing still Tbr2 at E16.5.

10) Lack of Cyclin D1 during radial migration leads to defects in the layer V organization at postnatal ages.

11) Abnormal limb reflex and clasping movements observed in Cyclin D1 deficient mice could be provoked by defects in the corticospinal tract, which connect the pyramidal neurons of layer V with spinal cord.

6.2 Future Outlook

The present work opens a door in the CycD1 knowledge beyond the nuclear functions related with the cell cycle progression or as transcription factor explained in the introduction chapter. The discovery of CycD1 cytoplasmic functions in the murine nervous system reveals that this protein may be an important regulator of the important molecular mechanisms that manage the radial migration. Although the results presented in this work show a clear cortical phenotype when CycD1 is overexpressed or absent, further experiments are necessaries to elucidate the molecular signaling pathways triggered by CycD1 that regulates radial migration and distribution within the developmental cortex.

Indeed, in our laboratory we are already performing *in vitro* studies as for example adhesion assays to demonstrate the adhesion role of CycD1 in neuronal cells. As explained in the introduction chapter, several reports (Bodeman BO, 2008; Body S, 2017; Drobnjak M, 2000; Fernández RMH, 2011; Fernández RMH, 2013; Fusté N, 2016; Li Z, 2006; Neumeister P, 2003; Rosse C, 2006; Shi J, 2006; Spiczka KJ, 2008; Zhong Z, 2010) show that CycD1 controls the motility of many types of cells through adhesion regulation, and we think that this regulation may occurs also in the radial migration. In this context, it would be interesting study the adhesion ability of $CycD1^{-/-}$ neuronal cells *in vitro* and their spread capacity in a Poly-D-Lysine/Laminin matrix. In addition, transfection of $CycD1^{K112\text{-}CAAX}$ and $CycD1^{CAAX}$ to $CycD1^{+/+}$ neurons could be performed. Accordingly to our hypothesis, $CycD1^{-/-}$ and $CycD1^{K112\text{-}CAAX}$ would be more attached to the matrix than cells $CycD1^{CAAX}$ –expressing cells.

In order to complement *in utero* electroporation results, we are improving *in utero* electroporation assay to inject $CycD1^{-/-}$ embryos the pCAGIG vector to observe the scattered Tbr2 positive cells morphology. Until now, we have been able to obtain electroporated $CycD1^{-/-}$ embryos satisfactorily due to their default sensibility and their abortion probability.

If this experiment was successful, given the $CycD1^{K112\text{-}CAAX}$ –expressing neurons morphology results, lack of CycD1 and the consequent decreased levels of CycD1-

CDK4/6 complex in the CycD1$^{-/-}$ embryos should induce that scattered Tbr2 positive cells have long leading processes, even some attached to BM. In addition, it would be interesting observe the other electroporated cells negative for Tbr2, to determine if the CycD1-CDK4/6 complex just affect to the IPCs differentiation, as observed in the *in utero* electroporation experiments.

In the same way, and with the same abortion problems, we are working in the CycD1$^{-/-}$ embryos electroporation with CycD1 and CycD1^{CAAX} constructs. The aim of this experiment is to rescue the observed Tbr2 phenotype in the CycD1$^{-/-}$ embryos overexpressing CycD1. The use of both vectors means that we would totally confirm that the abnormal localization of Tbr2 positive cells is provoked by the lack of cytoplasmic CycD1, and we hope that the rescue just will happen overexpressing CycD1^{CAAX} vector.

Finally, the design of nervous system-specific conditional CycD1 knock-out mice, would make us obtain a very useful tool to answer and confirm many emerged questions from this thesis. For instance, the life of these mice would reach to advanced ages, being able for behavior and neurological studies. Furthermore, Emx1-Cre conditional knock-out mice for CycD1 would allow us to determine if limb-reflex defect and clasping capacity observed in CycD1$^{-/-}$ postnatal mice are due to the analyzed disorganization of pyramidal neurons of layer V.

PUBLICATIONS AND MEETINGS

7. PUBLICATIONS AND MEETINGS

Fleitas C, Piñol-Ripol Gl, Marfull P, Rocandio D, Ferrer I, Rampon I, Egea J, Espinet C. 2018. proBDNF is modified by Radical Oxygen Species in Alzheimer's Disease and causes neuronal apoptosis by inducing p75 neurotrophin receptor processing. *Molecular Brain* 11 (1): 68.

The results of this thesis have been presented in:

- 16th SENC Natioanl Meeting, Granada (Spain), September 2015.
- 10th FENS Forum of Neuroscience, Copenhaguen (Denmark), July 2016.
- X Simposi of Catalan Biology Society, Barcelona (Spain), October 2016.
- 17th SENC National Meeting, Alicante (Spain), September 2017.

Publication ongoing in collaboration with Dr Eloi Gari and Dra Neus Pedraza from the Cell Cycle Group of IRB Lleida.

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