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TESIS DOCTORAL

Alteraciones celulares y funcionales derivadas de la manipulación del receptor cannabinoide CB_1 durante el desarrollo cerebral

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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ALTERACIONES CELULARES Y FUNCIONALES DERIVADAS DE LA MANIPULACIÓN DEL RECEPTOR CANNABINOIDE CB1 DURANTE EL DESARROLLO CEREBRAL

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Muy tarde por la noche el Mullah Nasrudin se encuentra dando vueltas alrededor de una farola, mirando hacia abajo. Pasa por allí un vecino.

—¿Qué estás haciendo Nasrudín, has perdido alguna cosa?
—Sí, estoy buscando mi llave.

El vecino se queda con él para ayudarle a buscar. Después de un rato, pasa una vecina.

—¿Qué estáis haciendo? —les pregunta.
—Estamos buscando la llave de Nasrudín.

Ella también quiere ayudarlos, así que se pone a buscar. Luego, otro vecino se une a ellos y juntos buscan, buscan y buscan. Habiendo buscado durante un largo rato acaban por cansarse y un vecino pregunta:

—Nasrudín, hemos buscado tu llave durante mucho tiempo, ¿estás seguro de haberla perdido en este lugar?
—No, dice Nasrudín
—¿dónde la perdiste, pues?
—Allí, en mi casa.
—Entonces, ¿¡por qué la estamos buscando aquí!?
—Pues porque aquí hay más luz, mi casa está muy oscura.

...

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ABBREVIATIONS

2-AG: 2-arachidonoylglicerol

5-HT: 5-hydroxytryptamine

AA: arachidonic acid

Akt: murine thimoma viral oncogene homolog

APN: associative projection neuron

BDNF: brain-derived neurotrophic factor

BrdU: bromo-deoxyuridine

CAM: cell adhesion molecule

CB1: cannabinoid receptor, type 1

CB2: cannabinoid receptor, type 2

CBD: cannabidiol

CBN: cannabinol

CBG: cannabigerol

CBR: cannabinoid receptors CB1 and CB2

CCK: cholecystokinin

CFuPN: corticofugal projection neuron

CGE: caudal ganglionic eminence

CNS: central nervous system

COX2: cyclooxygenase-2

CP: cortical plate

CPC: adherent cortical progenitor cell

CPN: commissural/callosal projection neuron

C-R: Cajal-Retzius cells

CREB: cAMP-response element binding

CRIP1A: cannabinoid receptor-interacting protein 1 a

CSMN: corticospinal motor neuron

CThPN: corticothalamic projection neuron

Ctip2: coup-TF interacting protein 2

DAGL: diacylglicerol lipase

DAPI: 4',6-diamidino-2-phenylindole

DIU: days *in utero*

DIV: days *in vitro*

DMSO: dimethyl sulfoxide

E: embryonic day

eCB: endocannabinoid

ECM: extracellular matrix

ECS: endocannabinoid system

EGF: epidermal growth factor

ERK: extracellular signal-regulated kinase

FAAH: fatty acid amide hydrolase

FCD: focal cortical dysplasia

FGF: fibroblast growth factor

GABA: gamma-aminobutyric acid

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

GPCR: G protein-coupled receptor

GTP: guanosine triphosphate

IKM: interkinetic nuclear migration

IP: intermediate progenitor

IP3: inositol trisphosphate

ISH: *in situ* hibridization

IUE: *in utero* electroporation

IZ: intermediate zone

LGE: lateral ganglionic eminence

LTD: synaptic long-term depression

MAGL: monoacylglicerol lipase

MAPK: mitogen-activated protein kinase

MCD: malformation of cortical development

MGE: medial ganglionic eminence

mTORC: mammalian target of rapamycin complex

MZ: marginal zone

NAPE-PLD: N-acylphosphatidylethanolamine-hydrolyzing phospholipase D

NEC: neuroepithelial cell

NSC: neural stem cell

NT: neurotrophin

P: postnatal day

Pax6: paired box protein 6

PCNA: proliferating cell nuclear antigen

PH: periventricular heterotopia

PI3K: phosphatidylinositol-3-kinase

PIP2: phosphatidylinositol bisphosphate

PKA: protein kinase A

PN: projection neuron

PP: preplate

PPAR: peroxisome proliferator-activated receptor

PTZ: pentylenetetrazole

qPCR: quantitative polymerase chain reaction

RGC: radial glia cell

RIPA: radioimmunoprecipitation assay

RMS: rostral migratory stream

SBH: subcortical band heterotopia

SCPN: subcerebral projection neuron

SEZ: subependymal zone

SGZ: subgranular zone

shRNA: short hairpin ribonucleic acid

siRNA: small interfering ribonucleic acid

SP: subplate

SPPN: subplate projection neuron

STD: synaptic short-term depression

SVZ: subventricular zone

TGF: transforming growth factor

THC: tetrahydrocannabinol

TRPV1: transient receptor potential cation channel subfamily V, type 1

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

VZ: ventricular zone

WM: white matter

RESUMEN

La neocorteza (comúnmente conocida como corteza cerebral) es la estructura más compleja y recientemente evolucionada del cerebro de mamíferos. Contiene cientos de tipos celulares ensamblados en sofisticados circuitos neurales que –integrando información del mundo interno y externo– posibilitan las extraordinarias capacidades cognitivas, sensoriales y motoras que nos hacen humanos, desde los precisos y delicados movimientos de un músico virtuoso al intrincado procesamiento verbal y emocional necesario para la poesía.

La organización básica de la corteza cerebral es producto de la formación de patrones durante el desarrollo embrionario. Dicha información está codificada en el genoma y es expresada mediante conservadas redes de regulación génica que dirigen la construcción de la estereotipada arquitectura cortical en el feto en desarrollo. Hoy en día, se cree que una gran proporción de trastornos neuropsiquiátricos –como las epilepsias refractarias, autismo o esquizofrenia– tienen su origen en el desarrollo cerebral embrionario. Por lo tanto, realizar una disección de los determinantes intrínsecos que subyacen al desarrollo cortical –en condiciones normales y en ciertas patologías– es una provechosa manera de comprender en profundidad la corteza cerebral *per se*, así como de ofrecer oportunidades terapéuticas a aquellos que sufren de patologías del neurodesarrollo.

La planta *Cannabis sativa* (conocida como marihuana) ha sido cultivada por el ser humano, con diferentes propósitos, desde el Neolítico. El compuesto psicoactivo más prominente del cannabis es el Δ^9-tetrahidrocannabinol (THC), cuyos efectos están mediados por su principal diana molecular, el receptor cannabinoide CB1 (CB1R). CB1R es muy abundante en el cerebro y muchos otros órganos, donde ejerce acciones pleiotrópicas en el control del metabolismo celular, fisiología y función. Este, junto a un segundo (CB2) receptor, sus ligandos endógenos así como las enzimas responsables de su síntesis y degradación conforman lo que se llama el Sistema Endocannabinoide (SEC).

El SEC, además de su conocido papel en la fisiología adulta de mamíferos, está presente y modula aspectos clave a lo largo de todo el desarrollo cerebral embrionario. Se ha visto que modula la motilidad del esperma y la maduración del oocito o el proceso de fertilización e implantación del óvulo fecundado. Más adelante, CB1R regula una plétora de eventos del desarrollo cortical, desde la proliferación y destino de progenitores neurales hasta la migración neuronal, especificación de motoneuronas corticoespinales (MNCS) y la morfogénesis final de neuronas de proyección.

El cannabis es, de lejos, la droga ilícita más ampliamente consumida en países occidentales, con especial prevalencia en mujeres gestantes. Hay profusa literatura científica sobre las consecuencias del consumo materno de cannabis en la descendencia, que incluyen déficits cognitivos o un aumento de la susceptibilidad a desarrollar trastornos neuropsiquiátricos como

la esquizofrenia, depresión o ansiedad. No obstante, en la mayoría de los casos, el sustrato celular y molecular que subyace a los defectos producidos por exposición prenatal a cannabinoides, así como las consecuencias funcionales de una disfunción embrionaria del SEC son ampliamente desconocidos.

Es por esto que una caracterización precisa de los aspectos del desarrollo fuertemente influenciados por CB1R y el SEC, además del impacto neurobiológico en la progenie de una disfunción embrionaria del SEC –causada por variaciones genéticas o por influencias ambientales como el consumo materno de marihuana– es una cuestión fundamental y sirve como marco conceptual para esta Tesis Doctoral.

En este contexto, hemos definido los siguientes objetivos para esta Tesis Doctoral:

Objetivo 1. Investigar el papel del SEC en el proceso de migración radial de neuronas piramidales durante el desarrollo cortical y evaluar las alteraciones celulares y funcionales a largo plazo derivadas de una disfunción de CB1R restringida al embrión.

Los resultados correspondientes han sido publicados en el siguiente artículo científico:

Díaz-Alonso J*, de Salas-Quiroga A*, Paraíso-Luna J, García-Rincón D, Garcez P, Parsons M, Andradas C, Sánchez C, Guillemot F, Guzmán M and Galve-Roperh I. (2016) 'Loss of Cannabinoid CB1 Receptors Induces Cortical Migration Malformations and Increases Seizure Susceptibility'. *Cereb. Cortex* 1-15.

Objetivo 2. Dilucidar el sustrato neurobiológico de la acción del Δ^9-THC durante el desarrollo embrionario cortical. En particular, explorar el impacto de la administración prenatal de este cannabinoide en la diferenciación neuronal, con especial atención a la especificación de MNCS.

Los resultados correspondientes han sido publicados en el siguiente artículo científico:

de Salas-Quiroga, A.*, Díaz-Alonso, J.*, García-Rincón, D., Remmers, F., Vega, D., Gómez-Cañas, M., Lutz, B., Guzmán, M., and Galve-Roperh, I. (2015) 'Prenatal exposure to cannabinoids evokes long-lasting functional alterations by targeting CB1 receptors on developing cortical neurons'. *Proc. Natl. Acad. Sci. USA*, 112(44), 13693–13698.

Para abordar el **Objetivo 1** de esta Tesis, trabajando con ratones, llevamos a cabo el *knockdown* transitorio de *CB1* con siRNA en neuronas piramidales recién generadas para revelar el impacto de la pérdida de función de *CB1* restringida y a corto plazo en la migración radial de neuronas de proyección y los déficits funcionales potenciales en la edad adulta. Observamos un bloqueo de la migración que conllevó fuertes alteraciones a largo plazo en el posicionamiento de las neuronas corticales, la formación de heterotopías neuronales y un incremento de la

susceptibilidad a desarrollar crisis epilépticas en ratones adultos. Análisis celulares y bioquímicos mostraron que la pérdida de función de CB1R llevaba a una acumulación aberrante de la GTPasa RhoA –crítica en el control del citoesqueleto de actina– en neuronas piramidales, que por tanto perjudicó la morfología de las neuronas migrantes. Notablemente, los déficits desencadenados por la disfunción de CB1R fueron completamente rescatados por el *knockdown* concomitante de RhoA.

En suma, nuestros resultados allanan el camino hacia una mejor comprensión de rol fisiológico del SEC en el cerebro en desarrollo y proveen una perspectiva mecanística sobre las Malformaciones del Desarrollo Cortical (MDC) causadas por alteraciones de la migración neuronal.

Alternativamente, al respecto del **Objetivo 2** de esta Tesis, modelizamos un consumo prenatal de cannabis en ratón para identificar el preciso sustrato del neurodesarrollo responsable de las alteraciones funcionales producidas por exposición a THC evidentes en el adulto. La administración de THC se llevó a cabo durante una ventana temporal restringida del desarrollo embrionario, coincidente con el periodo más activo de generación de neuronas de la corteza cerebral que condujo a una regulación a la baja del receptor. Significativamente, observamos un fallo en la generación de MNCS que se correlacionaba con alteraciones a largo plazo de la función motora fina y un incremento de la susceptibilidad a epilepsia. Para valorar la implicación de CB1R en dicho fenotipo, empleamos ratones deficientes en *CB1* de manera constitutiva, que resultaron resistentes a las alteraciones observadas dependientes de THC. A continuación, mediante el uso de una estrategia de rescate de la expresión de *CB1*, mediada por la recombinasa Cre de manera selectiva de linaje celular, en ratones desprovistos de *CB1*, fuimos capaces de rescatar selectivamente los déficits en el desarrollo de MNCS característicos de ratones carentes de *CB1* y, a su vez, restablecimos por completo la susceptibilidad a las alteraciones celulares y funcionales dependientes de THC en la descendencia adulta.

Por otro lado, observamos que la exposición prenatal a THC indujo un incremento en la susceptibilidad a convulsiones mediado por CB1Rs presentes tanto en neuronas piramidales del telencéfalo dorsal como en neuronas GABAérgicas prosencefálicas.

Por tanto, podemos concluir que afectar a CB1Rs presentes en una población neuronal determinada y una particular ventana temporal del desarrollo embrionario con el más abundante y prominente compuesto psicoactivo del cannabis puede desencadenar importantes alteraciones neurológicas en la descendencia.

ABSTRACT

The neocortex (commonly referred to as cerebral cortex) is the most complex and recently evolved structure in the mammalian brain. It contains hundreds of cell-types assembled into sophisticated neural circuits that –by integrating information from the external and internal world– enable the extraordinary cognitive and sensorimotor capacities that make us human, from the delicate and precise movements of a music virtuoso to the intricate verbal and emotional processing required for poetry.

The basic organization of the cerebral cortex is a product of developmental pattern formation, whose information is encoded in the genome and expressed by conserved genetic regulatory networks, which direct the construction of the stereotyped cortical architecture in the developing fetus. Nowadays, it is believed that a large proportion of neuropsychiatric disorders –as refractory epilepsies, autism or schizophrenia– have their origin in embryonic brain development. Therefore, dissecting the intrinsic determinants underlying –normal and abnormal– cortical development is a helpful path to fully comprehend the cerebral cortex itself and to offer therapeutical possibilities to those who suffer from neurodevelopmental pathologies.

The plant *Cannabis sativa* (commonly known as marijuana) has been cultivated, with several purposes, by humans since Neolithic times. The most prominent psychoactive compound of cannabis is the Δ^9-tetrahydrocannabinol (THC), whose effects are mediated by its main molecular target, the CB1 cannabinoid receptor (CB1R). CB1R is strikingly abundant in the brain and many other regions of the body, where it exerts pleiotropic actions in the control of cell metabolism, physiology and function. This, along with a second (CB2) receptor, their endogenous ligands and the enzymes responsible of their synthesis and degradation conform the so-called Endocannabinoid System (ECS).

The ECS, in addition to its well-known roles in adult mammalian physiology, is present and modulate key events throughout embryonic brain development. It has been shown to regulate sperm cell motility, oocyte maturation or the process of fertilization and implantation. Later, CB1R regulates a plethora of cortical developmental steps, ranging from neural progenitor cell proliferation and fate acquisition to neuronal migration, corticospinal motorneuron (CSMN) specification and eventual morphogenesis of projection neurons.

Cannabis is, by far, the most widely used illicit drug in Western countries, with a remarkable prevalence by pregnant women. There is profuse literature about the functional consequences of maternal cannabis use on the offspring, which include cognitive impairments and increased susceptibility to develop neuropsychiatric disorders such as schizophrenia, depression or anxiety. Nevertheless, in most cases, the particular molecular and cellular substrate underlying cannabinoid-dependent alterations, as well as the functional outcome of embryonic ECS dysfunction

are mostly unknown. Hence, a precise characterization of the developmental events deeply influenced by CB1R and the ECS, and the long-lasting neurobiological impact of developmental ECS malfunction in the adult progeny –caused by genetic variations or environmental insults such as prenatal marijuana exposure– is a major concern as serves as the conceptual framework for this Doctoral Thesis.

In this context, we defined the following Aims for this Doctoral Thesis:

Aim 1. To investigate the role of the ECS in the process of radial migration of newborn pyramidal neurons during cortical development and to evaluate long-lasting functional alterations derived of developmentally-restricted CB1R dysfunction.

The corresponding results have been published in the following paper:

Díaz-Alonso, J.*, de Salas-Quiroga, A.*, Paraíso-Luna, J., García-Rincón, D., Garcez, P., Parsons, M., Andradas, C., Sánchez, C., Guillemot, F., Guzmán, M. and Galve-Roperh, I. (2016) 'Loss of Cannabinoid CB1 Receptors Induces Cortical Migration Malformations and Increases Seizure Susceptibility'. *Cereb. Cortex* [Epub ahead of print].

Aim 2. To elucidate the neurobiological substrate of Δ^9-THC actions during embryonic cortical development. Particularly, to explore the impact of prenatal administration of this phytocannabinoid on neuronal differentiation, with special attention to CSMN specification.

The corresponding results have been published in the following paper:

de Salas-Quiroga A*, Díaz-Alonso J*, García-Rincón D, Remmers F, Vega D, Gómez-Cañas M, Lutz B, Guzmán M, and Galve-Roperh I. (2015) 'Prenatal exposure to cannabinoids evokes long-lasting functional alterations by targeting CB1 receptors on developing cortical neurons'. *Proc. Natl. Acad. Sci. USA*, 112(44), 13693–13698.

To address the **Aim 1** of this Thesis, working with mice, we performed transient siRNA-mediated *CB1* knockdown in newborn pyramidal neurons to reveal the impact of developmentally restricted, short-term *CB1* loss of function on radial migration of projection neurons and its potential functional deficits in adulthood. We observed a migration arrest that led to profound and long-lasting alterations in cortical neuron positioning, the formation of neuronal heterotopias and increased seizure susceptibility in adult mice. Cellular and biochemical analyses showed that loss of CB1R function led to abnormal accumulation of the GTPase RhoA –critical for the control of actin cytoskeleton– in newborn pyramidal neurons thereby disrupting the morphology of migrating cells. Remarkably, migration deficits elicited by CB1R dysfunction were fully rescued by concomitant RhoA knockdown.

Collectively, our findings pave the way toward a better understanding of the physiological role of the ECS in brain development and provide relevant molecular mechanistic insights into Malformations of Cortical Development (MCD) caused by altered neuronal migration.

Alternatively, regarding the **Aim 2** of this Thesis, we modeled prenatal cannabis consumption in mice to identify the particular neurodevelopmental substrate responsible for cannabinoid-induced functional alterations that remain overt in adulthood. Administration of THC was conducted during a restricted embryonic time window, coinciding with the active period of neuron generation in the cerebral cortex that induced a downregulation of the receptor. We found an impairment in CSMN generation that correlates with long-lasting skilled motor functional alterations and susceptibility to epilepsy. To unequivocally assess the role of CB1R signaling in THC-induced alterations, we employed constitutive *CB1*-deficient mice, which were resistant to THC-induced developmental alterations. Next, by using a Cre recombinase-mediated, lineage-specific, *CB1* expression-rescue strategy in a *CB1*-null background, we were able to selectively rescue the deficits in CSMN development characteristic of *CB1*-deficient mice and, in turn, fully restore the susceptibility to embryonic THC-induced cellular and functional deficits in adulthood.

Furthermore, we also found that prenatal THC exposure induced an increase in seizure susceptibility that was mediated by CB1Rs present in both developing dorsal telencephalic pyramidal neurons and forebrain GABAergic neurons.

Hence, targeting CB1Rs with the most prominent marijuana-derived psychoactive compound in a particular neuronal population and time frame during embryonic development can evoke remarkable long-lasting neurological alterations in the offspring.

INTRODUCTION

More than a century ago, the seminal work accomplished by Santiago Ramón y Cajal contributed to the recognition that the neuron was the fundamental brick of the brain. His research set the basis to understand, at the cellular level, how brain's structure is related to its complex function, giving rise to the field of neuroscience (Ramón y Cajal 1899; Ramón y Cajal 1909). Since then, astonishing advances have been made to unveil the genetic, molecular, cellular, anatomical and functional logic that underlies our most intriguing organ, although it is still far from understood.

The neocortex (commonly referred to as cerebral cortex) is the most complex and recently evolved structure in the mammalian brain. It contains hundreds of cell-types assembled into sophisticated neural circuits that, by integrating information from the external and internal world, enable the extraordinary cognitive and sensorimotor capacities that make us human. The cerebral cortex is essential for the consciousness and empowers sublime behaviors such as the delicate and precise movements of a music virtuoso or the intricate verbal and emotional processing required for poetry.

The human neocortex is a 3-4 mm thick, highly convoluted sheet of tissue with an approximate surface of 26 m^2. It contains ~16 x 10^9 neurons –and more than three times of glial cells– (Herculano-Houzel 2009; Azevedo et al. 2009) connected to each other by an estimated number of synapses of ~164 x 10^{12} within the cerebral cortex (Tang et al. 2001), what allows a virtually infinite computing potential (Grillner 2006; Bartol et al. 2015).

Neuronal number and diversity is at the core of brain function, where complex circuitries may be largely simplified as hierarchical networks of excitatory and inhibitory neurons. The cerebral cortex is mainly comprised of excitatory glutamatergic pyramidal neurons and inhibitory gamma-aminobutyric acidergic (GABAergic) interneurons highly organized into layers (I-VI) and columns. Roughly, most neurons present in one layer share overall connectivity patterns whereas those localized within the same column are typically interconnected across layers, what represents the functional unit of the cerebral cortex (Mountcastle 1957; Rakic 1988; Mountcastle 1997; Markram et al. 2015). These cortical columns are organized into functional modules specialized to operate certain tasks. It is conceivable that vertebrate's evolution has developed an extraordinary mechanism, a scalable functional architecture which –by amplifying and combining these functional templates– allows to adapt to such a variety of lifestyles and their corresponding challenges (Huang 2014; Hofman 2014). Pyramidal cells, which account for ~80% of the neuronal population in the cerebral cortex, usually extend long axons and are specialized in dispatching information between different cortical areas as well as to distant regions of the brain, hence they are also known as projection neurons (PNs). Cortical interneurons, in turn, establish local neuronal assemblies where, by coordinated inhibitory inputs, synchronize the firing of pyramidal cells and orchestrate electric functional oscillations (Ascoli et al. 2008; Buzsáki et al. 2012; Defelipe et al. 2013) (Figure 1). An appropriate balance between excitation and inhibition is essential for maintaining normal brain function and, consequently, crucial developmental and

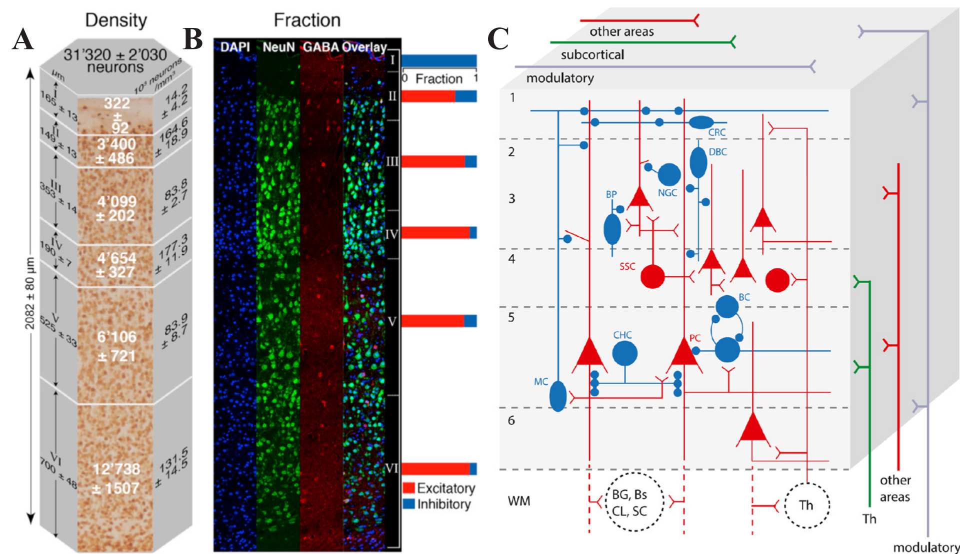

Figure 1. Graphic representation of the cortical column functional unit. (**A**) Neuron densities and numbers. (**B**) Neuronal fractions in a cortical column. DAPI labels all cells (blue). NeuN labels all neurons (green), GABA labels all GABAergic cells including glia (red), dual GABA⁺/NeuN⁺ cells depict interneurons. Bars to the right show fractions of excitatory (red) and inhibitory (blue) neurons in each layer. (**C**) Simplified schematic representation of the neocortical microcircuitry, including major cell types and their afferencies and efferencies. Excitatory (red) and inhibitory (blue) cells and synaptic connections are shown. ChC, Chandelier cell; BCs, basket cells; DBCs, double-bouquet cells; BPs, bipolar cells; NGCs, neurogliaform cells; MNCs, Martinotti cells;CRCs, Cajal-Retzius cells; Bs, brainstem; SC, spinal cord; BG, basal ganglia (BG); TH, thalamus; CL, claustrum; SSC, spiny stellate cell; WM, white matter. Adapted from Markram *et al.*, 2015 and Huang, 2014.

physiological mechanisms have evolved to safeguard this dynamic equilibrium.

It is convenient to note that, given the obvious ethical limitations to study the human central nervous system (CNS) at a profound level, what inevitably implies its manipulation, the great majority of our knowledge about brain's structure and physiology is coming from animal models. There is a wide spectrum of models used for research purposes which mainly depend on the level of complexity or the aim of the project, thus the fruit fly *Drosophila melanogaster* or the nematode *Caenorhabditis elegans* may be well-suited models for investigating highly conserved molecular mechanisms whereas for studying cognitive or social behaviors is frequent the use of rats or even monkeys. For the purposes of this Thesis, the mouse (*Mus musculus*) was used as animal model for almost the totality of the experiments performed. Despite the limitations from a translational perspective, this model provides very useful genetic tools to study mammalian brain development as well as a nourished conceptual frame given the deep knowledge that currently exists about its biology and its extended use in worldwide labs.

1. CEREBRAL CORTEX DEVELOPMENT

The basic organization of the cerebral cortex is a product of developmental pattern formation, whose information is encoded in the genome and expressed by conserved genetic regulatory networks, which direct the construction of the stereotyped cortical architecture in the developing fetus. Therefore, dissecting the intrinsic determinants underlying –normal and abnormal– cortical development is a helpful path to fully comprehend the cerebral cortex itself.

Despite the vast complexity of the adult CNS, it all begins as a simple neuroepithelium from a specialized region of the dorsal ectoderm, the neural plate. Early in development –at embryonic day 8.5 (E8.5) in the mouse– the neural plate undergoes a process termed neurulation, which consists in the shaping, folding and midline fusion of the neural plate, resulting in an outside-in hollow tube that extends along the longitudinal axis of the embryo and will generate the brain and spinal cord (Copp et al. 2003). Hereafter commences a topographic compartmentalization of the neural tube, where the rostrocaudal and dorsoventral axis are determined by precisely controlled gradients of morphogens that induce specific arrays of transcription factors (Rubenstein et al. 1998; Puelles & Rubenstein 2003; Hébert & Fishell 2008). This process results in the formation of the primary vesicles: prosencephalon, mesencephalon and rhombencephalon, the embryonic primordia of the forebrain, midbrain and hindbrain, respectively. The cerebral cortex specializes from the dorsal part of the telencephalon, the rostral-most end of the neural tube, that along with the diencephalon –which gives rise to thalamic and hypothalamic nuclei– outline the prosencephalon (Rallu et al. 2002; O'Leary et al. 2007) (Figure 2A). Following these early patterning events, by E10.5, the embryonic telencephalon is grossly divided into a dorsal ventricular zone or pallium and a subpallium or a series of ventral ganglionic eminences –lateral (LGE), medial (MGE) and caudal (CGE)– positioned along the rostrocaudal axis (Figure 2B). Each of these domains contains a proliferative compartment directly apposed to the cerebral ventricle and produces the various types of cells that eventually result in the mature telencephalon. The pallium generates three distinct structures in the adult brain: the neocortex, the archicortex (entorhinal and retrosplenial cortices, subiculum and hippocampus) and the paleocortex (olfactory piriform cortex). The subpallium, in turn, is responsible for the development of the basal ganglia and almost the totality of the forebrain GABAergic interneurons (Hansen et al. 2013; Huang 2014; Marín & Müller 2014). Noteworthy, this scenario might differ among species, since it has been proposed a pallial proliferative niche contributing to some subclasses of cortical interneurons in humans and other primates (Radonjić et al. 2014).

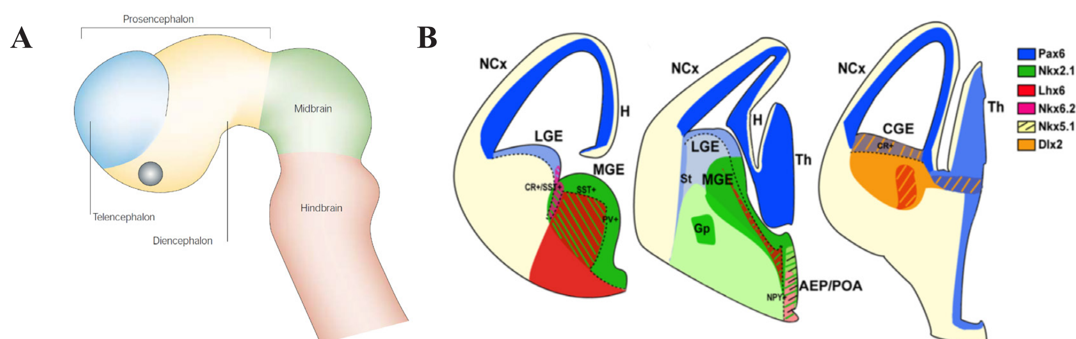

Figure 2. Main subdivisions of the embryonic mouse brain and gene expression in the telencephalon. (**A**) Side view of a mouse brain at around E10, showing the main compartmets. (**B**) Gene expression patterns in the telencephalon. Drawings of medial, intermediate and caudal coronal sections of E13.5 mouse brain showing the expression patterns of main transcription factors. Pax6 is predominant in the pallium, whereas Dlx2 or Nxk2.1 are characteristic of subpallial domains. Adapted from Rallu *et al.*, 2002 and Hernández-Miranda *et al.* 2010.

In the mouse, the entire embryonic development takes around twenty days, but the majority of neurons are born within the last week of gestation. During these days are settled the basis of the radial and tangential organization of the neocortex, following the theoretical framework established by the 'proto-map and radial unit hypothesis' that, decades ago, made an effort to reconcile available data on these two organizational axes of the cortex (Rakic 1988). According to this hypothesis; "*the ependymal layer of the embryonic cerebral ventricle consists of proliferative units that provide a proto-map of prospective cytoarchitectonic areas. The output of the proliferative units is translated via glial guides to the expanding cortex in the form of ontogenetic columns, whose final number for each area can be modified through interaction with afferent input*" (Figure 3).

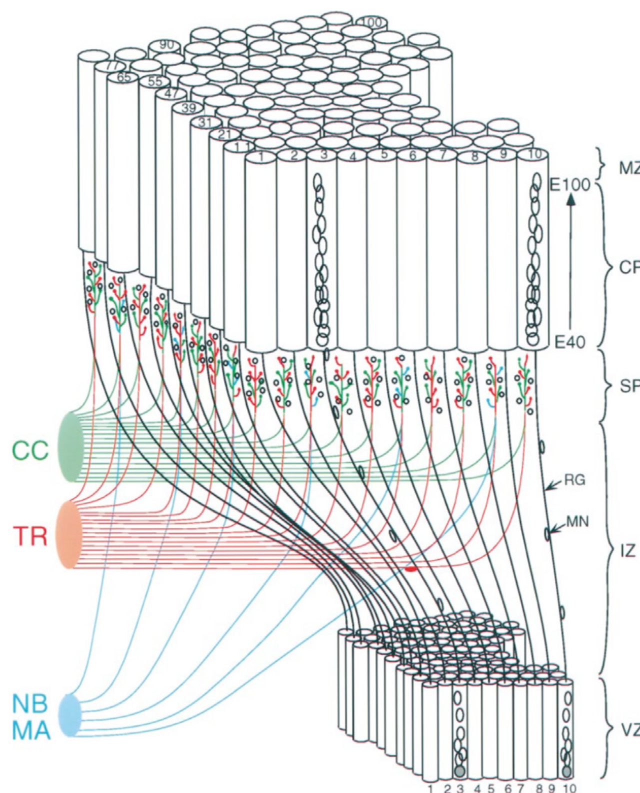

Figure 3. Representation of the 'proto-map and radial unit hypothesis'. A three-dimensional illustration of the developmental events occurring during early stages of corticognesis in the monkey. The drawing illustrates radial migration which underlies its columnar organization. After their last division, cohorts of migrating neurons (MN) traverse the intermediate zone (IZ) and the subplate (SP) where they may interact with afferents arriving sequentially from the nucleus basalis (NB), the monamine nuclei of the brainstem (MA), from the thalamic radiation (TR), and from several ipsilateral and contralateral corticocortical bundles (CC). Newly generated neurons bypass those generated earlier, and settle at the interface between the developing cortical plate (CP) and the marginal zone (MZ) in an inside-out fashion. Although some cells may detach from the cohort and move laterally, guided by an axonal bundle, most are gliophilic, have affinity for the glial surface, and obey the constraints imposed by transient radial glial (RG) cell scaffolding. This cellular arrangement preserves the relationship between the proliferative mosaic of the ventricular zone (VZ) and the corresponding map within the SP and CP, even though the cortical surface in primates shifts considerably during the massive cerebral growth in the mid-gestational period. The numerals refer to corresponding units in the VZ and CP. From Mountcastle, 1997.

1.1. Cortical progenitor diversity

The idea that the organization of the mature cortex depends on the proliferation of germinal cells lining the embryonic ventricles was cast by Wilhelm His as early as in the late 19th century (His 1874; Allen 1912). Nevertheless, it was not until the development of labeled analogs of thymidine (first H^3-thymidine and later halogen-labeled deoxyuridines as BrdU) that this theory was confirmed and led, one century later, to set a uniform nomenclature for neurogenic compartments by the Boulder Committee (The Boulder Committee 1970). In that revision, the committee proposed the following terms for the four fundamental cortical embryonic zones according to the form, behavior, and fate of its constituent cells: ventricular (VZ), subventricular (SVZ), intermediate (IZ) and

marginal (MZ) zones. Moreover, the VZ and SVZ are the mitotically active compartments where are located the embryonic cortical progenitors that give rise to most neurons and glia present in the mature cortex. There are several classes of cortical progenitors, regarding their morphological and spatiotemporal characteristics, their molecular profile or neurogenic fate (Kriegstein & Alvarez-Buylla 2009; Taverna et al. 2014; De Juan Romero & Borrell 2015). However, this is a changing topic subjected to intense study and debate, since progenitor populations vary among species and there are different hypothesis regarding their identity, location, dynamics and cell-fate pluripotency (Lui et al. 2011; Franco et al. 2012; Eckler et al. 2015; De Juan Romero & Borrell 2015). Nonetheless, we may roughly determine three –sequentially related– main classes of cortical progenitors during mouse development: neuroepithelial cells (NECs), apical progenitors or radial glia cells (RGCs) and basal or intermediate progenitors (IPs) (Figure 4).

1.1.1. Neuroepithelial cells

At early stages of cortical development, the telencephalic vesicle is uniformly composed of a simple neuroepithelium formed by elongated, bipolar cells spanning the entire thickness of the telencephalic wall (Sidman & Rakic 1973). NECs undergo a process called interkinetic nuclear migration (IKM), namely cell divisions take place in the apical –in contact to the ventricle– side of the telencephalic wall whereas during G1 they translocate their soma to the basal side to undergo

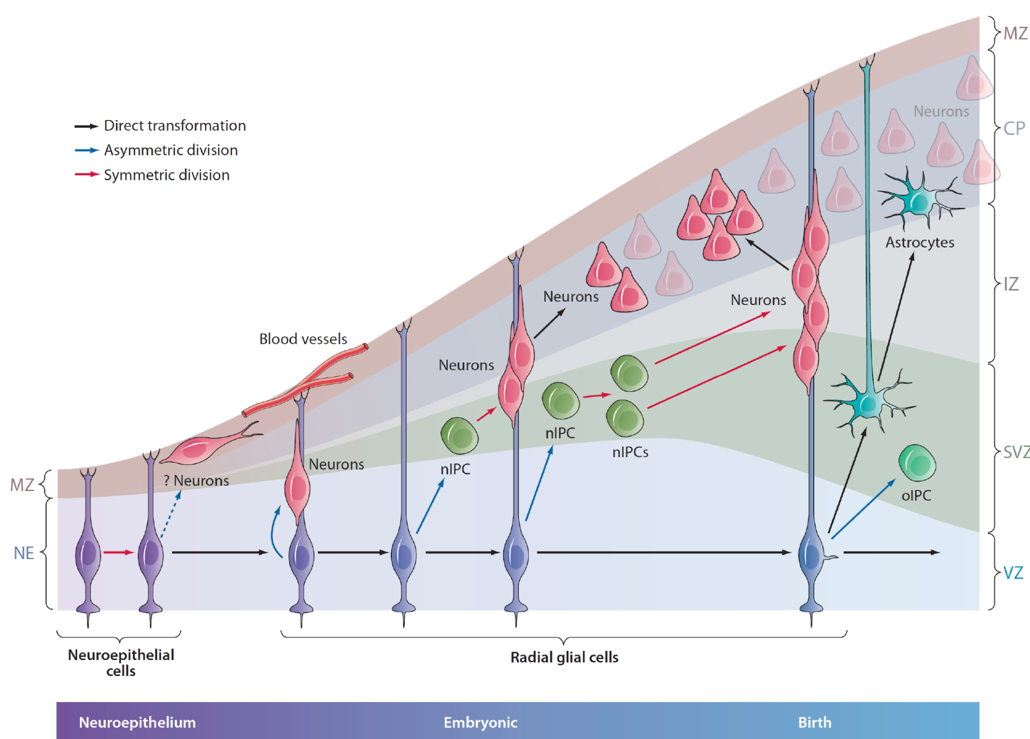

Figure 4. Major classes of cortical progenitors in mouse cortical development. The image schematizes the overall corticogenesis process and represents the transition between the main types of cortical progenitors (NECs, RGCs and IPs) and their respective developmental cortical compartments. IPs are segregated into neuronal IP cells (nIPCs) and oligodendroglial IP cells (oIPCs), which give rise to neurons and oligodendrocytes, respectively. CP cortical plate; IZ intermediate zone; MZ, marginal zone; SVZ, subventricular zone; VZ, ventricular zone. From Kriegstein & Álvarez-Buylla, 2009.

S-phase. G2 comes about while the soma is moved back to the apical wall where the cell divides again (Sauer 1935; Sauer & Walker 1959; Takahashi et al. 1993). NECs mainly divide symmetrically to amplify the neural stem cell (NSC) pool. Despite NECs are committed to neural lineage, they retain some features of epithelial cells, in particular their high apical-basal polarization. NECs are attached to the apical surface, where they form tight junctions to each other, and to the pial surface by integrins (Aaku-Saraste et al. 1996; Graus-Porta et al. 2001). Later in development NECs start to downregulate some epithelial traits and to exhibit astrocyte-typical markers in their transition to RGCs, coincident with the onset of neurogenesis (Kriegstein & Alvarez-Buylla 2009).

1.1.2. Radial glia cells

At approximately E10.5 of mouse development, with the appearance of RGCs, neurons begin to emerge within the cortical wall, which from a single, pseudostratified epithelium progressively becomes a multilayered structure. Like NECs, RGCs extend radial processes to the apical and basal edges –what granted their name– and undergo IKM, although tight junctions are replaced by adherens junctions (Martynoga et al. 2012). In addition, they share with NECs the expression of molecular markers as the transcription factor paired box protein 6 (*Pax6*), or cytoskeletal elements as nestin (*Nes*), radial cell 2 (*Rc2*) or vimentin (*Vim*), but unlike NECs, RGCs express some markers typical of astrocytes, such as glial fibrillary acidic protein (*Gfap*) or the astrocyte-specific glutamate/aspartate transporter (*Glast*) (Götz et al. 2002; Osumi et al. 2008). However, the major difference between both cell types resides in the ability of RGCs to undergo neurogenic divisions in addition to their well-established role as radial scaffold (Malatesta et al. 2000; Noctor et al. 2001; Miyata et al. 2001). Another, perhaps most remarkable, feature of RGCs is their capacity to bear, though not only, three main types of cell division: symmetric non-neurogenic division –yielding two identical RGCs–; asymmetric non-neurogenic division –that results in a RGC and a mitotically active IP–; and asymmetric neurogenic division –RGC self-renews and arise a postmitotic newborn neuron– (Noctor et al. 2004).

1.1.3. Intermediate progenitors

More than a century ago it was already described a second germinal layer present in the SVZ, adjacent to the marginal edge of the VZ, which was eventually named basal or intermediate progenitor pool (Allen 1912; The Boulder Committee 1970). IPs are non-epithelial progenitors arisen from an asymmetrical division of a RGC in the VZ which, remarkably, do not undergo IKM. The newly generated cell retracts its inherited apical process, translocates its soma to the SVZ and exhibits a multipolar morphology in their transition to IP (Noctor et al. 2008; Borrell et al. 2012; Wilsch-Brauninger et al. 2012). Concomitantly, IPs switch off the expression of epithelial progenitor-distinctive markers such as *Pax6* or nestin and trigger the expression of transcription factors as subventricular-expressed transcript 1 (*Svet1*) or, more importantly, T-box brain protein

2 (*Tbr2*)/*Eomes* (Tarabykin et al. 2001; Englund et al. 2005). In most cases IPs bear symmetrical divisions; non-neurogenic –at early stages of development– to further amplify the IP pool and self-consuming neurogenic divisions later in development (Takahashi et al. 1995; Noctor et al. 2004). Nowadays it is generally accepted that IPs are responsible for the genesis of most cortical pyramidal cells, disregarding their neuronal lineage (Kowalczyk et al. 2009; Mihalas et al. 2016).

1.2. Spatiotemporal dynamics of neocortex formation

The tightly regulated chain of spatiotemporal events that occurs during brain development is one of the most complex and fascinating biological processes ever faced, and is superbly rooted in the genome.

Pallial progenitors produce specific subsets –although overlapping– of pyramidal neurons in an inside-out fashion, *id est*, newly-generated neurons migrate along previously-born ones to settle in the outer-more, basal zone of the prospective cortex (Figure 5). Briefly, from E10 to E12, cortical progenitors divide asymmetrically to self-renew and generate few neurons that undergo RGC-aided radial migration and form the preplate (PP). Coincidently, the Cajal-Retzius (C-R) cells –crucial players for cortical development and laminarization– emerge from three different focal sources at the borders of the developing pallium and very quickly colonize the pallial surface by tangentially migrating through the MZ (Marín-Padilla 1998; Soriano & Del Río 2005; Borrell & Marín 2006). Around E12.5, a second neurogenic wave migrates radially sitting into the PP –splitting it in the superficial MZ and a deep subplate (SP)– and forms the cortical plate (CP), which will give rise the mature, six-layered neocortex. Concomitantly, superficial to the primary VZ develops the SVZ, providing a secondary germinal zone that will rapidly increase the number

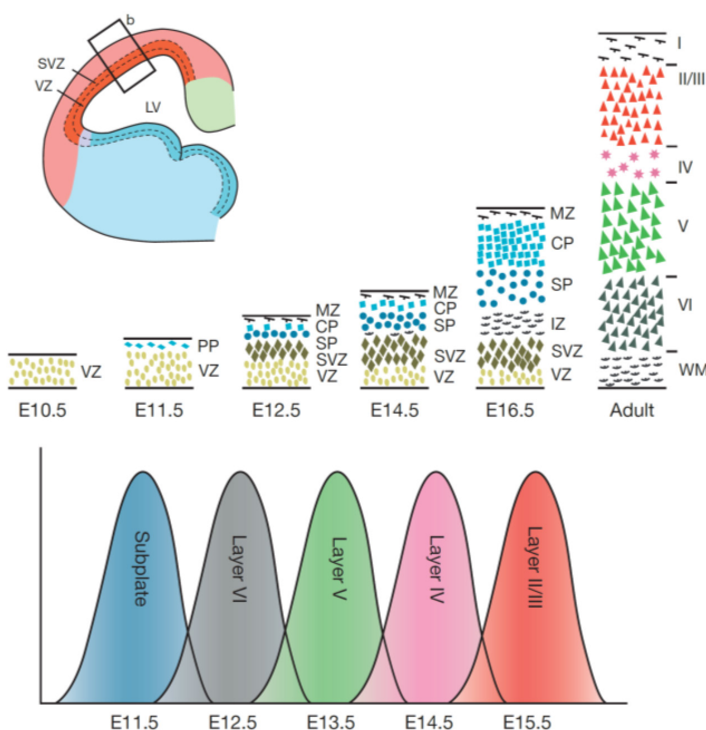

Figure 5. Spatiotemporal dynamics of neocortex formation. Progenitors residing in the VZ and SVZ produce projection neurons in an inside-out fashion. From approximately E10 to E12, pallial ventricular zone (VZ) progenitors divide asymmetrically and generate neurons that migrate radially and form the preplate (PP). At approximately E12.5, a second wave of postmitotic neurons migrates radially and intercalates into the preplate, splitting it into the superficial marginal zone (MZ) and a deep subplate (SP), forming the cortical plate (CP), which will expand over the next several days and the first postnatal week into the mature, six-layered neocortex. The subventricular zone (SVZ) develops superficial to the primary VZ, providing a secondary germinal zone composed of intermediate progenitors (IPs). Over the next approximately 5 days, diverse cortical projection neuron subtypes are born in sequential and overlapping waves. From McDonald *et al.*, 2013

of neurons, thus boosting the growth of the CP. Next, for the subsequent five days, diverse cortical PN subtypes are born in sequential and overlapping neurogenic waves (Figure 5). With a peak at E12.5, cortical PNs destined for layer VI are born, including corticothalamic PNs (CthPNs) and a subset of commissural/callosal PNs (CPNs). Next, peaking around E13.5, emerge an additional subpopulation of CPNs and subcerebral PNs (SCPNs) destined for layer V, including corticospinal motor neurons (CSMNs). One day later, at approximately E14.5, layer IV pyramidal neurons arise, including the genuine, locally projecting stellate cells. Finally, between E15.5 and E17.5, are generated heterogeneous subpopulations of CPN and other intracortical PNs destined for layers II/III (Martynoga et al. 2012; Greig et al. 2013; Huang 2014). The soon-to-be layer I (MZ) is occupied by C-R cells and migrating GABAergic interneurons during cortical development. In the mature cortex, the layer I lacks pyramidal cell somata, in contrast, it is characterized by abundant horizontal neurites and sparse interneurons.

Remarkably, the cell fate advent of the cortical progenitor-derived cells during corticogenesis seems to follow an internal clock. Hence, NSCs first generate neurons, followed by astrocytes and oligodendrocytes in a sequential manner (Abney et al. 1981; Qian et al. 2000). In the mouse, cortical astrocytes are first detected around E16 –albeit at low numbers– and oligodendrocytes around birth, but the vast majority of both cell types are produced during the first postnatal month (Misson et al. 1991; Cameron & Rakic 1991; Rowitch & Kriegstein 2010).

Importantly, based on previous observations from other groups (Parnavelas et al. 1991), the discovery by the group of John L.R. Rubenstein, twenty years ago, that cortical GABAergic interneurons originate outside the pallium –in the subpallial proliferative niches of the MGE and CGE– has revolutionized our understanding of the development of the cerebral cortex (Anderson et al. 1997; Marín & Rubenstein 2001). The notion that cortical pyramidal neurons and interneurons follow distinct developmental programs has challenged the rationale for how neurons assemble to constitute intricate neural networks, and still there is much to disclose (Marín & Müller 2014). Broadly, different subpopulations of GABAergic interneurons arise in discrete domains of the ventral telencephalon, in a spatiotemporally-regulated manner, from where they must tangentially migrate toward the developing cortex by an unprecedented long-distance migration (Anderson et al. 2001; Marín & Rubenstein 2001). There are three main migratory corridors followed by interneurons –with distinct preference by each subpopulation– to colonize the embryonic cortex: through the SVZ, the MZ and a slight stream through the SP (Antypa et al. 2011; Marín 2013; Bartolini et al. 2013). Once interneurons arrived to their corresponding cortical area, they must switch its tangential migratory behavior for a radial migration to invade the CP and eventually refine their final laminar allocation (Lopez-Bendito et al. 2008; Miyoshi & Fishell 2011; Bartolini et al. 2017).

1.3. Adult neurogenic niches

Even though the great majority of cells within the nervous system are born during embryonic and early postnatal periods, new glia and neurons are continuously added in certain regions of the adult mammalian brain throughout life (Altman & Das 1965; Altman & Das 1967; Eriksson et al. 1998; Frisén 2016). These newborn cells are derived from two locally-restricted niches containing relatively quiescent astrocyte-like NSCs: the postnatal subventricular/subependymal zone (SVZ/SEZ) and the dentate gyrus subgranular zone (SGZ) of the hippocampus (Kaplan & Hinds 1977; Johansson et al. 1999; Kriegstein & Alvarez-Buylla 2009). In spite some fundamental differences between both neurogenic niches can be recognized, they share a broad structure, features and functioning (Alvarez-Buylla & Lim 2004). Thus, in both niches astrocytes serve as NSC pool (type B cell and radial astrocyte/type I progenitor in the SVZ/SEZ and SGZ, respectively) which, under certain signals, divide into intermediate progenitors (type C cell and type IIa/D1 cell in SVZ/SEZ and SGZ, respectively) which, in turn, generate newborn oligodendrocytes or neurons (Alvarez-Buylla et al. 2002; Vadodaria & Gage 2014; Tong & Alvarez-Buylla 2014) (Figure 6). In addition, a common aspect between embryonic and adult NSCs is their molecular regulatory network, although some key regulators exert different roles in developmental or postnatal neurogenesis (Urbán & Guillemot 2014). Another fundamental feature is the presence of a basal lamina and concomitant specialized vasculature, essential components of both niches (Palmer et al. 2000; Shen et al. 2008; Tavazoie et al. 2008). Furthermore, adult oligodendrogenesis has been extensively described, in basal conditions as well as after brain damage, with important implications in brain function and repair (Frisén 2016).

Integrating intrinsic and niche-derived signals is crucial for the physiology of adult NSCs and there is extensive literature analyzing signals governing major processes as NSC expansion, quiescence maintenance or cell-fate decision (Song et al. 2012; Marqués-Torrejón et al. 2013;

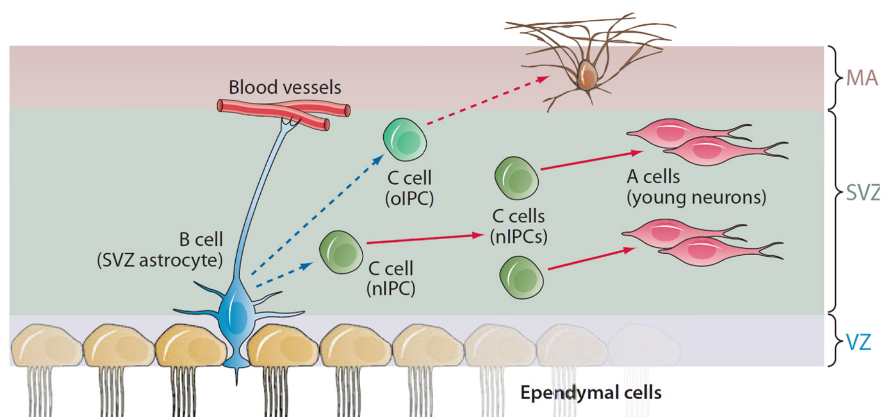

Figure 6. Scheme of progenitor types and lineages in the adult brain SVZ/SEZ. Adult neural NSC in the wall of the lateral ventricles of adult rodents correspond to type B cells (SVZ astrocytes). These cells retain epithelial properties, including the extension of a thin apical process that ends on the ventricle and a basal process ending on blood vessels. B cells give rise to C cells, which correspond to neuronal IP cells (nIPCs). B cells also generate oligodendrocytes through oligodendroglial IP cells (oIPCs). From Kriegstein & Álvarez-Buylla, 2009.

Porlan et al. 2014; Delgado et al. 2014; Urbán et al. 2016).

In the adult mouse brain, type A cells or neuroblasts born in the SVZ/SEZ migrate extensively through the rostral migratory stream (RMS) to reach the olfactory bulb where they integrate into the network, replacing at least six different interneuron subtypes (Lois & Alvarez-Buylla 1994; Tong & Alvarez-Buylla 2014). As regards to the type IIb/D2-4 cells or neuroblasts of the SGZ of the hippocampus, although most die before integrating in the circuits, they substantially contribute to the granule neuron pool of the dentate gyrus (Imayoshi et al. 2008; Vadodaria & Gage 2014).

Likewise, there are some differences in the ontogeny of these populations, while it is currently accepted that B cells localized at the SVZ/SEZ are derived from embryonic RGCs (Tramontin et al. 2003; Merkle et al. 2004), radial astrocytes or type I progenitors within the SGZ seem to initially originate from a population raised in the ventral hippocampus during late gestation which eventually relocates into the dorsal hippocampus (Li et al. 2013).

Other remarkable difference is the evolutionarily acquired contribution of each adult neurogenic niche to the overall effective neurogenesis throughout vertebrates (Kempermann 2012; Bergmann et al. 2015). For instance, although both compartments have been shown to retain proliferative capacity in humans, there are robust evidences that the SVZ/SEZ is not a postnatal source of neurons for the human olfactory bulb (Eriksson et al. 1998; Sanai et al. 2004; Sanai et al. 2011; Bergmann et al. 2012). In contrast, substantial postnatal neurogenesis has been elegantly proven for the human hippocampus and striatum, a trait that seems almost exclusive, since only barely neurogenesis has been detected in the striatum of other mammals (Bergmann et al. 2015).

The possibility to raise newborn cells on-demand constitute an exciting and unique form of structural plasticity whose evolutionary becoming and functional –thus clinical– implications are far from understood and so deserve further research (Parent et al. 2006; Imayoshi et al. 2008; Aimone et al. 2010; Berninger & Jessberger 2016).

2. NEURONAL MIGRATION IN THE TELENCEPHALON

As other organs, the CNS has managed to grow in size and complexity by using cell migration as a strategy to position cell types from different origins into precise –sometimes distant– areas. Cellular migration is a dynamic process in which, by sensing and integrating extracellular cues (diffuse, matrix-attached or cell-cell interactions) and intrinsic regulatory programs, the cell undergoes profound and locally-restricted cytoskeletal reorganizations, nucleokinesis and effective somal translocation that culminates with a net movement of the whole cell (Rakic 1972; Nguyen & Hippenmeyer 2014).

The cytoskeleton of a neuron –actin microfilaments, intermediate filaments and microtubules– is the major intrinsic determinant of its shape and migration mode. Actin filaments play a central role in every motile intracellular rearrangement, given the large driving force provided by the

actin meshwork for cell movement. In addition, the other cytoskeletal systems are also fundamental for cell migration, for instance, microtubules are key regulators of the elongation of the leading process and crucial for nucleokinesis (Heng et al. 2010; Govek et al. 2011) (Figure 7). Remarkably, many cytoskeletal or cytoskeleton-interacting proteins are regulated at transcriptional level by genetic programs triggered during neurogenesis (Hand et al. 2005; Ge et al. 2006; Pacary et al. 2011).

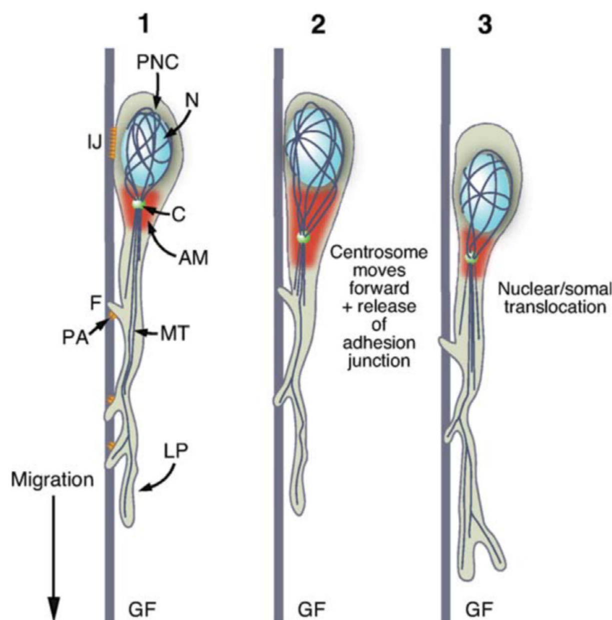

Figure 7. Importance of the cytoskeleton for neuronal migration. The example shows the cellular mechanisms of RGC-aided migration. (1) The neuron polarizes and forms a leading process (LP) in the direction of migration. The centrosome (C) is localized forward of the nucleus (N), which is enwrapped in a perinuclear tubulin cage (PNC). Microtubules (MT) extend from the centrosome into the leading process, and F-actin and actomyosin motors (AM) are enriched in the proximal portion of the leading process. A specialized interstitial adhesion junction (IJ) forms beneath the neuronal soma and punctae adherentia (PA) form beneath short filopodia (F) that protrude from the leading process and enwrap the glial fiber (GF). (2) The migration cycle involves forward movement of the centrosome (C) before nucleokinesis and soma translocation. Afterwards, the neuron takes a step along the glial fiber (3), a new interstitial adhesion junction forms and the migration cycle starts again so that migration continues in a cyclical, saltatory manner. From Govek *et al.* 2011.

As aforementioned, there are two main types of cellular migration in the developing telencephalon: radial and tangential migration. The former is characteristic of cortical PNs whereas the latter is typical of forebrain GABAergic interneurons.

2.1. Radial migration of pyramidal neurons

In 1972, pioneering work from Pasco Rakic showed that, in the fetal monkey cortex, neuronal somata are often juxtaposed to the radial glia fibers and hypothesized that newborn pyramidal neurons follow the guides offered by RGCs to reach the CP (Figure 8). Moreover, Rakic proposed that the entire neuron –and not only the neuronal soma, as it was previously suggested– migrates toward the CP, and also confirmed the inside-out pattern of radial migration, whereby later born neurons migrate through earlier born ones and settle into the more superficial strata of the CP (Angevine & Sidman 1961; Rakic 1972).

Three different types of radial migration are distinguishable during cortical development: somal translocation, multipolar migration and RGC-aided locomotion (Nadarajah et al. 2001; Tabata & Nakajima 2003; Kriegstein & Noctor 2004). At early stages of PP/CP formation –

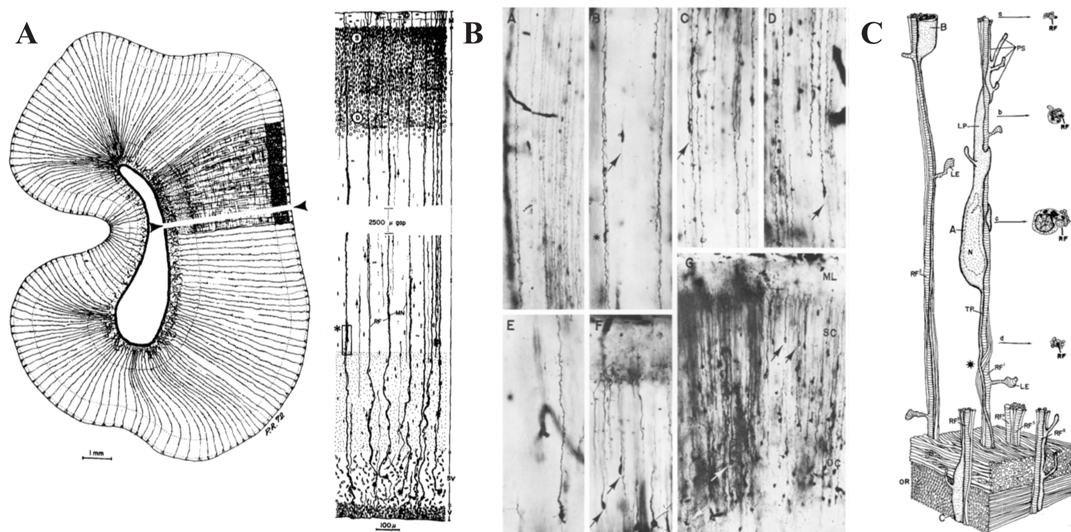

Figure 8. The process of radial glia cell-guided neuronal radial migration. (**A**) Camera lucida drawing of a Golgi-impregnated coronal section at the parieto-occipital level of the brain of a 97-day monkey fetus showing the radial fibers of RGCs. (**B**) Photomicrographs of the Golgi-impregnated telencephalon of 97 day monkey fetus showing the migrating neuronal somata intimately associated to radial processes. (**C**) Three dimensional reconstruction of the relationships between migrating cells and radial fibers in the intermediate zone. Adapted from Rakic, 1972.

between E10 and E14 in the mouse– the main mode of migration is somal translocation (Miyata et al. 2001; Nadarajah et al. 2001). Briefly, a cell undergoing somal translocation exhibits a long radial process that reaches the pial surface and a short trailing process that loses ventricular attachment. The continuous shrinkage of the basal process and the concomitant nucleokinesis determine a fast migratory behavior. Somal translocation is therefore independent from non-cell autonomous radial fibers. In addition, this mode of radial migration is sometimes found in RGCs, after a neurogenic final division or in their transition to other type of RGC, as basal RGCs (Noctor et al. 2004; Taverna et al. 2014). On the other hand, the RGC-aided locomotion strictly relies on the scaffold supplied by radial processes. It is predominant at later developmental stages, from E14, when the thickness of the cortical wall promptly increases (Rakic 1972; Noctor et al. 2001). Newborn pyramidal cells undergoing radial fiber-guided locomotion present a leading process and a short trailing tail –which are not attached to the pial surface nor the ventricle wall, respectively– that seem to *embrace* the radial fiber during their migration (Nguyen & Hippenmeyer 2014) (Figure 8C).

Last, multipolar migration is transiently observed in cells in the SVZ and deep IZ, when they detach from radial fibers and pause radial migration for some time. In this mode, the cell barely migrates and is often observed to shortly migrate tangentially, reaching a different radial process of that of its *mother* cell (Tabata & Nakajima 2003).

2.1.1. Steps of radial migration along the cortex

Radial migration is a multiphasic process in which neurons, as they progress from the VZ/SVZ to the CP, must go through rapid and striking changes in their morphology, cell polarity and migratory behavior (Noctor et al. 2004; Nguyen & Hippenmeyer 2014) (Figure 9). The steps of migration are

coordinated by extracellular cues and internal signaling mechanisms which allow the cell to form transient specialized structures responsible for cell polarization, protrusion, adhesion, retraction, etc (Heng et al. 2010). First, newborn PNs detach from the apical surface and move radially to the SVZ/lower IZ –where they sojourn for up to 24h– and acquire a characteristic multipolar morphology. During this phase, neurons actively extend and retract dynamic processes –likely in search of environmental cues– without a real net movement but, in some cases, retrograde migration toward the ventricular wall or slight tangential movements (Tabata & Nakajima 2003; Noctor et al. 2004). Next, neurons rapidly adopt a bipolar morphology and associate to a radial fiber in order to engage RGC-aided migration to traverse the IZ and invade the CP. This step is characterized by repetitive cycles of extension of the leading process, translocation of the nucleus, and retraction of the trailing process. Remarkably, this bipolar morphology and behavior crucially conditions cell polarity. Consequently, the axon-dendrite polarity of PNs is derived from the polarized emergence of the trailing and leading processes, respectively (Barnes & Polleux 2009). The regulatory mechanisms controlling the passage of locomoting neurons from the IZ into the CP, in spite they are mostly unknown, involve promigratory, instructive and permissive guidance cues that act as a *gatekeeper* for cortical PNs to enter their respective CP target zone. Ultimately, migrating PNs must break through the entire thickness of the CP, where they eventually detach from the RGC fiber and execute terminal somal translocation to conclude migration and settle in their appropriate position to properly integrate in the circuits (Figure 9).

2.1.2. Molecular mechanisms underlying radial migration of pyramidal neurons

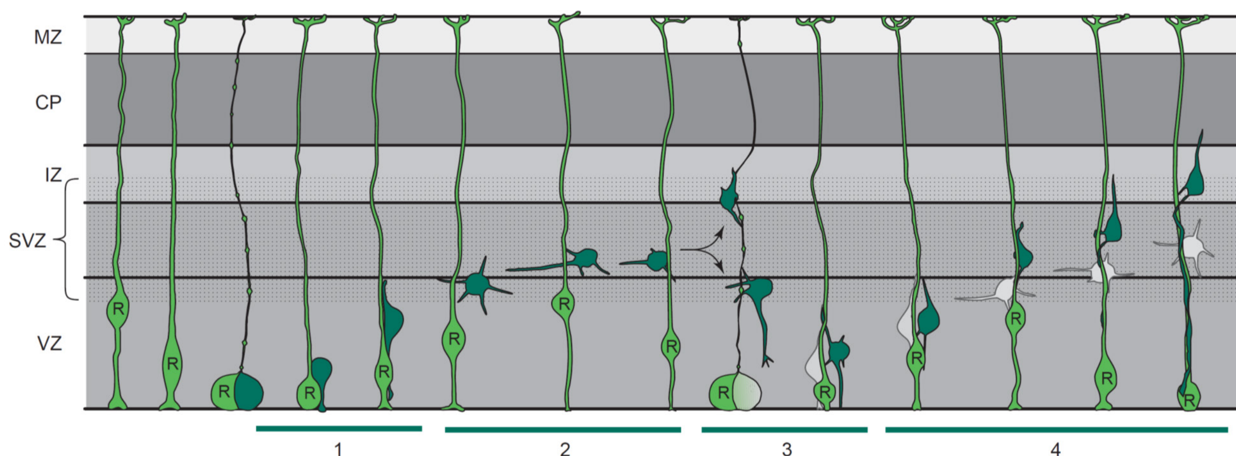

Figure 9. Different steps of the RGC-aided radial migration and subsequent morphological changes. Cortical pyramidal neurons undergo distinct phases of radial migration. Phase **1** involves radial movement of pyramidal neurons (dark green) from the site of origin at the ventricular surface to the subventricular zone (SVZ). In phase **2**, cells become multipolar and pause their migration in the lower intermediate zone (IZ) and subventricular zone (SVZ). Some neurons undergo phase **3**, which is characterized by lateral translocation or retrograde motion toward the ventricle. Phase **4** represents cortical plate (CP) invasion, supported by radial glia fibers, in which the neurons must adopt a bipolar morpholgy. Radial glia (light green) remain mitotic, undergo interkinetic nuclear migration, and generate an additional daughter cell (grey). From Kriegstein & Noctor, 2004.

As previously introduced, neuronal migration is tightly regulated by a large panoply of intrinsic determinants and extrinsic signals that ultimately converge in cytoskeletal reorganizations to control cell locomotion, speed and direction of migration. The impact of the intrinsic burden of transcription factors and membrane receptor endowment on respective stages of neuronal migration at the mechanistic level remains largely obscure and only recently have begun to be deciphered (Ge et al. 2006; Ishizuka et al. 2011; Tang et al. 2014; Nguyen & Hippenmeyer 2014; Wiegreffe et al. 2015). In addition, it is worth noting that particular genetic programs differentially regulate the migration and molecular differentiation of late- and early-born cortical PNs (Lai et al. 2008).

Nonetheless, extrinsic environmental cues are preeminent in controlling neuronal migration, although in most cases lack a mechanistic link explaining how they modulate the intrinsic effectors responsible for neuronal migration. The major extracellular signals modulating neuronal migration are the extracellular matrix (ECM), cell adhesion molecules (CAMs) and soluble or membrane-bound factors (Sobeih & Corfas 2002). The ECM exerts several roles, acting as a mechanical support, providing essential survival signals and tuning dynamics and directionality of neuronal migration. A noteworthy example of an ECM protein deeply affecting radial migration is reelin, a large extracellular glycoprotein released by C-R cells from the MZ. *Reln* gene is deleted in the *reeler* mutant mice and is responsible for its characteristic disrupted and inverted cortical layering (D'Arcangelo et al. 1995). Perturbation of any component of the reelin signaling axis yields a similar phenotype of that of *reeler* mice (Sobeih & Corfas 2002; Franco et al. 2011). In addition, heparan sulfate proteoglycans (HSPGs) and laminins are other pivotal ECM components that substantially affect migration.

CAMs are cell surface proteins that mediate cell-cell or cell-ECM interactions. The role of CAMs as integrins, nectins, cadherins or connexins has been shown to be crucial for RGC-dependent migration, as well as reelin signaling and C-R cell-dependent cortical organization (Franco et al. 2011; Valiente et al. 2011; Solecki 2012; Gil-Sanz et al. 2013). Likewise, soluble and membrane-bound molecules regulate many aspects of CNS development, from the initial steps of neural induction to the maintenance and plasticity of the adult nervous system. These factors induce diverse responses, including promotion of cell survival, cell-fate acquisition, cell-instructing events or morphogenesis. Members of the neurotrophin (NT) family –as brain-derived neurotrophic factor (BDNF) or NT-4– or ligands of the epidermal growth factor (EGF) receptor appear to play a significant role in neuronal migration (Sobeih & Corfas 2002). Additionally, guidance molecules –critical for axonal pathfinding– are necessary for proper radial migration of newborn PNs. For instance, ephrin/eph receptor forward signaling or semaphorins –acting through plexin receptors– modulate the activity of Rho GTPases to favor a promigratory cytoskeletal configuration (Azzarelli et al. 2014; Park & Lee 2015). However, the majority of extracellular promigratory or instructive cues profoundly influencing cortical building still remain elusive.

2.2. Rho GTPases in neuronal migration

The family of Ras homolog (Rho) GTPases belongs to the large Ras superfamily of small GTP-binding proteins. Rho GTPases are crucial regulators of cytoskeletal dynamics and affect many cellular processes, including cell polarity, migration, vesicle trafficking and cytokinesis (Heasman & Ridley 2008; Sit & Manser 2011). It comprises 20 intracellular signaling proteins well conserved in evolution, from plant or yeast to mammals. Most of them function by switching between active GTP-bound and inactive GDP-bound forms. The activity of Rho GTPases is controlled by three classes of molecules: guanine nucleotide-exchange factors (GEFs, facilitate the exchange of GDP for GTP), GTPase-activating proteins (GAPs, promote the hydrolysis of GTP into GDP) and guanine nucleotide-dissociation inhibitors (GDIs, sequester Rho-GDPs in the cytoplasm and protect them from degradation) (Boulter et al. 2010; Azzarelli et al. 2015) (Figure 10).

Several members of this protein family play a pivotal role in neuronal migration, specifically by controlling microtubule stability and actin polymerization (Govek et al. 2011; Azzarelli et al. 2015) (Figure 10). In particular, Ras-related C3 botulinum toxin substrate 1 (*Rac1*) and cell division cycle 42 (*Cdc42*) modulate different phases of cortical PN migration in a cell-autonomous fashion (Konno et al. 2005), while Ras homolog family member A (*RhoA*) exerts a key non-cell autonomous contribution to pyramidal neuron migration, since its expression is necessary in RGCs but dispensable in migrating neurons (Cappello et al. 2012). Moreover, *RhoA* gain of function has been shown to be detrimental for radial migration of pyramidal neurons (Tang et al. 2014). Hence, cell-autonomous inactivation of RhoA emerges as a critical requirement for cortical PN migration, and different intrinsic and extrinsic factors converge in RhoA inhibition to promote radial migration (Hand et al. 2005; Ge et al. 2006; Nguyen et al. 2006; Pacary et al. 2011; Tang et al. 2014; Azzarelli et al. 2014). Alternatively to balancing the active fraction (GTP-bound) of Rho GTPases, the role of proteasomal degradation has gained attention in recent years as a non-canonical mechanism controlling overall Rho GTPases signaling (Nethe & Hordijk 2010).

2.3. Neuronal migration disorders

Disruption of neuronal migration causes cortical malformations of varying degrees of severity. The consequences of such migration abnormalities include severe mental retardation, epilepsy and various intellectual disabilities (Guerrini & Parrini 2010; Barkovich et al. 2012). In some cases, neuronal migration is perturbed in early steps of radial migration, therefore neurons do not reach their correct location within the CP and form ectopic accumulations. The aberrant mass of neurons may remain either close to the ventricle, classified as periventricular heterotopia (PH), or embedded into the white matter, forming a double cortex or subcortical band heterotopia (SBH). In other cases, neurons are able to reach the cortical plate, but fail to properly position, thus giving rise to a very thick and disorganized cortex. As a consequence, the normal pattern

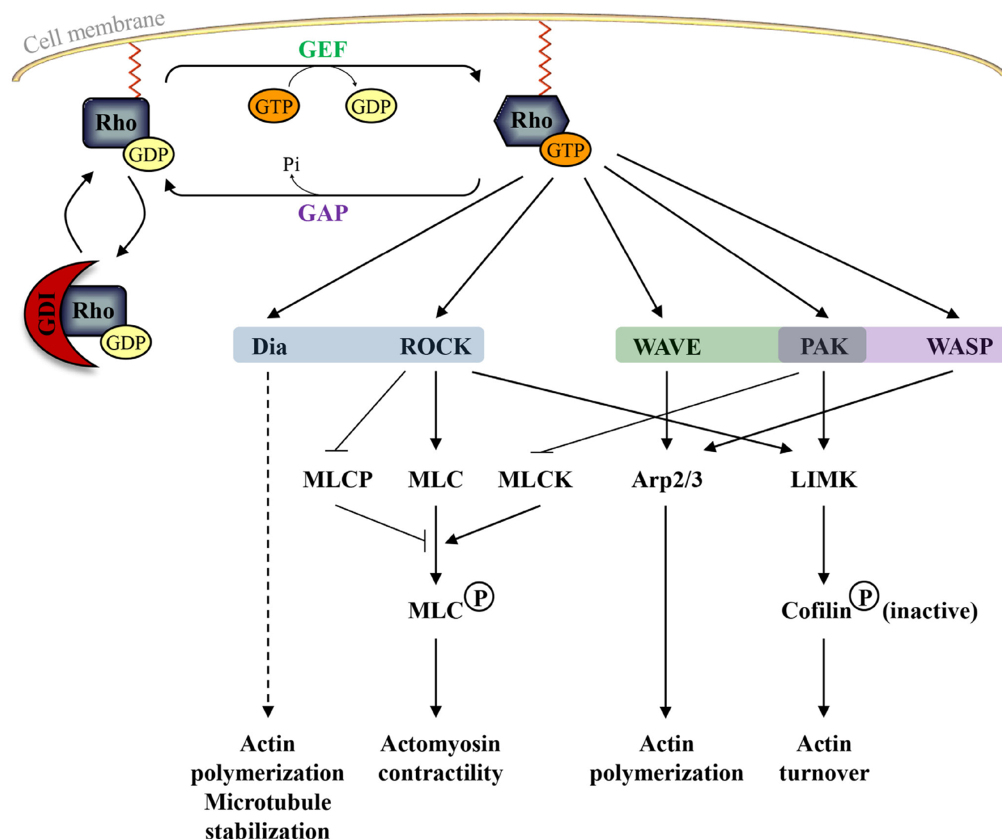

Figure 10. The classical Rho GTPase activation cycle and main pathways regulated by active Rho GTPases. Classical signaling of RhoA is shown (in blue), Rac1 (in green), and Cdc42 (in purple). Guanine nucleotide-exchange factors (GEFs) activate Rho GTPases by promoting the release of GDP and the binding of GTP. GTPase-activating proteins (GAPs) inactivate Rho GTPases by increasing the intrinsic GTPase activity of Rho proteins. Guanine nucleotide-dissociation inhibitors (GDIs) sequester RhoGTPase in their inactive state and protect them from degradation. In their active form, Rho GTPases can bind to different effector molecules. Dia, diaphanous-related formins; ROCK, Rho kinase; MLCP, myosin light chain phosphatase; MLC, myosin light chain; MLCK, myosin light chain kinase; WAVE, Wiskott–Aldrich syndrome protein family verprolin homolog; Arp2/3, actin-related proteins 2 and 3; PAK, p21-activated kinases; LIMK, Lin-11, Isl-1, and Mec-3 kinase; WASP, Wiskott-Aldrich syndrome protein. From Azzarelli *et al.*, 2015.

of connectivity and gyrification of the brain is disrupted, leading to a simplified (pachygyria) or absent (agyria) degree of convolutions, typical features of lissencephaly, which owes its name to the smooth appearance of the brain surface (Guerrini & Parrini 2010; Barkovich et al. 2012).

In recent years, the combination of high-resolution neuroimaging techniques and genetic studies brought to the identification of some genes mutated in human patients with malformations of cortical development (MCDs) related to migration deficits. Particularly, many disorders arise by mutations in genes that encode cytoskeletal proteins and their modifiers, such as tubulin alpha 1a (*TUBA1A*), doublecortin (*DCX*), lissencephaly-1 protein (*LIS*-1), and filamin A (*FLNA)*, whose mutations underlie severe human MCDs, including SBH, X-linked PH and lissencephaly (Guerrini & Parrini 2010; Barkovich et al. 2012). Accordingly, *RhoA* deficiency in murine RGCs dramatically affect radial migration and derive in double cortex formation (Cappello et al. 2012). Furthermore, mutations in *Gpr56* –an orphan G-protein-coupled receptor (GPCR)– cause polymicrogyria, a severe disorder also characterized by mental retardation and seizures (Bae et al. 2014). Last, the most prominent group of MCDs are the focal cortical dysplasias (FCDs), in which –besides cortical progenitor proliferation abnormalities– neuronal migration deficits are often involved.

To a large extent, FCDs are caused by somatic mutations in components of the mammalian target of rapamycin complex 1 (mTORC1) pathway and are characterized by dysplastic neurons, cortical delamination and refractory epilepsy (Blümcke et al. 2011; Lim et al. 2015).

3. POSTMITOTIC SPECIFICATION OF CORTICAL PROJECTION NEURONS

Even though PNs represent the great majority of neurons within the cortex, the conventional idea was that forebrain interneurons contributed more significantly to the cellular heterogeneity of the cerebral cortex. However, the concept that PNs are rather homogeneous within a given cortical layer has been challenged lately by studies combining gene expression and target specificity analyses, indicating that the diversity of PN subclasses is similar to or even larger than that of GABAergic interneurons (Gray et al. 2004; Sorensen et al. 2015).

In recent years, as a result of newly-developed high throughput techniques of single-cell transcriptome analysis, there has been an outstanding increase in the knowledge of molecular markers identifying specific subclasses of PNs as well as molecular mechanisms governing their specification, wiring and the maturation of their physiological traits (Macdonald et al. 2013; Molyneaux et al. 2015; Telley et al. 2015).

As newborn neurons quit cycling, cell-specific primordial transcriptional waves dynamically unfold and instruct the sequence to resolve lineage-choice decisions in the cortex (Telley et al. 2015). Today we know that many of these transcriptional regulators are coexpressed in PNs –at different levels depending on the prospective lineage– and strongly interact, often by reciprocally repressing one another (Srinivasan et al. 2012). This sets a molecular logic whereby each pyramidal subclass progressively refines its molecular identity and connectivity (Greig et al. 2013).

One of the most defining features of PNs is their axonal projection pattern. This hodology-based classification define three major groups of PNs according to whether they extend axons away from the cortex (corticofugal PNs), across the midline to the contralateral hemisphere (commissural/callosal PNs), or within one cortical hemisphere (associative PNs) (Macdonald et al. 2013) (Figure 11). Notably, PNs of a certain subtype located at different functional cortical areas (somatosensory, motor, visual or auditory) project to distinct functional targets.

3.1. Corticofugal projection neurons

Corticofugal PNs comprise subplate PNs (SPPNs), corticothalamic PNs (CThPNs) and subcerebral PNs (SCPNs), which reside in deep layers of the neocortex (V-VI) and are born sequentially early in corticogenesis (Figure 12).

3.1.1. Subplate PNs

SPPNs inhabit the deepest compartment of the layer VI and send pioneering axons to the

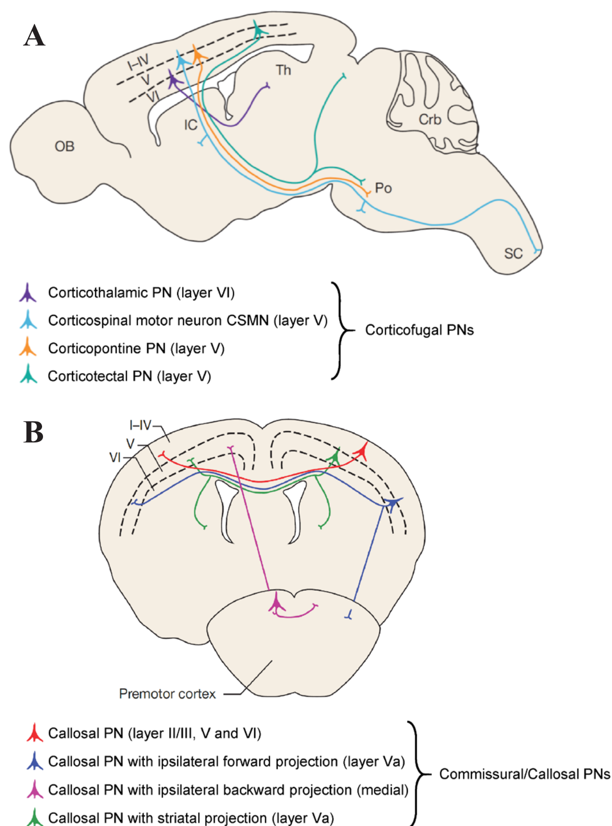

Figure 11. Major subtypes of projection neurons within the neocortex classified by hodology. There are three classes of cortical PNs: associative, commissural, and corticofugal. (**A**) Corticofugal (projections away from the cerebral cortex). Corticothalamic projection neurons: primarily located in layer VI, that project to different nuclei of the thalamus (purple). Subcerebral Projection Neurons: include neurons located in layer V and extend projections to the brainstem and the spinal cord. They are further subdivided into corticospinal motor neurons (CSMNs, light blue, located in the sensorimotor area of the cortex and maintain primary projections to the spinal cord, with some collaterals to the striatum); corticopontine PNs (orange) maintain primary projections to the pons; corticotectal PNs, located in the visual area of the cortex andmaintain primary projections to the superior colliculus, with secondary collateral projections to the rostral pons. (**B**) Commissural/Callosal PNs are primarily located in layers II/III (80%), V (20%), and VI (a small %) and extend axons across the corpus callosum. At least four major types of callosal neurons can be classified that maintain single projections to the contralateral cortex (red); dual projections to the contralateral cortex and the ipsilateral frontal cortex (blue); dual projections to the contralateral cortex and the ipsilateral caudal cortex (pink); dual projections to the contralateral cortex and the ipsilateral or contralateral striatum (green). The three last types are also considered associative PNs given their projections within one hemisphere. From McDonald *et al.*, 2013.

thalamus. These neurons express markers as complexin 3 (*Cplx3*), connective tissue growth factor (*Ctgf*), nuclear receptor-related 1 (*Nurr1*) and monooxygenase Dbh-like 1 (*Moxd1*) (Hoerder-Suabedissen et al. 2009) and play a pivotal role for thalamocortical innervation. Thereby, deviation of its development has been related to several pathologies (Hoerder-Suabedissen & Molnár 2015).

3.1.2. Corticothalamic PNs

CThPNs are located primarily in layer VI and project to different thalamic nuclei. SRY-box containing gene 5 (*Sox5*) and T-box brain protein 1 (*Tbr1*) are important genes involved in their specification. Subtype-specific expression of *Tbr1* or forkhead box P2 (*Foxp2*), is commonly used to mark CThPNs (Molyneaux et al. 2007).

3.1.3. Subcerebral PNs

SCPNs are nested in cortical layer V (Vb), present the largest somata in the cerebral cortex and project their axons to targets in the midbrain, hindbrain and spinal cord. SCPNs comprise several subpopulations such as corticospinal motor neurons (CSMNs), corticotectal PNs or corticopontine PNs, and each of these populations can be further divided into additional subclasses. CSMNs are large pyramidal neurons that reside in sensory and motor areas and extend their primary axon to the spinal cord, with some secondary collaterals to the striatum, red nucleus, caudal pons and medulla. Further, corticotectal PNs reside in the visual cortex and lengthen their primary axon to the superior colliculus in the midbrain. Last, corticopontine PNs prolong their primary axon to targets in the pons and medulla (Macdonald et al. 2013). The expression of the transcription factor FEZ family zinc-finger 2 (*Fezf2*) is necessary

and sufficient for the generation and specification of SCPNs (Molyneaux et al. 2005; Rouaux & Arlotta 2012). Downstream of *Fezf2*, CoupTF interacting protein 2 (*Ctip2*)/*Bcl11b* has also been shown to be crucial for the specification of CSMNs, as mice lacking this gene fail to send axons to the spinal cord and show striking fasciculation aberrations (Chen et al. 2005; Arlotta et al. 2005). Among the most common markers used to identify SCPNs are *Ctip2*, carboxyl-terminal LIM domain-binding protein 1 (*Clim1*), encephalopsin or ETS-related protein 81 (*Er81*) (Molyneaux et al. 2007).

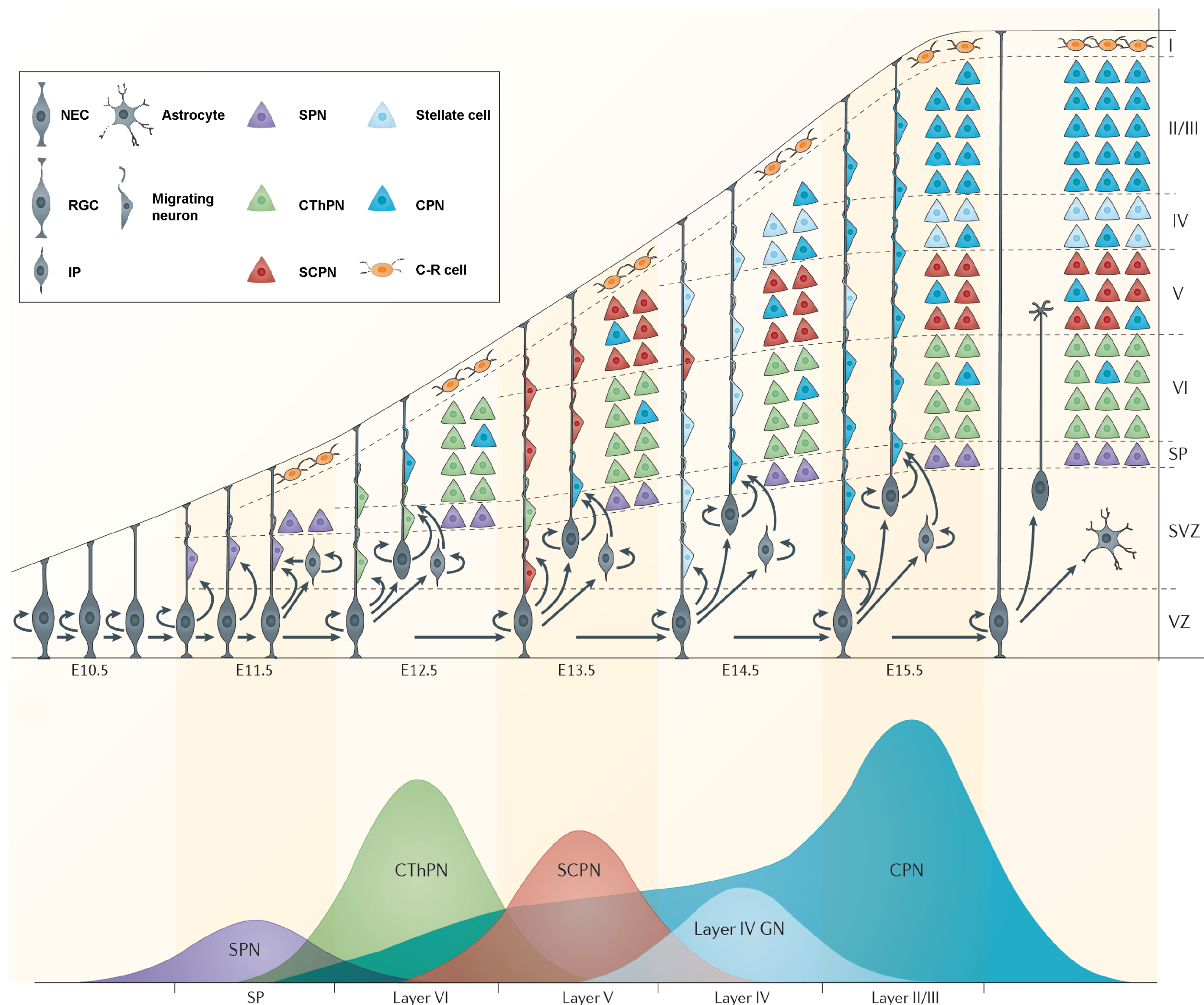

Figure 12. Neocortical projection neurons are generated in sequentially cortical progenitors in the VZ and SVZ. The schematic depicts the sequential generation of neocortical PN subtypes and their migration to appropriate layers over the course of mouse embryonic development. (**A**) Transition of neuroepithelial cells (NEC) to radial glia cells (RGC) in the ventricular zone (VZ). Around E11.5, RGCs start generating intermediate progenitors (IPs), which establish the subventricular zone (SVZ) and act as transit-amplifying cells. Cajal-Retzius (C-R) cells primarily migrate into prospective layer I, whereas PNs are born in the neocortical VZ and/or SVZ and migrate along radial glial processes to reach their final laminar destinations. (**B**) Distinct PN subtypes are born in sequential waves over the course of neurogenesis. The peak birth of subplate neurons (SPN) occurs around E11.5; the peak birth of corticothalamic PNs (CThPN) and subcerebral projection neurons (SCPN) occurs at E12.5 and E13.5, respectively. Layer IV stellate cells are born around E14.5. Some callosal projection neurons (CPN) are born starting at E12.5, which are born concurrently with CThPN and SCPN and also migrate to deep layers. Subsequently, most CPN are born between E14.5 and E16.5, and these late-born CPN migrate to superficial cortical layers. Peak sizes are proportional to the approximate number of neurons of each subtype born on each day. By birth, after neurogenesis is complete, neural progenitors transition to a gliogenic mode, generating astrocytes and oligodendrocytes (not shown). Adapted from Greig *et al.*, 2013.

3.2. Commissural/callosal projection neurons

Commissural/callosal PNs (CPNs) are cells of small to medium pyramidal size and considerably heterogeneous attending to their birth dates and eventual laminar destinations. CPNs are predominantly located in layers II/III –albeit considerable numbers are also found in layer V and VI– and extend axons across the corpus callosum or the anterior commissure, even though several subtypes with distinct projection patterns can be distinguished. Currently, at least four major types of CPNs are classified depending on complex projections to the ipsilateral and contralateral striatum and to other ipsilateral cortical areas (Fame et al. 2011) (Figure 11B).

The postmitotic specification of CPNs markedly relies on a transcriptional regulation machinery that operates by suppressing the SCPN program. The homeodomain-containing special AT-rich sequence-binding protein 2 (*Satb2*), emerges as a key mediator of this transcriptional repression. It is well-established that, together with chromatin remodeling partners like the nucleosome remodeling and deacetylase (NURD) complex (Britanova et al. 2008) or Ski (Baranek et al. 2012), Satb2 binds to the *Ctip2* promoter region, repressing its expression and, therefore, CSMN specification. In the lack of *Satb2*, upper layer neurons are unable to cross the midline, begin to express SCPN markers and extend their axons through the internal capsule (Alcamo et al. 2008). Other upper-layer neuron specific genes, such as POU domain, class 3, transcription factor 2 and 3 (*Pou3f2/3*) and cut-like homeobox 1 and 2 (*Cux1/2*), also play a pivotal role in CPN specification and –along with *Satb2*– are routinely used as CPN markers (Molyneaux et al. 2009). Additionally, LIM homeodomain 4 (*Lmo4*) constitutes a useful marker of CPNs located in layer V (Arlotta et al. 2005).

3.3. Associative projection neurons

APNs are present throughout the neocortex and project axons to other areas within the same cortical hemisphere. APNs include short-distance intrahemispheric PNs, which extend axons within a single cortical column or to nearby cortical columns –such as layer IV stellate neurons– and long-distance intrahemispheric PNs, which extend axons to adjacent or distant cortical areas –such as forward and backward PNs– (Greig et al. 2013). Particularly, stellate neurons –identified by the expression of RAR-related orphan receptor beta (*Rorβ*) (Schaeren-Wiemers et al. 1997)– seem to play an outstanding role in brain function, since layer IV receives most thalamocortical and intrahemispheric afferents. Given the number of cortical areas and their potential connectivity, the number of APN subtypes is likely to be large, especially in more complex species, but this issue remains to be explored.

4. CANNABIS AND THE ENDOCANNABINOID SYSTEM

The plant *Cannabis sativa* (commonly known as cannabis, marijuana, ganja or hemp) has been cultivated by humans since Neolithic times. The earliest record of hemp use is a fiber cordage found in the Czech Republic, dated at *ca*. 26980 to 24870 BP, which means between 25030 to 22920 years old (Fleming & Clarke 1998). Around five millennia ago, in the ancient China, –in addition to its textile uses– cannabis was prescribed for a multitude of maladies, although "when taken in excess it could cause seeing devils" (Mechoulam et al. 2014). Cannabis was also extensively used by the Assyrians, from 2000 BC to 600 BC, who made use of its inebriating and medical properties. It was named either *gan-zi-gun-nu* ("the drug that takes away the mind") or *azzalu*, which was apparently a drug for "depression of spirits" (Mechoulam & Parker 2011). Over the last millennium, most cannabis use became localized from Middle East to India –where it was called *ganjika*, in Sanskrit– and was intimately associated to their cultures and spiritual practices. After the French Campaign in Egypt and Syria (1798-1801) leaded by Napoleon Bonaparte, returning soldiers import knowledge of cannabis usage to Europe. Similarly, British physicians destined for India brought back to Europe both recreational and medical practices (Mechoulam & Parker 2011). For centuries, cannabis industry was of major importance in Europe and North America, where most mooring and ropes –crucial for sailing– were made of hemp fibers. Therefore, our culture stood a long tradition of industrial, recreational and medical cannabis usage, until the Single Convention on Narcotic Drugs (New York, 1961) applied the same restrictions on cannabis that on heroin or cocaine, which made of it the most widely used illicit drug.

Among the approximately 300000 species of vascular plants described nowadays (Christenhusz & Byng 2016), so far, *Cannabis sativa* is the only one to produce the bioactive compounds known as cannabinoids or phytocannabinoids. These are present in cannabis as a mixture of over 100 closely related lipidic constituents, from which we may highlight the Δ^9-tetrahydrocannabinol (Δ^9-THC or THC) and the cannabidiol (CBD) as the most abundant and physiologically relevant (Mechoulam et al. 2014) (Figure 13). Both compounds –and many other cannabinoids– were characterized by the group of Raphael Mechoulam during the 1960's (Mechoulam & Shvo 1963; Gaoni & Mechoulam 1964).

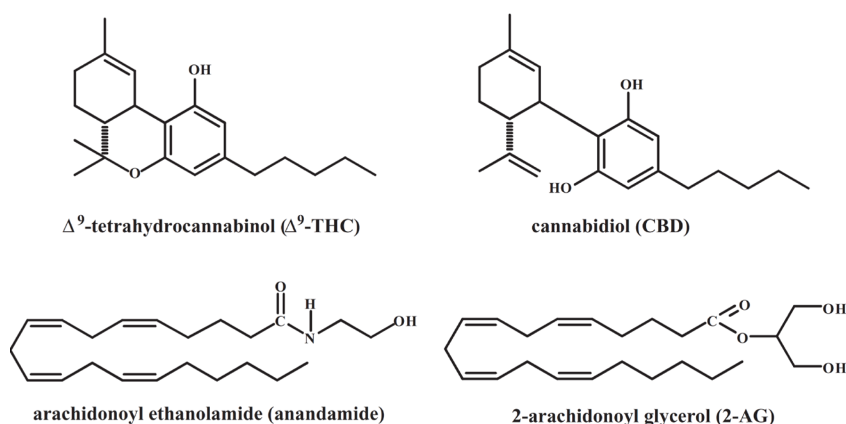

Figure 13. Major phyto- and endocannabinoids. Chemical structures of the plant cannabinoids Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) and of the endogenous cannabinoids anandamide and 2-arachidonoyl glycerol (2-AG). From Mechoulam & Parker, 2011.

However, it was not until 30 years later that the molecular substrate of THC action was first cloned and characterized in rat brain (Matsuda et al. 1990). Shortly after, its human (Gérard et al. 1991) and murine (Chakrabarti et al. 1995) orthologs were also described. The gene identified encodes a GPCR, which was lately named cannabinoid receptor type 1 (*Cnr1* or *CB1*), since in following years a gene encoding a second –peripheral– cannabinoid receptor was also discovered in spleen (Munro et al. 1993) and thus named cannabinoid receptor type 2 (*Cnr2* or *CB2*).

Two years after the discovery of the CB1 cannabinoid receptor (CB1R), Mechoulam's lab isolated and characterized an endogenous brain constituent that bound to CB1Rs. It was an arachidonic acid (AA) derivative named anandamide, after the Sanskrit word *ananda* (meaning bliss) and the chemical nature of the compound (Devane et al. 1992). This finding confirmed –as it was expected– that cannabinoid receptors (CBRs) were not evolutionarily acquired for binding phytocannabinoids from cannabis, but endogenous ligands (endocannabinoids), and simultaneously opened a new field of study on an endogenous system of transduction of extracellular signals, the so-called Endocannabinoid System (ECS) (Mechoulam & Parker 2011; Pertwee 2015).

4.1. Main components of the Endocannabinoid System

Overall, the ECS is composed of the CBRs, the endocannabinoids (eCBs) and the enzymes responsible of their metabolism (synthesis and degradation). It has been proposed the existence of eCB intracellular transporters as well as carrier proteins (Fowler 2013), which might belong to the ECS, but these remain to be confirmed and thus are not further introduced.

4.1.1. Cannabinoid receptors

CBRs are not restricted to mammals, not even vertebrates, since several orthologs have been described across deuterostomes, including protochordates as cephalochordates –amphioxus (Elphick 2007)– or urochordates –tunicates or sea squirts (Elphick et al. 2003)–, and echinoderms –sea urchins (Buznikov et al. 2010)–. For some time it was believed that cannabinoid signaling started in deuterostomes, given that not orthologous had been found in protostomes. However, recently, two putative CBRs orthologs have been described in the nematode *Caenorhadbditis elegans* –which respond to anandamide and whose functions are rescued by expression of human *CB1* (Pastuhov et al. 2016; Oakes et al. 2017)–. This fact, along with evidence from coevolutionary analysis of most ECS genes orthologs in nine phylogenetically distant species (McPartland et al. 2007), point to an early origin of the ECS in ancestral metazoans.

More importantly, there are two canonic, well-characterized mammalian CBRs: CB1R and CB2R. These CBRs belong to the class A GPCRs family. Thus, they are integral membrane proteins with extracellular N-terminal region, followed by seven transmembrane domains with three extracellular and three intracellular loops that end with the C-terminal domain toward the

cytoplam. CBRs exhibit 42% amino acid sequence identity in human (Shao et al. 2016), reaching almost 70% in the transmembrane residues, involved in ligand binding (Munro et al. 1993). Both CBRs typically signal through $G\alpha_{i/o}$ heterotrimeric G proteins –which inhibit adenylyl cyclase and decreases cAMP levels (Howlett 1985)–; activate mitogen-activated protein kinase (MAPK) signaling (Howlett et al. 2004) –e.g. by protein kinase A (PKA) inhibition (Davis et al. 2003) or by βγ-dependent activation of PI3K (Galve-Roperh et al. 2002)–; and interact with β-arrestins (Jin et al. 1999) to signal and internalize. Figure 14 exemplifies the main signaling cascades triggered by CB1R activation. Nonetheless, CBR signaling is highly pleiotropic and deeply dependent on the cellular context and the nature of the ligand (Turu & Hunyady 2010). Hence, CBRs are also capable of signal through Gα (Howlett et al. 2010), $G\alpha_{q/11}$ (Lauckner et al. 2005), transactivate tyrosine kinase receptors or regulate serine/threonine kinases (Dalton & Howlett 2012) to list a few. Additionally, CBRs have been shown to homo- and heteromerize (Mackie 2005; Callén et al. 2012), as well as with other GPCRs (Moreno et al. 2017) or even tyrosine kinase receptors (Pérez-Gómez et al. 2015), which turns largely difficult to specifically address their signaling cascades. Recently, the inverse agonist-bound structure of CB1R has been crystalized (Hua et al. 2016; Shao et al. 2016), what undoubtedly will shed light to fully understand the complexity and potential of CBR signaling (Figure 14).

Figure 14. Main signaling cascades triggered by CB1R activation and functional outcomes. Several pathways activated by CB1R signaling are depicted. To denote some remarkable examples, CB1Rs can inhibit voltage-dependent Ca^{2+} channels (VDCC) or activate G protein-coupled inward rectifying potassium channels (GIRKs) through βγ subunits. β-arrestins participate in CB1R internalization and desensitization, while GPCR-associated sorting protein 1 (GASP1) directs this receptor toward lysosomal degradation. The canonic activation of MAPKs (MEK, Erk1/2) by βγ subunits and inhibition of adenylate cyclase (AC) and protein kinase A (PKA) by $G\alpha_{i/o}$ are shown. Alternatively, CB1Rs can activate mTORC1 signaling to regulate protein translation by βγ-dependent recruitment of PI3K-Akt. From Keimpema *et al.*, 2011.

The CB1R is the most abundant GPCR in mammalian brain (Herkenham et al. 1990), although it is also highly expressed throughout the body and the ontogeny (Galve-Roperh et al. 2013; Maccarrone et al. 2014). The capacity of THC –and other cannabinoids– to activate CB1R signaling is responsible for the psychoactivity of cannabis, thereby, cannabinoid compounds which not bind to CB1Rs lack the typical physiological effects of cannabis intoxication. In the mid 1980's, it was develop an *in vivo* assay aimed at easily measuring the magnitude of the behavioral effects of cannabinoids, termed "the cannabinoid tetrad assay" (Pertwee 2006), consisting in four behavioral tests that allow to assess the cannabinoid-dependent hypothermia, hypokinesia, analgesia and catalepsy. Importantly, the pharmacokinetics and pharmacodynamics of CB1R signaling largely influence the overall physiological response to cannabinoids (Grotenhermen 2003) and, in turn, are conditioned by the nature of the ligand (Mechoulam et al. 2014); the status of glycosylation/acylation/phosphorilation of the receptor (Jin et al. 1999; Daigle et al. 2008; Shim 2010; Howlett et al. 2010; Oddi et al. 2012); the presence of CBR-regulatory proteins as cannabinoid receptor interacting protein 1 a (CRIP1A) (Niehaus et al. 2007) or β-arrestins (Smith et al. 2010); and allosteric modulators as hemopressin (Bomar & Galande 2013) or pregnenolone (Bellocchio et al. 2014). All these mechanisms exert a key role in the control of the intracellular trafficking, desensitization, downregulation, signal transduction and constitutive activity of CB1Rs.

Unlike *CB1*, *CB2* is mainly expressed in the periphery, where for long time it was believed to be exclusive of immune cells (Miller & Stella 2008; Mechoulam & Parker 2011). However, CB2Rs have now been found within the brain, particularly in microglial cells and embryonic and adult neural progenitors, with important implications in health and disease (Palazuelos et al. 2009; Palazuelos et al. 2012; Schmöle et al. 2015). In turn, the expression of *CB2* in neuronal cells of the adult brain is an issue of intense debate (Atwood & Mackie 2010), although there are recent functional evidences that suggest that CB2Rs are present –and functional– in neuronal cells (Stempel et al. 2016).

In addition to the canonical CBRs, there are other proteins targeted by eCBs at submicromolar concentrations, normally referred to as non-cannabinoid receptors (Pertwee et al. 2010; Pertwee 2015). Among them, we may distinguish GPCRs (e.g. GPR55); transient receptor potential channels (e.g. vanilloid receptor or TRPV1); nuclear receptors (e.g. peroxisome proliferator-activated receptors PPARs); ligand-gated ion channels (e.g. 5-HT_3 receptors); and some voltage-gated Ca^{2+} and K^+ channels (Pertwee et al. 2010; Pertwee 2015).

4.1.2. Cannabinoids

Rigorously speaking, a *cannabinoid* is a molecule able to orthosterically bind to a CBR. Therefore, well-accepted cannabinoids as the CBD would fall apart of this category. Then, generally speaking, cannabinoids are those compounds chemically related to each other that, in most cases, affect the ECS directly or indirectly. There are three classes of cannabinoids, depending on their origin:

phytocannabinoids (those from *Cannabis sativa*); endocannabinoids (produced by metazoans); and synthetic cannabinoids (artificial derivatives not found in nature) (Figure 15).

As phytocannabinoids, we may recognize the THC, CBD, cannabinol (CBN), Δ^8-THC or cannabigerol (CBG), with distinct affinities for CBRs, to name a few.

Next, the two well-established eCBs are AA derivatives, the anandamide and the 2-arachidonoyl glycerol (2-AG). Despite a few additional eCBs have been reported, none of them has been confirmed to date (Matias et al. 2006; Mechoulam et al. 2014). 2-AG was discovered shortly after CB2R, in seek of a peripheral eCB lipid ligand (Mechoulam et al. 1995). Remarkably, anandamide is a partial agonist at CB1R and CB2R –with slightly better affinity for the former– whereas 2-AG acts as a full agonist, with higher affinity for both CBRs than anandamide (Mechoulam et al. 2014).

Last, there is a plethora of synthetic cannabinoids. Engineered derivatives are aim to increase the affinity for CBRs, to design selective inverse agonists (antagonists), to bestow selectivity by a given receptor or receptor pool (e.g. central or peripheral receptor pool), to delay their metabolism, etc (Pertwee et al. 2010). Furthermore, although they cannot be considered proper cannabinoids, also have been designed allosteric modulators (Stornaiuolo et al. 2015) and drugs altering the functioning of the eCB-metabolizing enzymes (Blankman & Cravatt 2013). It is noteworthy to present some synthetic cannabinoids that appear in the Results section of this Thesis, as the HU-210 (dual agonist with higher affinity at CB1Rs); WIN55,212-2 and CP55,940 (dual agonists); and SR141716 or rimonabant (selective CB1R inverse agonist) (Pertwee et al. 2010).

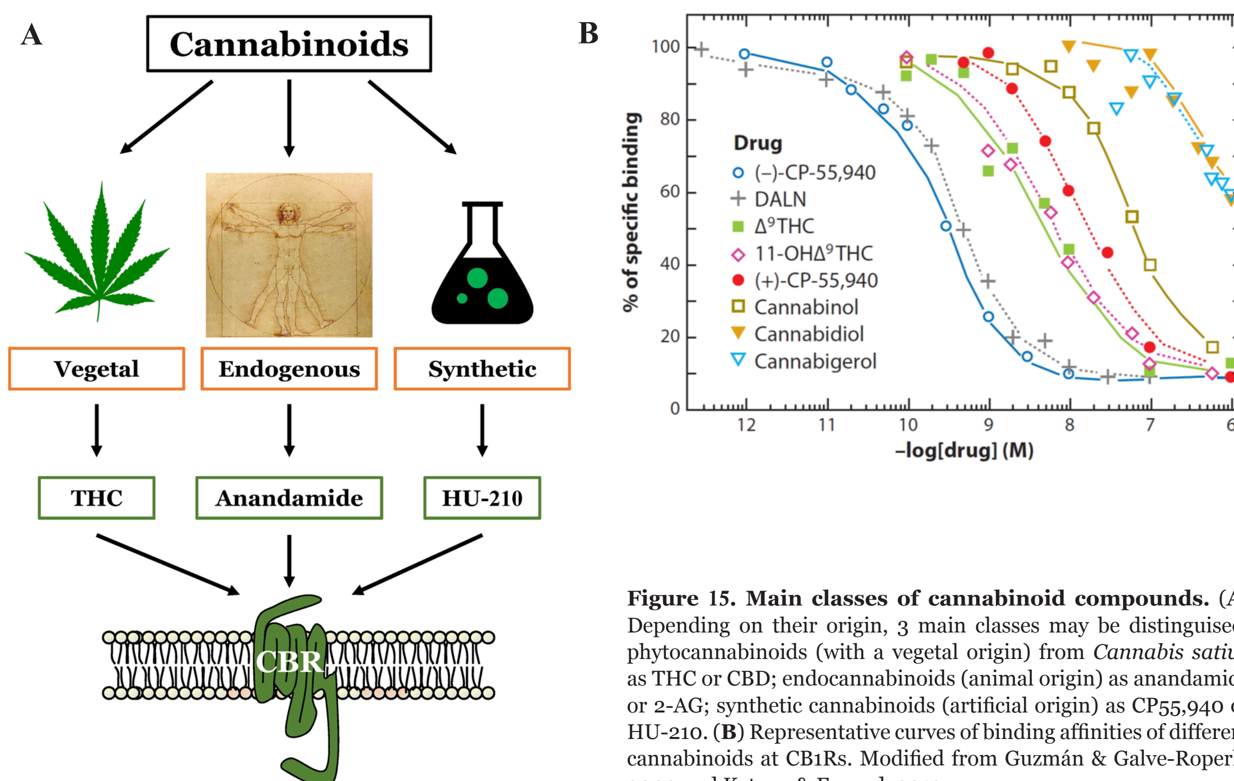

Figure 15. Main classes of cannabinoid compounds. **(A)** Depending on their origin, 3 main classes may be distinguised: phytocannabinoids (with a vegetal origin) from *Cannabis sativa* as THC or CBD; endocannabinoids (animal origin) as anandamide or 2-AG; synthetic cannabinoids (artificial origin) as CP55,940 or HU-210. **(B)** Representative curves of binding affinities of different cannabinoids at CB1Rs. Modified from Guzmán & Galve-Roperh, 2009 and Katona & Freund, 2012.

4.1.3. Endocannabinoid metabolism

There is a remarkable feature of the ECS that makes a difference with almost the rest of neurotransmitter-based systems: eCBs are synthetized *on demand* from lipidic moieties present in cellular membranes. This fact implies the constant bioavailability of the substrate and the permanence of metabolic enzymes in an active state. For instance, anandamide and 2-AG in neurons are synthetized by calcium-sensitive enzymes activated upon stimulus (e.g. membrane depolarization and strong Ca^{2+} influx), which also triggers $G_{q/11}$-coupled metabotropic glutamate receptors that, in turn, further activate eCB-synthetic enzymes and contribute to cannabinoid signaling (Katona & Freund 2008; Kano et al. 2009). The metabolism of eCBs is a complex process, since there are numerous enzymes participating in the synthesis and degradation of eCBs and, consequently, a great variety of metabolic pathways, many of which are well characterized nowadays (Ueda et al. 2013).

The canonic biosynthetic pathway of 2-AG involves a diacylglicerol lipase (DAGL), of which two isoforms (α and β) have been described (Bisogno et al. 2003). DAGL takes DAG –e.g. from the breaking of phosphatidylinositol bisphosphate (PIP2) into inositol trisphosphate (IP3) and DAG– and releases the *sn*-1 acyl chain to obtain 2-AG (Ueda et al. 2013) (Figure 16A). In contrast,

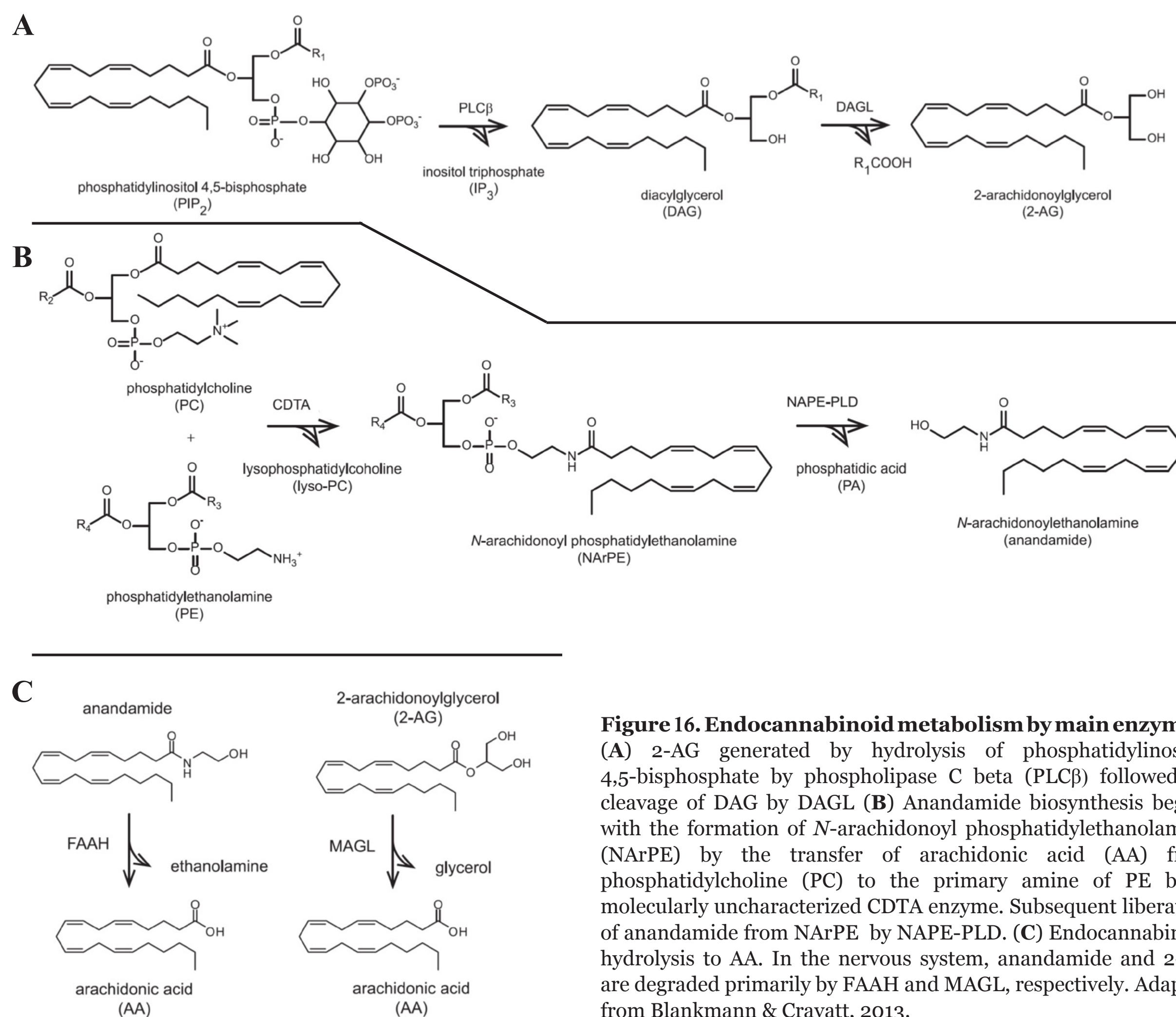

Figure 16. Endocannabinoid metabolism by main enzymes. (**A**) 2-AG generated by hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C beta (PLCβ) followed by cleavage of DAG by DAGL (**B**) Anandamide biosynthesis begins with the formation of *N*-arachidonoyl phosphatidylethanolamine (NArPE) by the transfer of arachidonic acid (AA) from phosphatidylcholine (PC) to the primary amine of PE by a molecularly uncharacterized CDTA enzyme. Subsequent liberation of anandamide from NArPE by NAPE-PLD. (**C**) Endocannabinoid hydrolysis to AA. In the nervous system, anandamide and 2-AG are degraded primarily by FAAH and MAGL, respectively. Adapted from Blankmann & Cravatt, 2013.

the typical biosynthesis of anandamide is catalyzed by the *N*-acylphosphatidylethanolamine-hydrolizing phospholipase D (NAPE-PLD), which hydrolyzes NAPEs –derived from phosphatidylethanolamine and phosphatidylcholine– to yield anandamide and a phosphatidic acid (Marzo et al. 1994) (Figure 16B).

Regarding the eCB degrading pathways, despite there are several enzymes implicated, mainly converge in the formation of AA, with important implications for the metabolism of prostaglandins and other eicosanoids (Ueda et al. 2013) (Figure 16C). Within the CNS, the main eCB-selective degrading enzymes are the fatty acid amide hydrolase (FAAH) (Marzo et al. 1994) –which hydrolizes anandamide– and the monoacylglicerol lipase (MAGL) (Dinh et al. 2002) –which degrades 2-AG–, but there are others –as the non-selective cyclooxygenase-2 (COX2)– with important biological implications (Kano et al. 2009; Muccioli 2010).

4.2. CB1 cannabinoid receptor in the CNS

The CB1R is broadly expressed in numerous neuronal populations throughout the CNS. It is mainly localized in the presynapsis, where it exerts a crucial neuromodulatory role based on the retrograde transmission of eCB signals. Hence, it is noteworthy pointing that, often, there is a discrepancy between the distribution of *CB1 mRNA* and protein in neuronal populations across brain areas, especially when those are PNs (e.g. despite medium spiny neurons highly express *CB1* there is barely CB1R protein within the striatum, in contrast, the protein is mainly localized to its anatomical targets).

On the one hand, CB1R protein in the murine brain is highly detected (by immunostaing or binding of synthetic radioactively-labeled ligands) in the olfactory bulb, hippocampus, lateral part of the striatum and its main target nuclei (i.e. globus pallidus, entopeduncular nucleus, substantia nigra pars reticulata) and cerebellar molecular layer (Kano et al. 2009) (Figure 17A-E). However, substantial CB1R signal is detected in other important brain areas as the cerebral cortex, septum, amygdala, hypothalamus, some nuclei of the brainstem and the dorsal horn of the spinal cord.

On the other hand, regarding *CB1 mRNA*, two distinct patterns of *CB1* expression are distinguished. There is a uniform labeling (resulting from CB1 expression in principal cells) found in the striatum, thalamus, hypothalamus, cerebellum and lower brainstem, whereas a non-uniform signal is observed in the cerebral cortex, hippocampus and amygdala, corresponding to interspersed *CB1* highly-expressing cells (Kano et al. 2009) (Figure 17F, G).

Additionally, CB1Rs have also been found, at much lower levels, in astrocytes (Sánchez et al. 1998) –where are key players of the astrocyte-dependent neuronal plasticity or *tripartite* synapse (Navarrete & Araque 2008)–; oligodendrocytes (Molina-Holgado et al. 2002); microglia (Stella 2010); and adult neural progenitors (Jin et al. 2004; Aguado et al. 2005).

Within the neocortex and hippocampus, CB1Rs are largely enriched in GABAergic interneurons

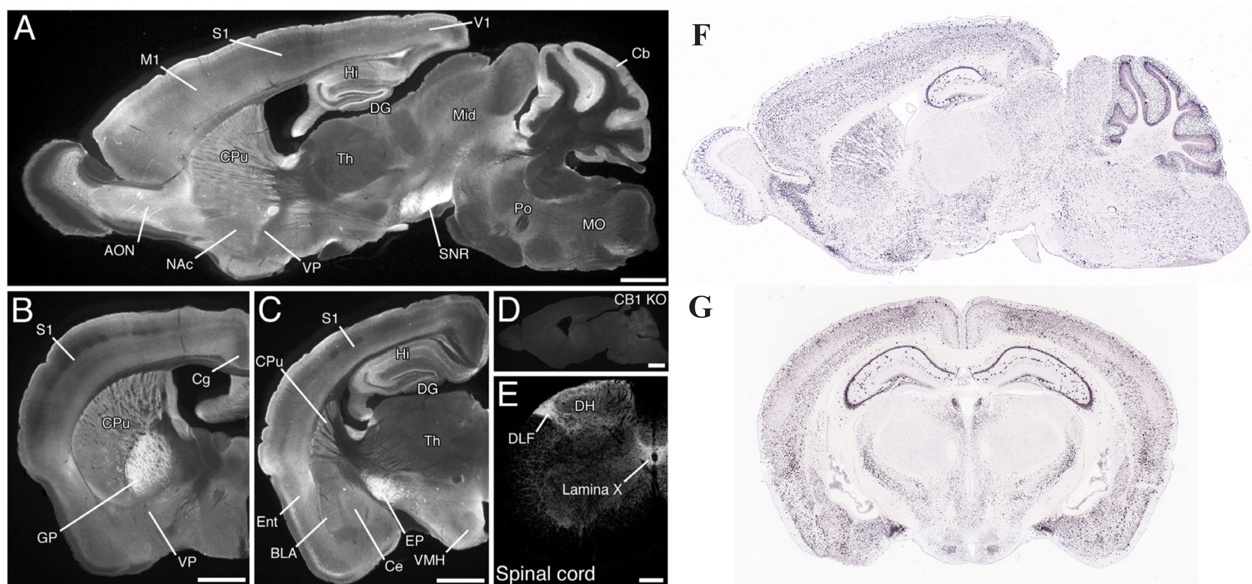

Figure 17. Distribution of CB1 protein and mRNA in the central nervous system of adult mice. (**A**-**D**) Overall distribution in sagittal (**A**, **D**) and coronal (**B**, **C**) brain sections of wild-type (**A**-**C**) and *CB1*-knockout (*CB1-KO*) (**D**) mice immunolabeled with a polyclonal antibody against mouse CB1R. CB1R immunoreactivity is highest along striatal output pathways, including the substantia nigra pars reticulata (SNR), globus pallidus (GP), and entopeduncular nucleus (EP). High levels are also observed in the hippocampus (Hi), dentate gyrus (DG), and cerebral cortex, such as the primary somatosensory cortex (S1), primary motor cortex (M1), primary visual cortex (V1), cingulate cortex (Cg), and entorhinal cortex (Ent). High levels are also noted in the basolateral amygdaloid nucleus (BLA), anterior olfactory nucleus (AON), caudate putamen (CPu), ventromedial hypothalamus (VMH), and cerebellar cortex (Cb). Virtual lack of immunostaining in *CB1*-knockout (KO) mice indicates the specificity of the CB1R immunolabeling. (**E**) CB1R immunolabeling in the spinal cord. Note that striking CB1R immunoreactivity is seen in the superficial dorsal horn (DH). (**F**,**G**) Representative images showing CB1 in situ hybridization in a sagittal (**F**) or coronal (**G**) section of adult mouse brain. From Kano, 2009 and Allen Brain Atlas.

–particularly, in cholecystokinin (CCK)-containing basket cells and a subset of calbindin D28K– whereas low but consistent and appreciable expression is detected in PNs (Marsicano & Lutz 1999). Nevertheless, an important consideration regarding CB1R function is that the amount of CB1Rs not necessarily suits its overall effective signaling, as it has been shown that the efficiency of the coupling to G proteins differs among CB1R-expressing neuronal populations (Steindel et al. 2013).

The subcellular distribution of CB1Rs in diverse and dependent on the cellular type and the pharmacodynamics of CB1R ligands (Thibault et al. 2013), although it is generally accepted that CB1Rs are localized to the plasma membrane at the presynaptic compartment (Castillo et al. 2012; Dudok et al. 2014). Nevertheless, there are reports pointing to a CB1R pool placed at the postsynapsis (Maroso et al. 2016) or even at the outer mitochondrial membrane (Bénard et al. 2012), with striking functional implications for memory consolidation (Hebert-Chatelain et al. 2016). There is extensive literature on the molecular and cellular mechanisms controlled by the ECS that, despite being outstanding for brain function and animal behavior, fall apart of the main scope of this Thesis and therefore are not further introduced. For a glimpse of the canonical role of the ECS as a retrograde messenger-based neuromodulatory system, and an overview of the two main plastic processes –synaptic short- (STD) and long-term depression (LTD)– in which it is involved, see (Figure 18). For extensive review see (Katona & Freund 2008; Kano et al. 2009; Castillo et al. 2012).

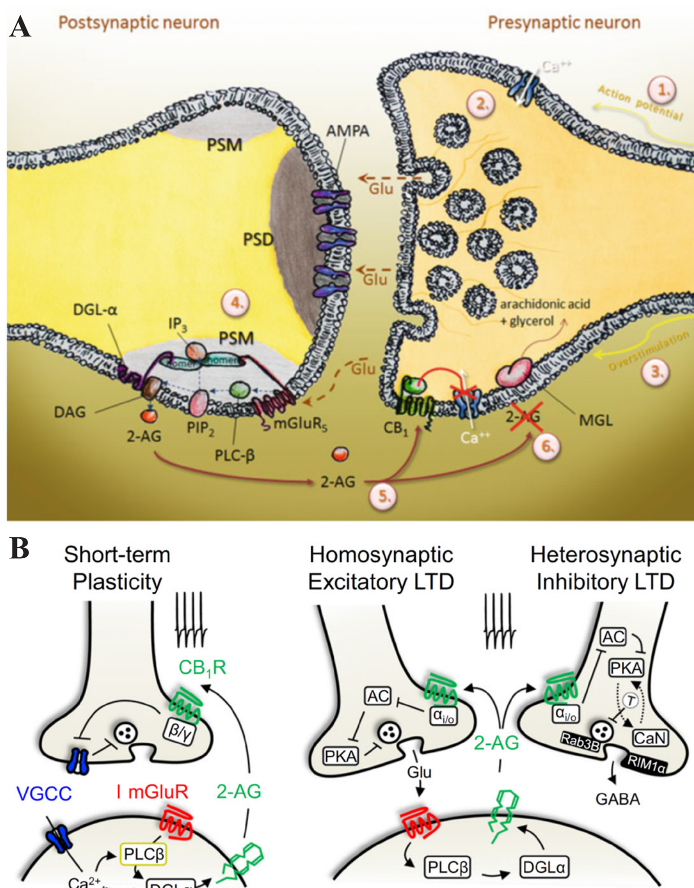

Figure 18. Schematic of the model of the ECS as retrograde messenger-based neuromodulatory ensemble and its major roles. (**A**) Representation of a glutamatergic synapse and the major events involved in the circuit-breaker role of CB1R. Under basal conditions, a single action potential (**1**) triggers the opening of voltage-gated calcium channels, and the resulting calcium transient evokes glutamate release from synaptic vesicles into the synaptic cleft (**2**). Upon presynaptic hyperactivity, such as may occur during epileptic seizures (**3**), an exceedingly high concentration of glutamate spills over from the synaptic cleft and reaches perisynaptically located metabotropic glutamate receptors such as mGluR5. This event triggers $G_{q/11}$ signaling and then PLCβ activity (**4**), both of which are also located at the perisynaptic zone integrated into the perisynaptic machinery (PSM) adjacent to the postsynaptic density (PSD) that contains ionotropic receptors. PLCβ splits PIP2, and when a larger amount of DAG is produced, perisynaptically accumulated DAGLα, converts DAG to 2-AG. This then travels backwards through the synapse (**5**) and mediates feedback inhibition of glutamate release via activation of presynaptic CB1Rs and subsequent closure of voltage-gated calcium channels (**6**). The whole process is tightly regulated by MAGL, which inactivates 2-AG by catalyzing its hydrolysis into AA and glycerol. (**B**) Simple representation of two plastic processes mediated by CB1Rs and its main molecular effectors: short- (STD) and long-term (LTD) synaptic depression. STD is mediated by βγ-dependent inhibition of VGCC while LTD requires $G\alpha_{i/o}$-dependent inhibition of AC (thus PKA). Both phenomenons lead to decreased levels of neurotransmitter released to the cleft. Adapted from Castillo, 2012 and Katona, 2015.

Similarly, there is copious evidence that the ECS controls an endless repertoire of physiological and behavioral manifestations. It is crucial for memory (Puighermanal et al. 2009) or cognitive (Puighermanal et al. 2012) and motor learning (Kishimoto & Kano 2006); extinction of aversive memories (Marsicano et al. 2002); tuning of stress and anxiety (Jenniches et al. 2014; Morena et al. 2016); protection against excitotoxicity (Marsicano et al. 2003; Monory et al. 2006; Bravo-Ferrer et al. 2016) and neurodegeneration (Fernández-Ruiz et al. 2015); control of nociception –in the periphery, along with CB2R (Cravatt & Lichtman 2004)–; energy balance (Quarta et al. 2010); or feeding behavior (Bellocchio et al. 2010; Soria-Gómez et al. 2014), to denote a few (Figure 19).

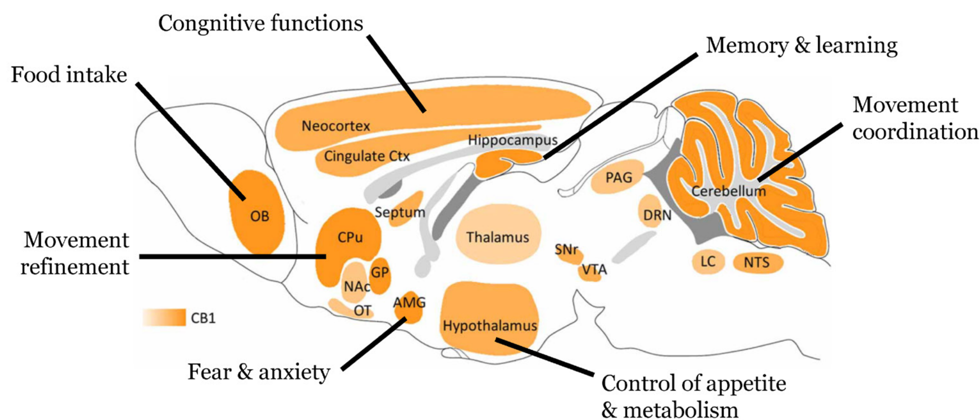

Figure 19. Some important physiological functions controlled by CB1R present in the mammalian brain. Given the broad distribution of CB1Rs and its largely pleiotropic signaling, there is a vast array of physiological functions regulated by CB1Rs present in the brain. Adapted from Flores *et al.*, 2013.

4.3. The ECS in mammalian brain development

The ECS controls embryonic development even before it begins, since it has been shown to regulate sperm cell motility (Agirregoitia et al. 2010) and oocyte maturation (Agirregoitia et al. 2015). Later, anandamide plays a key role in the fertilization (Harkany et al. 2007), periimplantation biology (Sun & Dey 2008) and subsequent early development (Galve-Roperh et al. 2013), and THC has been shown to disrupt neurulation in chick (Psychoyos et al. 2008). After the first published evidences on the presence of CB1R during human (Mailleux & Vanderhaeghen 1992; Glass et al. 1997) and rat (McLaughlin & Abood 1993) development, the profuse work accomplished by the group of Fernández-Ruiz and Ramos in the Complutense University, substantially contributed to characterize the expression, distribution and functionality of many components of the ECS throughout mammalian brain development (Berrendero et al. 1998; Berrendero et al. 1999; Fernandez-Ruiz et al. 2000) (Figure 20). The high levels of 2-AG (comparable to those in the adult brain) and the atypical CB1R protein distribution –exclusively localized to the white matter (WM), which lacks detectable *CB1* expression– during the last days of gestation (Romero et al. 1997), led to hypothesize a different, developmental-specific role of the ECS. In subsequent years, several papers came out describing strikingly diverse actions of the ECS –ranging from the control of neural progenitors to neuronal specification or morphogenesis– that prompt the emergence of a new developmental research field. For extensive review see (Harkany et al. 2007; Galve-Roperh et al. 2013; Maccarrone et al. 2014).

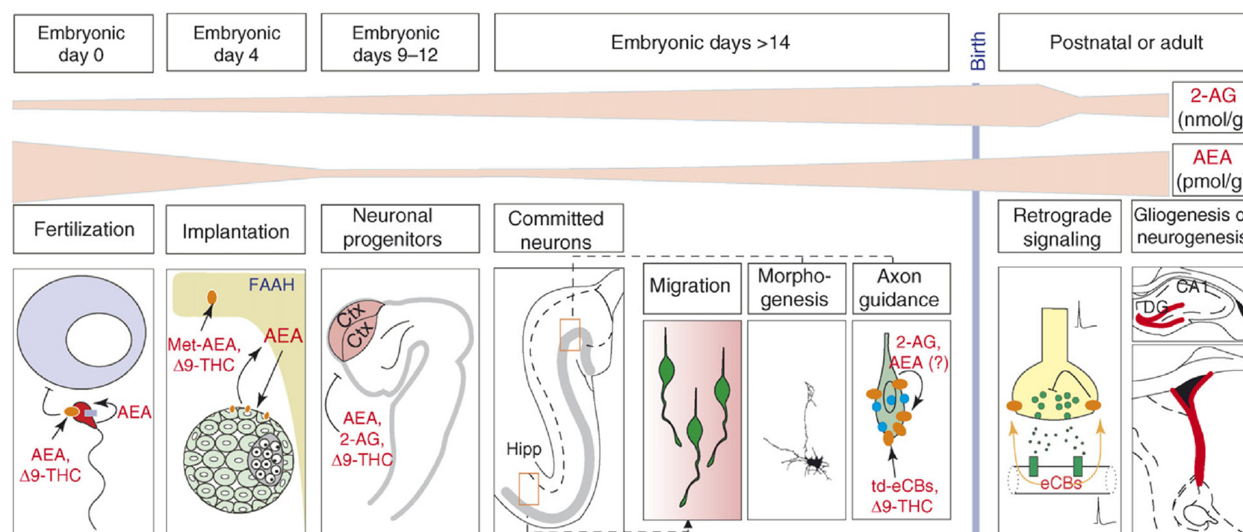

Figure 20. Major developmental roles of the endocannabinoid system along the ontogeny. Top panel shows temporal changes in available quantities of the two major endocannabinoids, 2- AG and anandamide, at the indicated developmental stages. Concentrations of 2-AG generally exceed those of anandamide in the developing brain. Bottom panels illustrate the major events of embryogenesis that are regulated by endocannabinoid signaling through CB1Rs (in orange).The actions of the endocannabinoids and those processes potentially affected by prenatal exposure to THC are shown. The term 'td-eCBs' refers to target-derived endocannabinoids, potentially released from putative postsynaptic target cells during axon guidance. From Harkany *et al.*, 2007.

4.3.1. Role of the ECS in neural progenitor proliferation

The first evidences of the role of the ECS in the control of neural progenitor proliferation and neurogenesis came up more than 10 years ago, first in *in vitro* systems (Rueda et al. 2002), then in progenitors of the adult brain *in vivo* (Jin et al. 2004) –where CB2Rs also play an important role (Goncalves et al. 2008; Palazuelos et al. 2012; Bravo-Ferrer et al. 2016)–, and afterwards in embryonic neural progenitors (Aguado et al. 2005). In the latter study, it is shown that neural progenitors have the ability to produce both anandamide and 2-AG upon stimulation, raising the possibility of auto- and paracrine eCB signaling in neural progenitors.

Despite the above-mentioned functional read-outs, a precise characterization of *CB1* expression in embryonic cortical progenitors remained elusive given its low levels of expression compared to postmitotic cells, and some existent methodological limitations (e.g. unspecificity of commercially available antibodies)(Morozov et al. 2013). Nevertheless, few years ago –taking advantage of newly developed genetic models– we published an exhaustive and reliable determination of *CB1* expression across different compartments, proving evidence that CB1Rs are present, albeit at low levels, in proliferative niches of the embryonic cortex (Díaz-Alonso et al. 2014) (Figure 21A-C).

Engagement of CB1Rs has been proven to be positively coupled to neural progenitor proliferation (Aguado et al. 2005; Mulder et al. 2008), in normal conditions, and after an excitotoxic insult (Aguado et al. 2007). Consequently, *CB1* knockout mice (*CB1*$^{-/-}$) display impaired neural progenitor proliferation in the embryonic VZ/SVZ (Mulder et al. 2008; Díaz-Alonso et al. 2014) as well as in

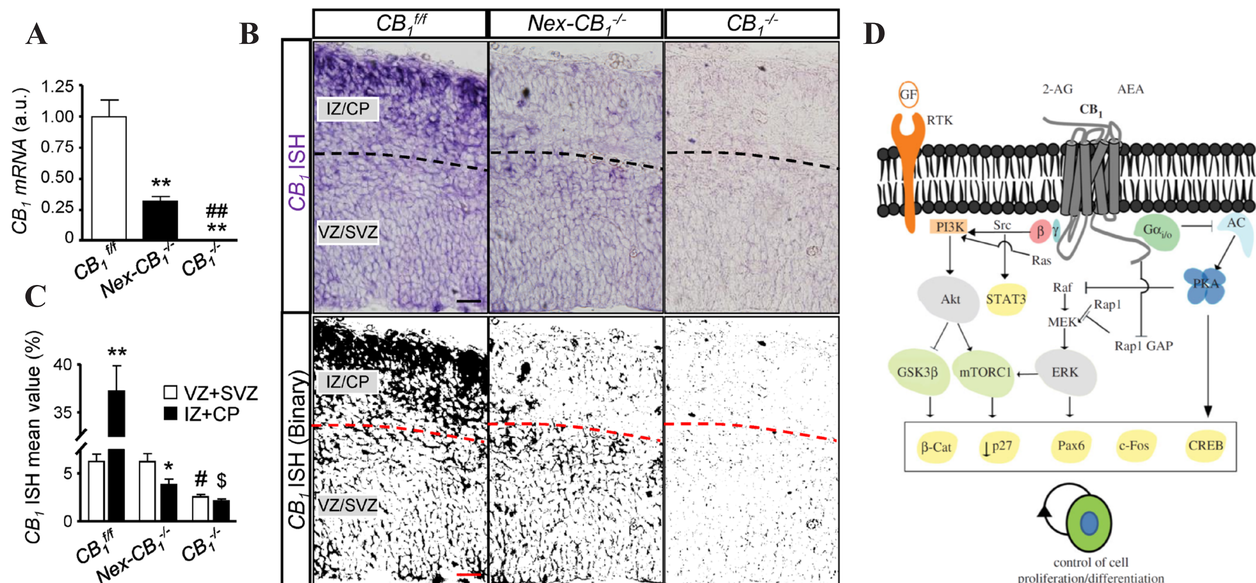

Figure 21. Expression and signaling pathways triggered by CB1Rs in embryonic cortical progenitors. *CB1* is expressed, albeit at low levels, in proliferative areas of the developing mouse cortex. (**A**) *CB1 mRNA* (quantified by qPCR) in E13.5 cortical extracts of *CB1*$^{f/f}$, *Nex-CB1*$^{-/-}$, and *CB1*$^{-/-}$ embryos demonstrate a low, but signifficant, *CB1* expression in cortical progenitors. (**B**, **C**) Representative raw and binary ISH images of the same genotypes at E14.5. Semiquantitative analysis of ISH signal shows the relative presence of *CB1* transcripts in preeminently proliferative (VZ+SVZ) and postmitotic (IZ/CP) compartments. (**D**) Principal signaling pathways activated by CB1R engagement in neural progenitors. Activation of PI3K/Akt/mTORC1 and MAPKs or AC inhibition converge in the regulation of some molecular effectors (β-catenin, Pax6 or CREB) which in turn tune cortical progenitor proliferation and cell fate. Adapted from Díaz-Alonso et al., 2012b and Díaz-Alonso *et al.* , 2014.

the adult SVZ/SEZ and SGZ (Jin et al. 2004). In contrast *FAAH* deficient mice (*FAAH*$^{-/-}$) –with increased eCB tone– present the opposite phenotype (Aguado et al. 2005; Mulder et al. 2008).

The intracellular signaling cascades triggered by CB1Rs in neural progenitors are diverse (e.g. inhibition of PKA and activation of MAPKs or PI3K/Akt) and converge in the modulation of cell cycle regulators, determinants of neural progenitor's fate or master transcriptional regulators, as for instance β-catenin, cAMP-response element binding (CREB) or Pax6 (Diaz-Alonso et al. 2012) (Figure 21D). As a remarkable example, we have described a key role of CB1Rs in the transition from apical (Pax6^{+}) to basal (Tbr2^{+}) cortical progenitors in a mechanism that involves the expression of *Tbr2/eomes* under the activation of the mTORC1/Pax6 signaling axis (Díaz-Alonso et al. 2014).

4.3.2. Role of the ECS in neuronal migration

The ECS, particularly the CB1R, has been involved in the regulation of the process of neuronal migration in different cellular contexts. Leaving apart the inconsistency of some results, the engagement of CB1Rs –by transactivating the TK receptor TrkB– has been shown to be positively coupled to the migration rate of CB1R^{+}/CCK-containing interneurons (Berghuis et al. 2005). Accordingly, chronic prenatal THC administration increases the number of CCK^{+} interneurons in the dentate gyrus of the hippocampus. In contrast to these results, prenatal treatment with THC diminishes the number of 5-HT_{3A}^{+}/CCK^{+} hippocampal interneurons (Vargish et al. 2016), and reduces the amount of CB1R+ basket cell innervation in the CA1 pyramidal layer (Tortoriello et al. 2014). In agreement, embryonic exposure to WIN55,212-2, seems to disrupt tangential migration (Saez et al. 2014), although the molecular mechanisms controlling all the above effects are still open questions.

Alternatively, eCB signaling has been shown to affect the migration of SVZ/SEZ-derived neuroblasts toward the olfactory bulb (Oudin et al. 2011), in a mechanism that seems to involve PKC-dependent phosphorylation of fascin to control the actin cytoskeleton (Sonego et al. 2013).

4.3.3. Role of the ECS in cell-fate specification

The ECS participates in the process of cell-fate acquisition from early stages of development, from the above-mentioned role in the transition between cortical progenitor populations to promote astroglial differentiation –through the expression of astroglial markers as GFAP (Aguado et al. 2006) or activation of CREB (Soltys et al. 2010)– as well as neuronal differentiation and maturation. Remarkably, CB1 exerts a crucial role in the proper specification and maturation of long-range pyramidal neurons (Mulder et al. 2008), particularly, in the CSMN cell-fate acquisition (Díaz-Alonso et al. 2012a). In the latter study, Díaz-Alonso et al. describe a sophisticated mechanism whereby CB1R signaling in newborn pyramidal neurons favors Ctip2 transcriptional activation and proper specification of CSMNs. Accordingly, conditional knockout mice deficient in CB1 selectively in postmitotic cells display increased numbers of Satb2+ cells, decrease CSMNs

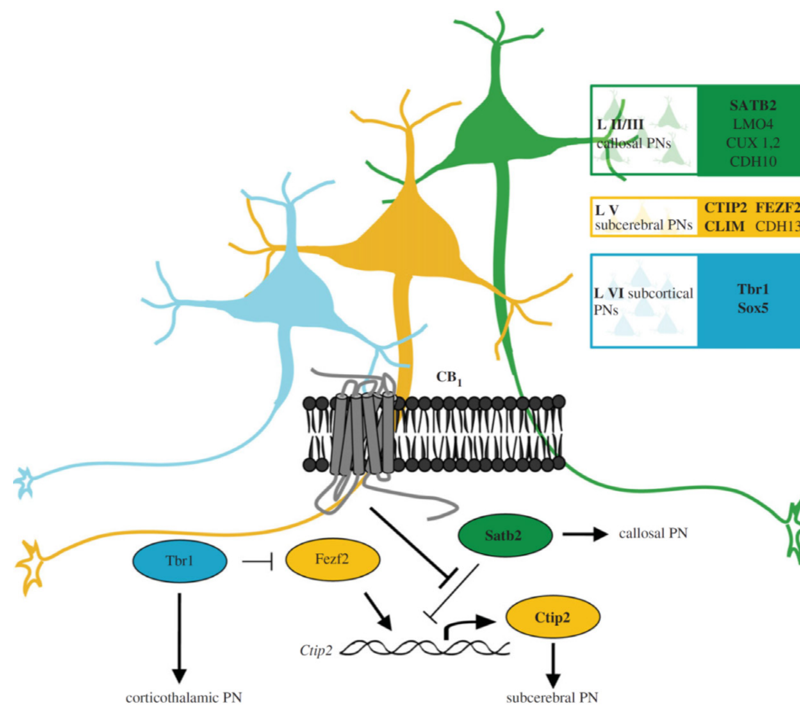

Figure 22. CB1 cannabinoid receptor signalling and CSMN specification. CB1R activity in differentiating cortical neurons is coupled to the modulation of the neurogenic transcriptional factor axis Ctip2/Satb2. CB1R signaling is positively coupled to Ctip2 transcriptional function and negatively to Satb2-mediated repression of Ctip2. Thus, *CB1* tunes the transcriptional neurogenic programme responsible for upper and lower cortical neuron differentiation. Transcription factors involved in cortical laminar specification regulated by CB1 receptor are indicated in bold letters. From Díaz-Alonso *et al.* 2012b.

and present evident deficits in some behavioral tests aimed at assessing the CSMN-involved skilled motor function (i.e. skilled reaching and staircase tests)(Díaz-Alonso et al. 2012a) (Figure 22).

4.3.4. Role of the ECS in neuronal morphogenesis

The ECS affects several aspects of neuronal morphogenesis. First, activation of CB1Rs has been shown to trigger neurite outgrowth (Jordan et al. 2005), though it also has been reported an opposite effect (Berghuis et al. 2005; Vitalis et al. 2008). In particular, attending to the axon, CB1Rs control the specialization and molecular composition of the axon initial segment (Tapia et al. 2017), while results regarding axonal outgrowth are contradictory (Williams et al. 2003; Berghuis et al. 2005). However, it is clearer that CB1R signaling is coupled to axonal growth cone collapse (P. Berghuis et al. 2007; Argaw et al. 2011) with important implications for axonal pathfinding and final brain wiring (P. Berghuis et al. 2007). Furthermore, it has been suggested that eCBs may act as auto- and paracrine chemotactic cues, with an astringent spatiotemporal control by metabolic enzymes (Keimpema et al. 2010). In agreement, dysfunction of CB1Rs – either by genetic or pharmacological disruption– during development leads to remarkable fasciculation deficits (Mulder et al. 2008; Wu et al. 2010; Tortoriello et al. 2014), although a plausible involvement of CB2Rs present in oligodendrocytes has also been suggested (Alpár et al. 2014).

4.4. Prenatal exposure to cannabinoids

Cannabis is, by far, the most widely used illicit drug among pregnant women, especially in Western countries. Notably, taking into consideration the lipophilic nature of cannabinoids –what facilitates cross-placental transfer and blood-brain barrier traversing (Hurd et al. 2005)– and the crucial functions endured by the ECS during embryonic development made of it an issue of outstanding relevance.

The aforementioned developmental alterations caused by ECS malfunction might be responsible of some of the functional impairments associated to prenatal cannabinoid exposure (Jutras-Aswad et al. 2009; Keimpema et al. 2011; Calvigioni et al. 2014; Volkow et al. 2014). Additionally, it is noteworthy the ability of developmental cannabinoid administration to induce long-lasting –even cross-generational– epigenetic modifications (Szutorisz & Hurd 2016), which may be relevant for drug-seeking behaviors in the offspring (Szutorisz et al. 2016). There is profuse literature about the functional consequences of maternal cannabis use on the offspring, which include cognitive impairments (Huizink 2014) and increased susceptibility to develop neuropsychiatric disorders such as schizophrenia, depression or anxiety (Jutras-Aswad et al. 2009; Mechoulam & Parker 2011; Volkow et al. 2014). Nonetheless, it is important to note that many human longitudinal studies present potential confounding factors (further discussed in the Discussion section), and that most of our knowledge on the consequences of prenatal cannabinoid exposure comes from animal models (Schneider 2009). Anyhow, whether embryonic cannabinoid administration evokes an overactivation of the ECS or, in contrast, its dysfunction, as well as the precise cellular and molecular substrates responsible and the long-term functional outcome of prenatal cannabinoid exposure remain largely understood.

AIMS OF THIS THESIS

The CB1R is the most abundant GPCR in mammalian brain, with widespread distribution and remarkable pleiotropic actions depending on the cellular context. Previous findings from our group contributed to delineate a key role of CB1R signaling in the proliferation and fate-acquisition of cortical progenitors, as well as in the migration, CSMN specification, axonal navigation and morphogenesis of cortical PNs. However, the precise cellular and molecular mechanisms underlying the developmental role of the ECS remain to be fully explored. In addition, a precise characterization of the long-lasting neurobiological impact of developmental ECS malfunction in the adult progeny –caused by genetic variations or environmental insults such as prenatal marijuana exposure– is a major concern.

In this context, we defined the following Aims for this Doctoral Thesis:

Aim 1. To investigate the role of the ECS in the process of radial migration of newborn pyramidal neurons during cortical development and to evaluate long-lasting functional alterations derived of developmentally-restricted CB1R dysfunction.

Aim 2. To elucidate the neurobiological substrate of Δ^9-THC actions during embryonic cortical development. Particularly, to explore the impact of prenatal administration of this phytocannabinoid on neuronal differentiation, with special attention to CSMN specification.

MATERIALS & METHODS

Constructs

pCIG2-DCX-CRE-IRES-EGFP expression vector, *pCAG-DAGLα-GFP-V5* expression vector, *pCAG-Cofilin*^{S3A} expression vector and *CB1 in situ* hybridization (ISH) probes (sense and antisense) were kindly provided by Dr. Ulrich Müller (Scripps Research Institute, La Jolla, CA), Dr. Patrick Doherty (Wolfson Age Research Center, London, UK), Dr. François Guillemot (MRC National Institute for Medical Research, Mill Hill, London) and Prof. Beat Lutz (Johannes Gutemberg University, Mainz, Germany), respectively.

Animals

Experimental designs and procedures were approved by the Complutense University Animal Research Committee in accordance with the European Commission regulations. All efforts were made to minimize the number of animals and their suffering throughout the experiments. *CB1*$^{-/-}$, *Nex-CB1*$^{-/-}$, *Dlx5/6-CB1*$^{-/-}$ and CB1R-rescue colony-founding mice were provided by Prof. Beat Lutz (Johannes Gutenberg University, Mainz, Germany). Mice were maintained in standard conditions, keeping littermates grouped in breeding cages, at a constant temperature (20 ± 2 °C) on a 12 h light/dark cycle with food and water *ad libitum*. The generation and genotyping of *CB1*$^{-/-}$, *Nex-CB1*$^{-/-}$ and *CB1*$^{f/f}$ littermates has been reported elsewhere, and was performed accordingly (Monory et al. 2007). Mouse embryonic tissues were obtained upon timed mating as assessed by vaginal plug observation (E0.5).
Rescue of CB1R expression in *Stop-CB1* mice was performed as described by using *Nex-Cre* (Ruehle et al. 2013), *Dlx5/6-Cre* (Monory et al. 2006), and *EIIa-Cre*–driven recombination (Ruehle et al. 2013). Immunohistological characterization of different CB1R-rescue mice was performed in P2.5 and P20 brain samples. Corticospinal motor function as well as seizure susceptibility were assessed in THC- and vehicle-treated 2 month-old CB1R-rescue mice.

THC administration

THC (≥99% HPLC; THC Pharm) was diluted in 0.9% NaCl (saline) solution containing 3% (vol/vol) DMSO and 2% (vol/vol) Tween-80 and administered intraperitoneally (i.p.) at a final dose of 3 mg/kg to pregnant females for 5 consecutive days, from E12.5 to E16.5. Control mice were injected with vehicle solution.

Immunofluorescence and confocal microscopy

Cell proliferation was determined after i.p. bromo-deoxyuridine (BrdU) injection (50 μg/g body weight) of pregnant females at E14.5. Coronal embryonic and postnatal brain slices (14 and 30 μm-thick, respectively) were processed as previously described (Díaz-Alonso et al. 2012). Cortical layers were identified by their discrete cell densities as visualized by DAPI counterstaining.

Immunofluorescence was performed, after blockade with 5% (vol/vol) goat serum in PBS 0.25% (vol/vol) Triton X-100, by overnight incubation at 4°C with the indicated primary antibodies: anti-CB1R (1:500; Af530-1/Af380-1; Frontier Institute), anti-ER81 (1:500; ab81086; Abcam), anti-VGLUT1 (1:1000; 135303C3; Synaptic Systems), anti-VGAT (1:1000; 131003; Synaptic Systems), anti-Pax6 (1:200; ab2237; Millipore), anti-Tuj1 (1:2000; T8660; Sigma-Aldrich), anti-Satb2 (1:200; ab51502; Abcam), anti-GFP (1:2000; 4745-1051; AbD Serotec), anti-RhoA (1:1000; ARH03; Cytoskeleton), anti-cleaved caspase-3 (1:500; #9661; Cell Signaling), anti-GFAP-Cy3 (1:2000; C9205; Sigma-Aldrich), anti-Ki67 (1:1000; RM-9106; Thermo Scientific), anti-Cre (1:500; 69050-3; Millipore), anti-PCNA (1:1000; sc-56; Santa Cruz) and anti-BrdU (1:200; ab6326; Abcam). Samples were subsequently incubated for 1.5 h at room temperature with the appropriate highly cross-adsorbed AlexaFluor secondary antibodies (Invitrogen). Confocal fluorescence images were acquired by using a Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with 2 passes by Kalman filter and a 1024x1024 collection box. Immunofluorescence of cortical sections was performed, and labeled cells were quantified in a 300 μm-wide cortical column of equivalent sections from the mediolateral area of the motor/somatosensory cortex. At least five independent cortical columns were analyzed per mice. Immunofluorescence data were obtained in a blinded manner.

In *CB1* knockdown experiments by *in utero* electroporation, cell migration was measured by assessing the position of GFP-positive cells along the cortical wall, assigning each cell to the corresponding cortical compartment attending to histological criteria defined by DAPI staining.

RhoA immunoreactivity quantification in primary cortical culture experiments was performed with ImageJ software, as previously described (Díaz-Alonso et al. 2012). Targeted cells were identified and their contour was delimited by GFP fluorescence. The number and length of primary neurites was calculated with the NeuronJ plugin of ImageJ.

In utero electroporation (IUE)

The indicated siRNAs or expression constructs were electroporated at a final concentration of 10 μM or 1 μg/μl, respectively. The siRNA/DNA solution was mixed with Fast Green (0.1 mg/ml; Sigma-Aldrich) and ~1 μl of the solution was injected into the lateral ventricle of E13.5 or E14.5 embryos as described previously (Borrell et al. 2005). Unless otherwise is stated, all electroporations shown include a constitutive GFP overexpression plasmid (*pCAG-EGFP*) to allow proper visualization. For *CB1* knockdown experiments, the small interfering RNA (siRNA) SMARTpool ON-TARGETplus *Cnr1* siRNA (target sequences: 5'-GGUAGUCCCUUCCAAGAAA-3'; 5'-CCACAGAAAUUCCCUCUAA-3'; 5'-GGGAAGAUGAACAAGCUUA-3'; 5'-GUGUUUGCCUUCUGUAGUA-3') or control (scrambled) siRNA (Thermo Scientific), and mouse *Cnr1* short hairpin RNA (sh*CB1*; 5'-CTGTTAAGATCGCCAAGGTGACCATGTCT-3') or control (scrambled) shRNA (Origene) were used. For *CB1* ablation in *CB1*$^{f/f}$ embryos, the following *Cre* expression vectors were employed:

pCAG-CRE-GFP (Addgene) and *pCIG2-DCX-CRE-IRES-EGFP* (Franco et al. 2011). Additionally, some experiments involved the coexpression of either an shRNA directed against *RhoA mRNA* (sh*RhoA*; 5'- CAAGAAGGACCTTCGGAATGACGAGCACA-3'; Origene) or a *pCAG-Cofilin*^{S3A} construct (Pacary et al. 2011). *In utero* electroporated embryos were analyzed at E16.5, E17.5, P2, P10 or P60, as indicated.

CSMN retrograde labeling

Deeply anesthetized mice were injected with 0.5 µL of red fluorescent microspheres (Retrobeads; Lumafluor Inc.) into the dorsal funiculus of the cervical spinal cord at P10 and perfused at P15. Brains were sectioned coronally at 30 µm, and CSMNs in a 300 µm-wide cortical column of the sensorimotor and lateral sensory cortex were counted on every sixth section, across the entire rostrocaudal extent of the cortex, and referred to a 1 mm-wide cortical column.

Behavioral determinations of skilled motor function

THC or vehicle-exposed *CB1*$^{-/-}$ and *CB1*$^{+/-}$ littermates (10 week of age) were tested for skilled-reaching and staircase tests as previously described (Díaz-Alonso et al. 2012). Control determinations, including the number of trials and the success ratio in unskilled conditions (*i.e.*, the ability to retrieve a pellet at a tongue-reaching distance), were performed. All tests were video-recorded for subsequent analysis and blind quantification. Results shown correspond to the average of two trials for each test. Additional characterization of general motor activity and exploration was performed with ActiTrack (Panlab), which evidenced an absence of major impairments in global motor function.

Pentylenetetrazole (PTZ)-induced seizures assay

PTZ (Sigma-Aldrich) was dissolved in 0.9% saline and administered i.p. to mice at P60 at a concentration of 22.5 mg/kg (at 10 µl/g) every 10 min until generalized seizures occurred. Mice were placed in Plexiglas cages and injected every 10 min, by an experimenter blinded to their treatment and genotype, until generalized seizures occurred, what was considered the end of the experiment. All the procedure was video-recorded and analyzed later by an experimenter blinded to the experimental groups, who determined the precise moment of generalized seizure onset. There was no statistically significant difference in weight or sex ratio between the different groups of mice.

Cortical explant migration assay

Cortical explants were prepared from E14.5 embryonic forebrain slices prepared as previously described (Mulder et al. 2008). Particularly, we started from 250 µm-thick embryonic brain slices, from which cortical fragments of ~300 µm diameter were dissected with scalpel and incubated

for 1 h in Eagle's Minimal Essential Medium (EMEM; Lonza) containing 10% FBS, 1% glucose at 37 °C in 5% CO2. COS7 cell aggregates expressing GFP-alone, or together with *pCAG-DAGLα-GFP-V5* or *pCMV-NAPE-PLD* (Origene) expressing plasmids, were prepared by diluting a pellet of transfected cells with Matrigel (BD) in a 1:1 proportion. After jellification, cell aggregates were cut with a scalpel in small cubes of ~600 μm long. Subsequently, cortical explants were placed in a Matrigel three-dimensional matrix facing corresponding COS7-transfected cell aggregates. Cocultures were maintained in Neurobasal culture medium (Gibco), supplemented with N2 (Millipore) and B-27 (Invitrogen) for 72 h, and then fixed. Cell migration from the explants was analyzed in 4 quadrants by quantification of cell nuclei counterstained with DAPI, and the proximal/distal (P/D) ratio with respect to the corresponding cell aggregate was calculated.

In situ hybridization (ISH)

Coronal sections (20 μm) of E16.5 and E17.5 embryonic mouse brains were obtained and processed for ISH as described (Marsicano & Lutz 1999). Antisense and sense *Cnr1 (CB1)* riboprobes were a kind gift from Prof. Beat Lutz (Johannes Gutenberg University, Mainz, Germany). In some cases, *CB1* ISH was followed by immunofluorescence of GFP or Satb2 protein in the same samples.

Binding assay of radioactively-labeled ligand

Binding analysis was performed as described (Ruehle et al. 2013). Briefly, perinatal sections were incubated for 3 h at 30 °C in 50 mM Tris·HCl pH 7.4, containing 5% (wt/vol) defatted BSA and 5 nM of the CB1R synthetic agonist [^{3}H]CP55,940 (124 Ci/mmol; Perkin-Elmer). Nonspecific binding was determined by incubating adjacent sections in the presence of 10 μM cold CP55,940 (Tocris Bioscience). After incubation, sections were washed, briefly dipped in distilled water, and dried overnight. Tritium-sensitive phosphor screens were exposed to slides for 3.5 d and scanned using a Cyclone Plus Storage Phosphor System (Perkin-Elmer). Ligand binding to CB1R was quantified from the standard curve compiled by using a tritium standard (American Radiolabeled Chemicals) and Optiquant software (Perkin-Elmer). A minimum of four sections per mice were quantified, and after subtraction of nonspecific labeling, the average density was calculated for each animal. Mean values for each condition were expressed relative to those from vehicle-treated animals.

Adherent cortical progenitor cell (CPC) culture

Primary CPC cultures were obtained from wild-type E14.5 embryos. Dorsal telencephalic tissue was dissected, subjected to mild enzymatic digestion with Accutase (Sigma-Aldrich), mechanically homogenized and grown in laminin-coated flasks containing Euromed medium (Euroclone) supplemented with 0.1% BSA (Sigma-Aldrich), 1% N2, laminin (2 μg/ml; Sigma-Aldrich), EGF and FGF (20 ng/ml; Gibco). Modulation of RhoA levels by CB1R signaling was assessed by treating

differentiating CPCs with the CB1R synthetic agonist HU-210 (50 nM; Tocris) with or without the CB1R inverse agonist SR141716 (1 μM; Sanofi-Aventis) or the proteasome inhibitor MG-132 (10 μM; Sigma-Aldrich) for 6 hours. Differentiation was achieved by switching to Neurobasal medium supplemented with B-27 and N2. Adherent CPC cultures were characterized by qPCR and immunofluorescence analyses (Fig. S1.5). Transcript levels of *CB1*, nestin and *RhoA* were quantified in proliferating and differentiating conditions. Immunodetection of Pax6 and Tuj-1 was used to assess RGC identity and postmitotic neurons, respectively.

RhoA activity measurement

Dorsal telencephali from E17.5 *Nex-CB1*$^{-/-}$ and *CB1*$^{f/f}$ littermates were carefully dissected and directly processed for active RhoA quantification with the G-LISA kit (Cytoskeleton Inc.), following manufacturer instructions.

Immunoblot assays

Embryonic cortical tissue, collected at E17.5 or P2.5, and CPC protein extracts were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitors. Equal amounts of protein samples were electrophoretically separated and transferred to PVDF membranes. After blocking with nonfat dry milk, membranes were incubated overnight at 4 °C with anti-CB1R (1:1000; Af380-1; Frontier Institute), anti-RhoA (1:1000; ARH03; Cytoskeleton), anti-RhoB (1:1000; #2098; Cell Signaling), anti-β-actin (1:5000; A5441; Sigma) or anti-α-tubulin (1:5000; T9026; Sigma) primary antibodies. Subsequently, membranes were incubated with the corresponding secondary antibodies coupled to horseradish peroxidase for 1.5 h at room temperature. The optical density of the relevant immunoreactive bands was quantified with the gel quantification plugin of ImageJ software and normalized to those of α-tubulin or β-actin for the corresponding samples in the same membranes.

Quantitative PCR

RNA was isolated using RNeasy Plus kit (Quiagen). cDNA was obtained with Transcriptor (Roche). Real-time quantitative PCR (qPCR) assays were performed using the FastStart Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library Set (Roche). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA and β-actin levels as reference.

Statistical analysis

All variables were first tested for normality (Kolmogorov–Smirnov) and homoscedasticity (Levene's). When variables satisfied these conditions, one-way ANOVA and LSD Fisher *post hoc*

tests were used to assess differences between groups. In other cases, the differences were analyzed by nonparametric (Kruskal–Wallis) tests, and *post hoc* comparisons by means of Mann–Whitney Wilcoxon test were used to assess differences between groups. Significance level was set below 0.05 in all cases. Results are shown as mean ± SEM, and the number of experiments is indicated in every case. All analyses were carried out using Statgraphics Centurion XVII software (Statpoint Technologies Inc.).

RESULTS

AIM 1: SUMMARY

Proper radial migration of PNs is essential as it establishes the basis for the subsequent wiring of the cortical circuitry. Not surprisingly, a wide spectrum of brain abnormalities emerge as a consequence of disrupted neuronal migration, which can have devastating consequences on adult brain function, including mental retardation, cognitive disorders and epilepsy.

The CB1R is expressed in the developing cerebral cortex, where it controls the proliferation and phenotype of cortical neural precursor cells (Díaz-Alonso et al. 2014), the specification of pyramidal neurons (Díaz-Alonso et al. 2012), and axon guidance and synaptogenesis (Mulder et al. 2008; Vitalis et al. 2008; Keimpema et al. 2010; Argaw et al. 2011). In addition, the CB1R regulates neuronal migration in the embryonic brain (Mulder et al. 2008), and eCB signaling promotes migration of newborn neurons along the RMS in the postnatal mouse brain (Oudin et al. 2011). Deciphering how CB1Rs signal in the developing brain is critical for understanding the neurobiological processes affected by developmental CB1R malfunctioning.

In this study, we performed transient siRNA-mediated *CB1* knockdown in newborn pyramidal neurons to reveal the impact of developmentally restricted, short-term *CB1* loss of function on radial migration and potential functional deficits in adulthood. We observed a migration arrest that led to profound and long-lasting alterations in cortical neuron positioning, the formation of neuronal heterotopias and increased seizure susceptibility in adult mice. Biochemical and cellular analyses showed that loss of CB1R function led to abnormal RhoA accumulation in newborn pyramidal neurons, thereby disrupting the morphology of migrating cells. Remarkably, migration deficits elicited by CB1R dysfunction were fully rescued by RhoA knockdown. Collectively, our findings pave the way toward a better understanding of the physiological role of the ECS in brain development, and provide relevant molecular mechanistic insights into human MCDs caused by altered neuronal migration.

AIM1. ROLE OF THE ECS ON PYRAMIDAL NEURON RADIAL MIGRATION AND LONG-LASTING CONSEQUENCES OF DEVELOPMENTAL CB1R DYSFUNCTION

R1.1. Acute *CB1* knockdown blocks radial migration

To evaluate the role of *CB1* in neuronal migration we initially confirmed its expression in the developing cortex at E17.5. Both CB1R *mRNA* and protein show a gradient with increased levels in postmitotic compartments of the developing cortex, *i.e.* the IZ and the CP (Fig. R1.1A-C), suggesting that *CB1* expression is upregulated in newborn pyramidal neurons after cell cycle exit and radial migration initiation (Díaz-Alonso et al. 2014). We also explored the presence of CB1Rs in developing pyramidal neurons by combining *CB1* ISH with Satb2 immunofluorescence (Fig. R1.1A, right panel). Confocal analysis further confirmed CB1R protein expression in immature Satb2^{+} pyramidal neurons in E17.5 embryonic cortices, a large proportion of which are undergoing radial migration at this developmental stage (Fig. R1.1C). In brain development, CB1R protein is enriched in axons of immature PNs (Fig. R1.1C) (Berrendero et al. 1999; Mulder et al. 2008), making extremely difficult to estimate the contribution of somatic CB1R immunostaining in migrating neurons to the overall staining. Thus, to recapitulate *CB1* expression in migrating pyramidal neurons, we used ISH of *CB1* transcripts in GFP-positive cells in E16.5 embryonic cortices subjected to IUE at E14.5 (Fig. R1.1B). As developing GABAergic interneurons also express CB1Rs (Paul Berghuis et al. 2007; Morozov et al. 2009), to unequivocally assess the presence of CB1Rs in pyramidal neurons we took advantage of the *Dlx5/6*-driven forebrain GABAergic neuron-selective *CB1*-deficient mice (Monory et al. 2007). Thus, in *Dlx5/6-CB1*$^{-/-}$ embryonic cortices, CB1R immunostaining was hardly distinguishable from their *CB1*$^{f/f}$ littermates, while in the developing hippocampus a clear reduction of CB1Rs was evident (Fig. R1.1D). Overall, these results confirm the expression of *CB1* in radially migrating pyramidal neurons, in addition to its presence in developing interneurons.

We then assessed the cell-autonomous role of CB1R signaling in PN migration during cortical development. To this end, we acutely knocked-down *CB1* in radially-migrating neurons at E14.5 by IUE of a pool of siRNAs directed against 4 sequences of *CB1 mRNA* (hereafter si*CB1*) together with a GFP expression construct, and subsequently analyzed the distribution of radially-migrating cells at different time points. In our hands, transfection of cortical cells with this siRNA pool reduces *CB1* expression below 40% (Díaz-Alonso et al. 2014). *CB1* knockdown significantly impaired newborn pyramidal cell migration (Fig. R1.2A-C). Remarkably, after either 2 or 3 DIU (Fig. S1.1A, B and Fig. R1.2A,B, respectively), we observed a reduced colonization of the CP by *CB1*-deficient cells that, instead, appeared stacked in the IZ and the VZ/SVZ. Moreover, when we restricted our analyses to the intracortical migration (*i.e.* cells within the CP), we also found a significant delay in si*CB1*-electroporated cells, that were less abundant in upper cortical layers of

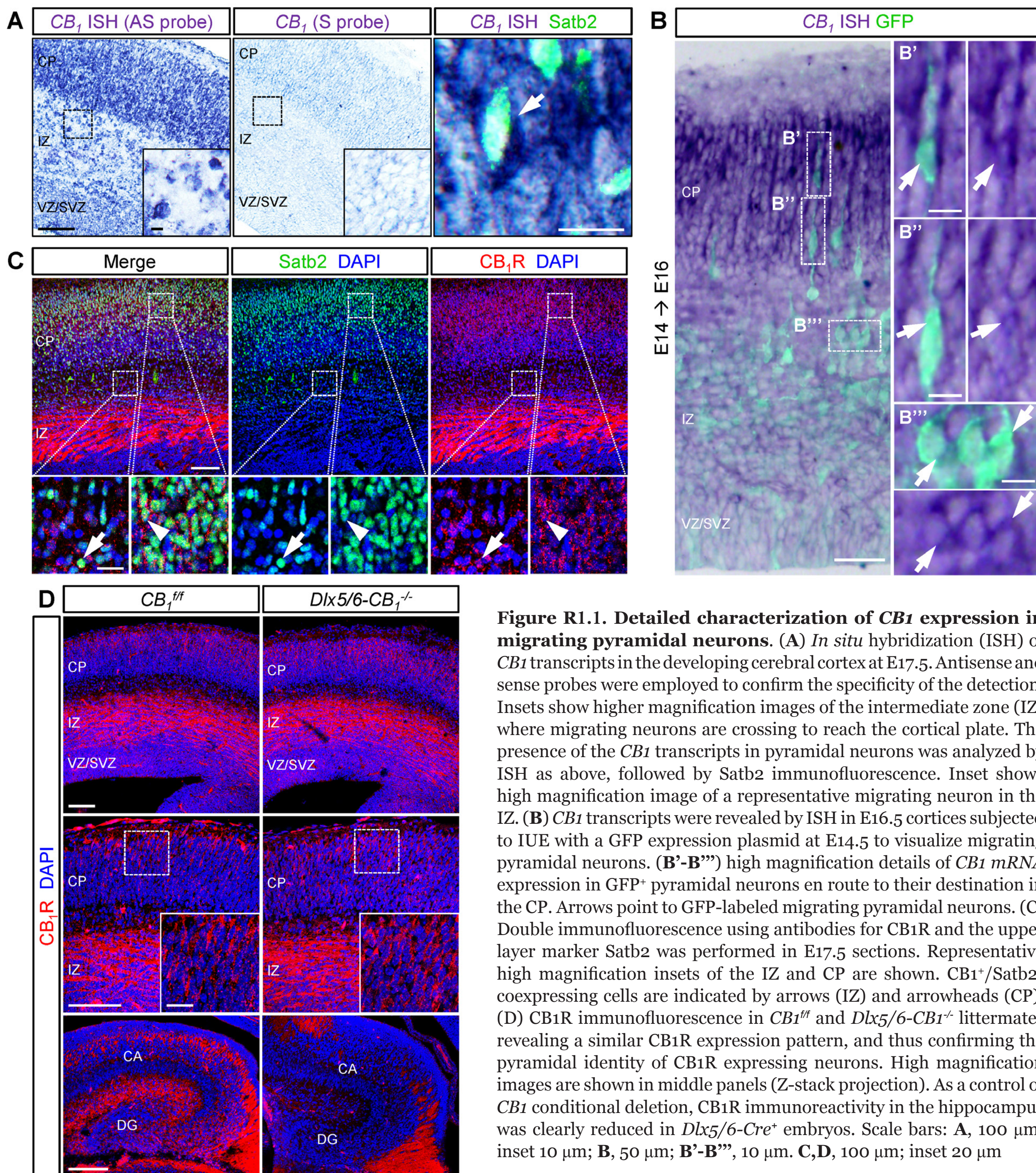

Figure R1.1. Detailed characterization of *CB1* expression in migrating pyramidal neurons. (**A**) *In situ* hybridization (ISH) of *CB1* transcripts in the developing cerebral cortex at E17.5. Antisense and sense probes were employed to confirm the specificity of the detection. Insets show higher magnification images of the intermediate zone (IZ) where migrating neurons are crossing to reach the cortical plate. The presence of the *CB1* transcripts in pyramidal neurons was analyzed by ISH as above, followed by Satb2 immunofluorescence. Inset shows high magnification image of a representative migrating neuron in the IZ. (**B**) *CB1* transcripts were revealed by ISH in E16.5 cortices subjected to IUE with a GFP expression plasmid at E14.5 to visualize migrating pyramidal neurons. (**B'-B'''**) high magnification details of *CB1 mRNA* expression in GFP^+ pyramidal neurons en route to their destination in the CP. Arrows point to GFP-labeled migrating pyramidal neurons. (C) Double immunofluorescence using antibodies for CB1R and the upper layer marker Satb2 was performed in E17.5 sections. Representative high magnification insets of the IZ and CP are shown. $CB1^+$/$Satb2^+$ coexpressing cells are indicated by arrows (IZ) and arrowheads (CP). (D) CB1R immunofluorescence in *CB1*$^{f/f}$ and *Dlx5/6-CB1*$^{-/-}$ littermates revealing a similar CB1R expression pattern, and thus confirming the pyramidal identity of CB1R expressing neurons. High magnification images are shown in middle panels (Z-stack projection). As a control of *CB1* conditional deletion, CB1R immunoreactivity in the hippocampus was clearly reduced in *Dlx5/6-Cre*$^+$ embryos. Scale bars: **A**, 100 µm; inset 10 µm; **B**, 50 µm; **B'-B'''**, 10 µm. **C,D**, 100 µm; inset 20 µm

the cortex, where E14.5-born neurons are committed to migrate (Greig et al. 2013) (Fig. R1.2C). We confirmed that acute *CB1* silencing impairs radial migration by using additional strategies: both a *CB1*-directed short-hairpin RNA (*CB1* shRNA, not shown) and Cre recombinase electroporation in *CB1*$^{f/f}$ embryos (Fig. S1.1C,D) perturbed migration of cortical projection neurons at very similar extent than siRNA-mediated *CB1* knockdown.

To invade the CP, migrating neurons must undergo a morphological switch from their characteristic multipolar shape, adopted to explore the SVZ and IZ environment, to a bipolar morphology, which enables their RGC-aided migration into the CP (Heng et al. 2008; Pacary et al. 2011). Acute *CB1* knockdown increased the number of primary neurites in cultured E14.5 cortical

neurons (Fig. S1.1E-G). Hence, we analyzed whether silencing of *CB1* affects the morphology of migrating neurons *in vivo*. We quantified the number of GFP⁺ cells with more than 2 primary processes in the CP of control siRNA and si*CB1*-transfected brains, and found a 2-fold increase in the proportion of cells with this aberrant morphology in si*CB1*-electroporated cortices (Fig. R1.2D,E), thereby indicating that radial migration blockade occurs concurrently with an

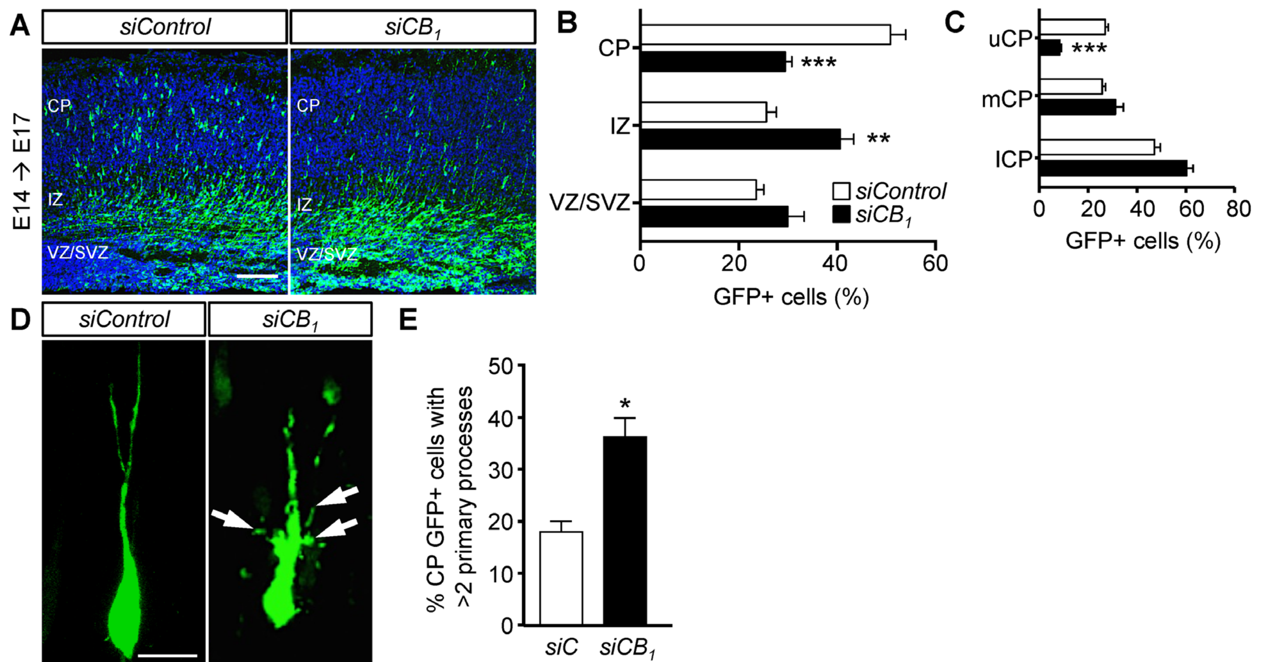

Figure R1.2. Cannabinoid *CB1* knockdown impairs pyramidal neuron migration. (**A-C**) Analysis of the migration of cortical neurons electroporated *in utero* with *CB1* siRNA or a control siRNA together with a GFP expression plasmid at E14.5 and analyzed 3 days later, at E17.5. Representative images are shown (**A**). The distribution of GFP⁺ cells in the ventricular/subventricular zone (VZ/SVZ), intermediate zone (IZ) and CP of the developing cerebral cortex in both conditions was quantified (**B**). The migration of targeted cells within the CP (uCP, upper CP; mCP, median CP; lCP, lower CP) was also analyzed (**C**). (**D,E**) The morphology of CP migrating cells was analyzed, and we quantified the proportion of cells with more than 2 primary processes. Arrows point to abnormal primary processes in *CB1*-knockdown cells. *n*= at least 3 different embryos from different litters per condition. Graphs represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Scale bars: **A**, 100 μm; **D**: 10 μm.

impairment of the multipolar to bipolar transition.

R1.2. Transient *CB1* knockdown long-lastingly perturbs neuronal migration and generates SBH

To assess whether *CB1*-deficient cells suffer from a transient delay in their radial migration but finally reach their appropriate cortical layer if given enough time, as is the case following the silencing of other important regulators of cell migration (Creppe et al. 2009; Manent et al. 2009), we extended our E14.5 IUE experiments until early postnatal life (P2 and P10), when the siRNA is no longer active. In P2 brains, the migration arrest observed upon CB1R downregulation was still present (Fig. R1.3A,B), thus confirming that temporally restricted *CB1* loss of function compromises radial migration of newborn neurons in the developing cortex. In P10 brains we observed a larger proportion of GFP⁺ cells in the upper layers of si*CB1*-electroporated cortices,

although we consistently found GFP+ cells in deep cortical layers, and extraordinarily, we also observed accumulations of GFP+ cells stacked into the WM (Fig. R1.3C,D). Furthermore, siRNA-electroporated mice that develop until young adults (P60) were assessed for the definitive positioning of transfected cells. Strikingly, we consistently found subcortical accumulations of GFP+ neurons in the majority of si*CB1*-electroporated brains (Fig. R1.3G-I).

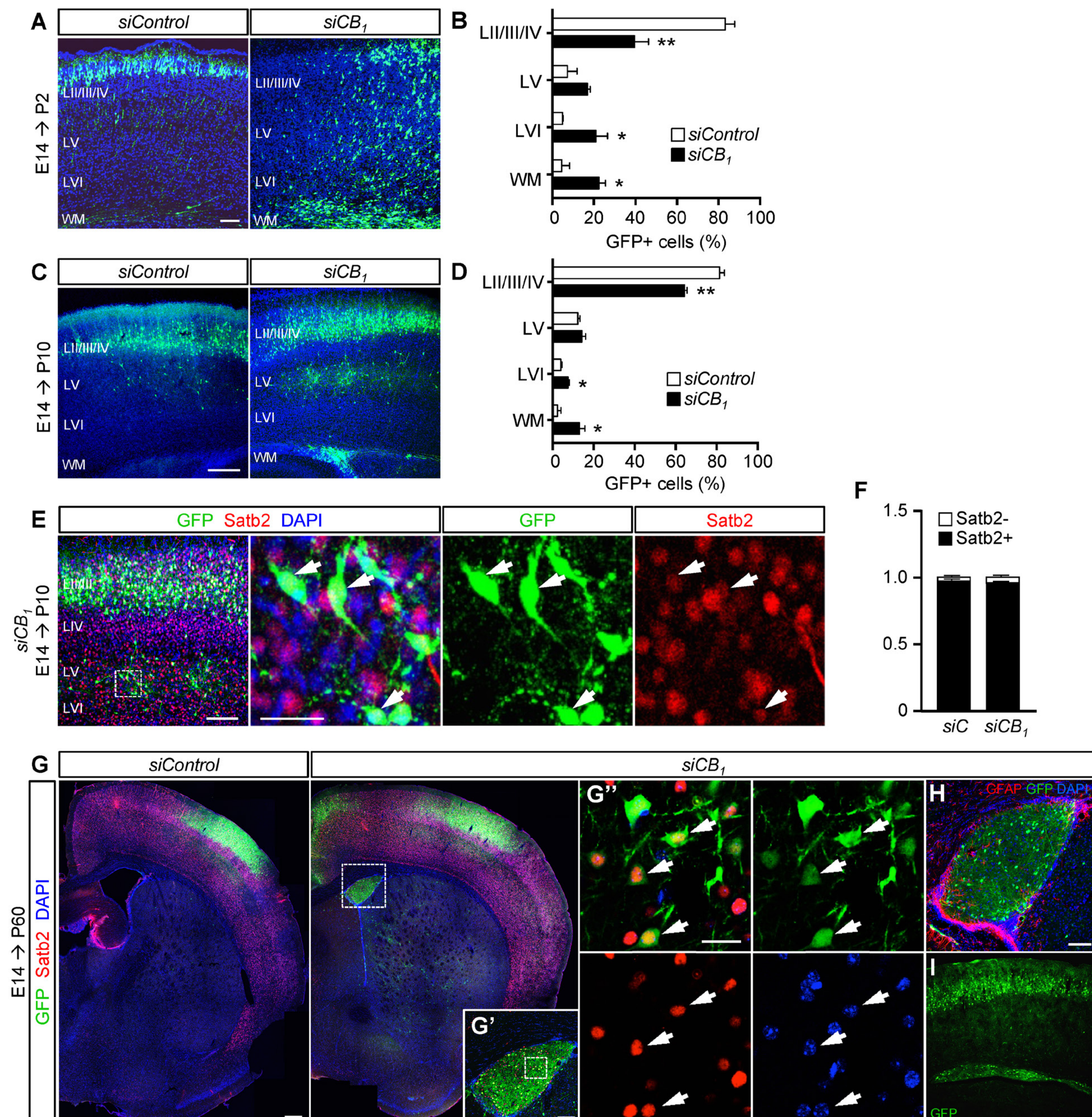

Figure R1.3. Transient *CB1* knockdown generates long-lasting aberrant neuronal positioning and subcortical band heterotopia (SBH). (**A,B**) Migration analysis of cortical neurons electroporated *in utero* with si*CB1* or a control siRNA together with a GFP expression plasmid at E14.5 and analyzed at P2. Electroporated cell distribution was analyzed in the indicated cortical layers and white matter (WM). (**C,D**) Brains electroporated at E14.5 were analyzed as above at P10. (**E,F**) Migration-arrested GFP+ cells from electroporated P10 brains were stained with the callosal projection neuron marker Satb2. The specification of targeted cells was quantified in control and si*CB1* electroporated cortices (**F**). (**G**) Subcortical accumulations of pyramidal neurons were evident in si*CB1*-electroporated brains at P60. (**G', G''**) detail of the expression of the callosal projection neuron marker Satb2 in subcortical heterotopias (SBH). Arrows point to Satb2+/GFP+ cells. (**H**) The SBH nature of the lesions was confirmed by the identification of a glial GFAP-immunoreactive capsule around the ectopic cell accumulations. (**I**) Example of a SBH-like lesion in another P60 si*CB1*-electroporated brain. *n*= at least 3 different embryos from different litters per condition. Graphs represent mean ± SEM. **P* < 0.05; ***P* < 0.01. Scale bars: **A**, **E**, 100μm (insets, 20 μm); **C**, 200 μm; **G'**, **H**, 200 μm; **G''**, 25 μm; **I**, 100 μm.

We next determined the identity of GFP$^+$ *CB1*-knocked-down cells at postnatal stages. Cells found in deep cortical layers of si*CB1*-transfected brains at P10 were immunoreactive for the callosal PN specification marker Satb2 (Alcamo et al. 2008) (Fig. R1.3E,F). Additionally, we found that the vast majority of GFP$^+$ cells within the subcortical heterotopias at P60 were also immunopositive for Satb2 (Fig. R1.3G-G''), even though the expression of this transcriptional regulator in the telencephalon is normally confined to the cerebral cortex (Alcamo et al. 2008). This observation indicates that arrested cells retain their corresponding callosal specification program even in ectopic locations. We then asked about the nature of the ectopic neuronal accumulations present in si*CB1*-transfected brains. SBH can be distinguished from PH because neuronal accumulations are surrounded by a glial capsule in SBH but not in PH. Hence, we stained samples from si*CB1*-electroporated P60 brains for the astrocytic marker GFAP and confirmed that the neuronal accumulations correspond to the SBH type (Fig. R1.3H).

We then explored whether the observed CB1R-dependent promotion of radial migration was an exclusive mechanism of E14.5-born cells. To this end, we performed IUE at E13.5 and conducted analyses 3 days later (Fig. S1.1H-I). Similarly to our previous manipulations at E14.5, when targeting E13.5-born PNs an overall delay in GFP$^+$ cell migration was observed in *CB1*-knocked-down brains after 3 DIU. Likewise, perturbed radial migration was still evident at P2 in *CB1*$^{f/f}$ mice electroporated at E13.5 with a *Cre*-expressing vector (Fig. S1.1C,D).

R1.3. CB1Rs present in postmitotic pyramidal neurons promote radial migration

CB1 plays an active role in the regulation of cortical progenitor cell proliferation and identity (Mulder et al. 2008; Díaz-Alonso et al. 2014). Hence, we determined whether the observed *CB1*-dependent promotion of pyramidal neuron migration involves CB1Rs located on cortical progenitor cells. To test this possibility, we electroporated a *Cre*-encoding construct driven by the postmitotic neuron-specific promoter *Dcx* (Fig. S1.2) (Franco et al. 2011) in E14.5 *CB1*$^{f/f}$ embryos, and analyzed the position of targeted cells at E17.5 (Fig. R1.4A-C). A clear migration defect was evident upon *CB1* ablation exclusively in postmitotic neurons. Importantly, this manipulation also affected the morphology of migrating GFP$^+$ cells (Fig. R1.4D,E). Moreover, we tested whether postmitotic *CB1* removal results in a transient or permanent migration impairment by allowing the electroporated embryos to develop until P2. Although many of the electroporated cells were found in the upper cortical layers at this stage, we consistently found accumulations of neurons ectopically located in the WM (Fig. R1.4F). These observations demonstrate that CB1R signaling cell-autonomously promotes radial migration in postmitotic neurons independently of the cortical progenitor cell pool.

We sought for additional support to the postmitotic nature of the CB1R promigratory role by analyzing the migration of postmitotic neurons in *CB1*$^{-/-}$ embryos. *CB1* constitutive deletion has

been shown to affect several aspects of PN development (Mulder et al. 2008), although its precise impact on radial migration remains unclear. Thus, we performed birthdate labeling experiments in *CB1*-knockout embryos and control littermates by administering BrdU at E14.5 followed by double immunofluorescence for BrdU and Ki67 at E16.5, in order to recognize cells that were still proliferating. $BrdU^{+}Ki67^{-}$ cells –*i.e.* the cells that had exited cell cycle between E14.5 and E16.5– were significantly delayed in their radial migration in $CB1^{-/-}$ embryos when compared to WT littermates (Fig. S1.3A,B).

As the CB1R is known to trigger prosurvival mechanisms in neurons (Galve-Roperh et al. 2008), we assessed whether its knockdown interferes with neuronal migration by affecting cell viability. Hence, we analyzed the apoptosis marker cleaved caspase-3 in cortices subjected to IUE with the siRNAs. We did not find significant differences in apoptotic cells in the developing cortex of si*CB1*-electroporated and control siRNA-electroporated cortices (Fig. S1.3C). Moreover, no differences were observed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in si*CB1*-electroporated versus control cortices (Fig. S1.3D). Thus ruling out a contribution of neuronal survival in the promigratory effect exerted by CB1Rs.

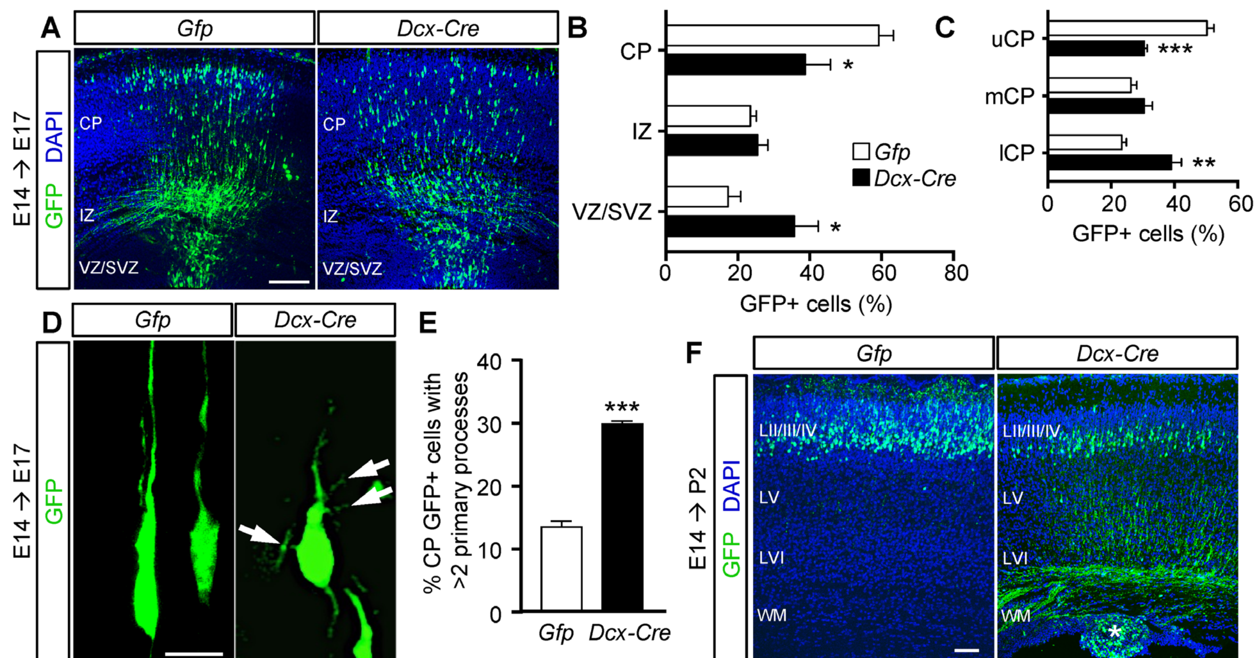

Figure R1.4. CB1R signaling promotes radial migration postmitotically. (**A-C**) Migration of cortical neurons was analyzed in E17.5 $CB1^{f/f}$ embryos electroporated *in utero* at E14.5 with a *Dcx-Cre* expression vector, to ablate the CB1R in postmitotic neurons, or *Gfp* as a control (see Materials and Methods section). The distribution of GFP^{+} cells along the developing cerebral cortex in E17.5 is indicated in (**B**). Quantification of the migration of targeted cells within the CP is shown (**C**). (**D, E**) The morphology of migrating cells was analyzed in the CP, and the proportion of cells with more than 2 processes was quantified. Arrows point to abnormal primary processes in *CB1*-deficient cells. (**F**) Migration analysis of cortical neurons electroporated *in utero* with *Dcx-Cre* or *Gfp* at E14.5 and analyzed at P2. In *Dcx-Cre*-electroporated cortices, heterotopic GFP^{+} cells accumulations were consistently found in subcortical areas (asterisk). *n*= at least 3 different embryos from different litters per condition. Graphs represent mean ± SEM. Scale bars: **A**, **F**, 100 µm; **D**, 10 µm. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

R1.4. The eCBs 2-AG and anandamide act as chemoattractant cues for newborn PNs

Given that developmental DAGLα-dependent intercellular eCB-signaling has been suggested (Keimpema et al. 2013), to better understand the mechanism of CB1R-driven neuronal migration, we investigated the ability of the eCBs to modulate pyramidal neuron migration. We prepared E14.5 cortical explants and exposed them to a confined source of eCBs. DAGLα is the key enzyme involved in 2-AG synthesis, and mice lacking this enzyme show impaired eCB-mediated neuromodulation and compromised adult neurogenesis. Neurons arising from cortical explants showed a marked preference to migrate toward DAGLα-overexpressing COS7 cells when compared with control, GFP-transfected cells (Fig. R1.5A,B). Alternatively, anandamide biosynthesis is mainly achieved through the action of NAPE-PLD. Likewise, the migration of neurons from cortical explants was favored towards heterologous COS7 cells overexpressing NAPE-PLD. Importantly, the promigratory effect of 2-AG and anandamide was blocked by coincubation with the CB1R inverse agonist SR141716. Thus, both eCBs promote pyramidal neuron migration by engaging CB1Rs.

The aforementioned observations could reflect two different mechanisms: eCBs might promote neuronal motility and/or instruct the directionality of neuronal migration. To dissect these two processes we overexpressed DAGLα *in utero* –normally upregulated exclusively in the CP– at E14.5 to disrupt the gradient of 2-AG, and analyzed neuronal migration at E17.5. DAGLα-overexpressing migrating neurons showed a mild –yet significant– delay compared to GFP-electroporated cells, suggesting that blurring the cortical 2-AG gradient misleads neuronal migration (Fig. R1.5B). We then evaluated the consequences of disturbing the cortical gradient of 2-AG on the subsequent waves of migrating neurons. Hence, we injected BrdU 12h after the IUE to label proliferating cells, and then tracked their migration at E17.5. BrdU-labelled cells accumulated aberrantly in the VZ of DAGLα-electroporated brains, where DAGLα-overexpressing cells were most abundant (Fig. R1.5C-E), thus indicating that abnormally high levels of 2-AG in the apical side of the cortex prevent proper PN radial migration. These findings suggest that eCBs may act as instructive, spatially regulated cues for the adequate migration of pyramidal neurons in the developing cortex.

R1.5. CB1R signaling promotes RhoA degradation in migrating pyramidal neurons

Several signaling pathways involved in the regulation of neuronal migration converge in the modulation of the activity of the small GTPase protein RhoA (Cappello et al. 2012; Pacary et al. 2013; Azzarelli et al. 2014). Given the morphological alterations found in *CB1*-deficient migrating cells, and the previous evidence suggesting a functional link between CB1R signaling and RhoA activity in both neuronal (Paul Berghuis et al. 2007) and non-neural (Nithipatikom et al. 2012; Mai et al. 2015) cells, we hypothesized that the promigratory effect of the CB1R in newborn pyramidal cells could rely on the modulation of actin cytoskeleton through RhoA. To investigate whether CB1R signaling regulates RhoA activity *in vivo*, we measured the amount

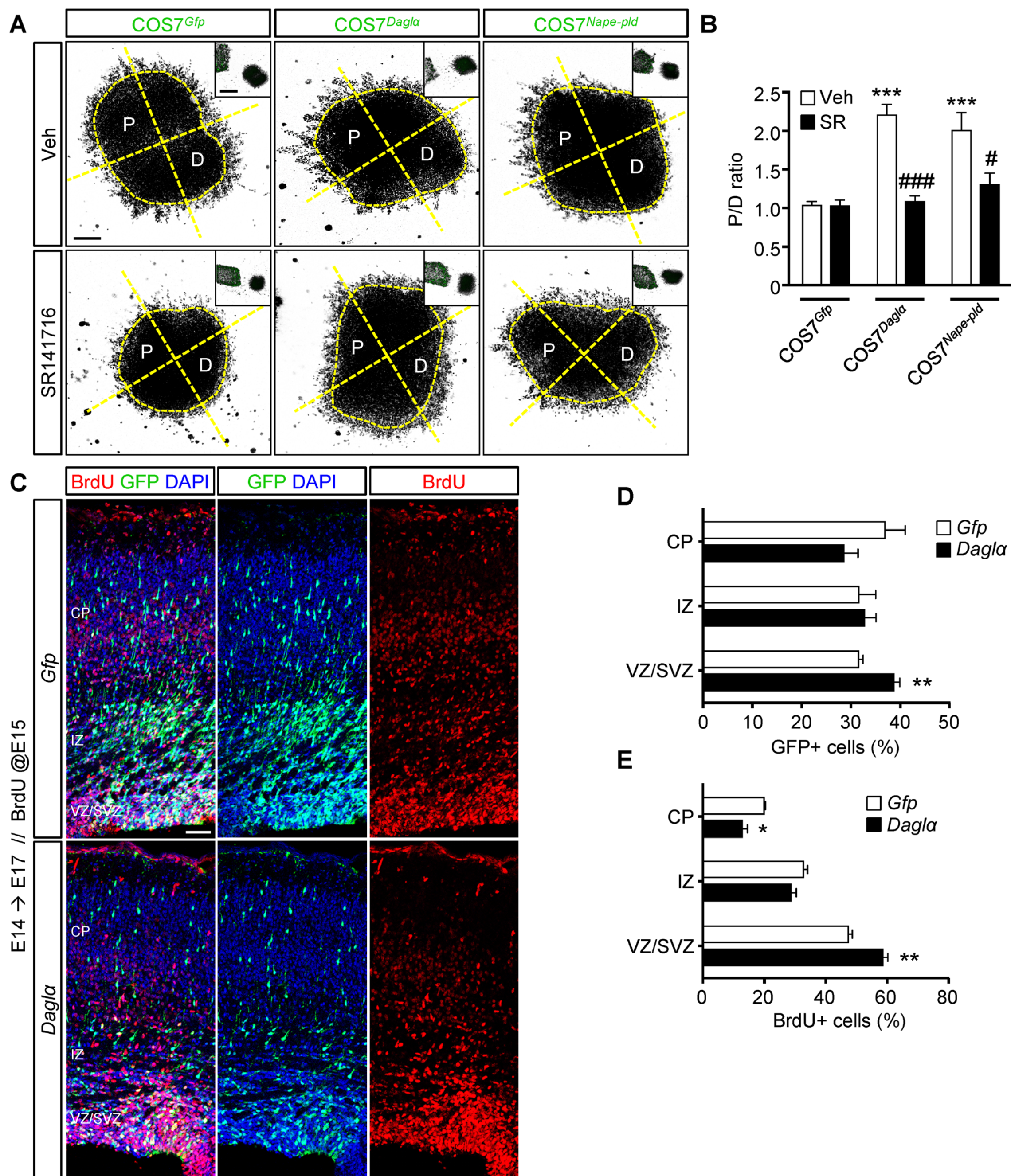

Figure R1.5. The endocannabinoids 2-AG and AEA act as promigratory cues for newborn cortical neurons. (**A**) Representative images of pyramidal neuronal migration assays from E14.5 cortical explants in response to *Daglα*-, *Nape-pld*- or mock (*Gfp*)- transfected COS7 cells. (**B**) The promigratory action of eCBs was quantified by determining the ratio of neurons in the proximal and distal (P/D) quadrants. The involvement of CB1Rs was determined by including in the medium the CB1R inverse agonist SR141716 (SR, 10 μM or vehicle. *n*= 3 independent experiments with at least 5 explants per condition. (**C-E**) Migration analysis of cortical neurons electroporated *in utero* with *Dagla* together with a *Gfp* expression or a control *Gfp*-only plasmid at E14.5 and analyzed at E17.5. BrdU was injected 12 h after IUE to label the subsequent wave of migrating cells. Representative images are shown. Quantification of GFP$^+$ cell distribution in the indicated cortical compartments is shown (D). Analysis of BrdU-labeled cells at E17.5 (E). *n*= at least 3 different embryos from different litters per condition. Graphs represent mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. $^{***}P < 0.001$ *vs Gfp*/Vehicle-treated explants of *Gfp* electroporated embryos; #*P* < 0.05; ###*P* < 0.001 *vs* the corresponding vehicle-treated explants. Scale bars: **A,** 100 μm; inset 500 μm; **C,** 100 μm.

of GTP-bound RhoA in cortical tissue extracts from E17.5 embryos, when a large proportion of neurons are undergoing radial migration. We employed a dorsal telencephalic glutamatergic neuron-specific *CB1* conditional knockout (*Nex-CB1*$^{-/-}$) to determine the regulation of RhoA activity by CB1R signaling specifically in postmitotic cortical pyramidal neurons. We found that

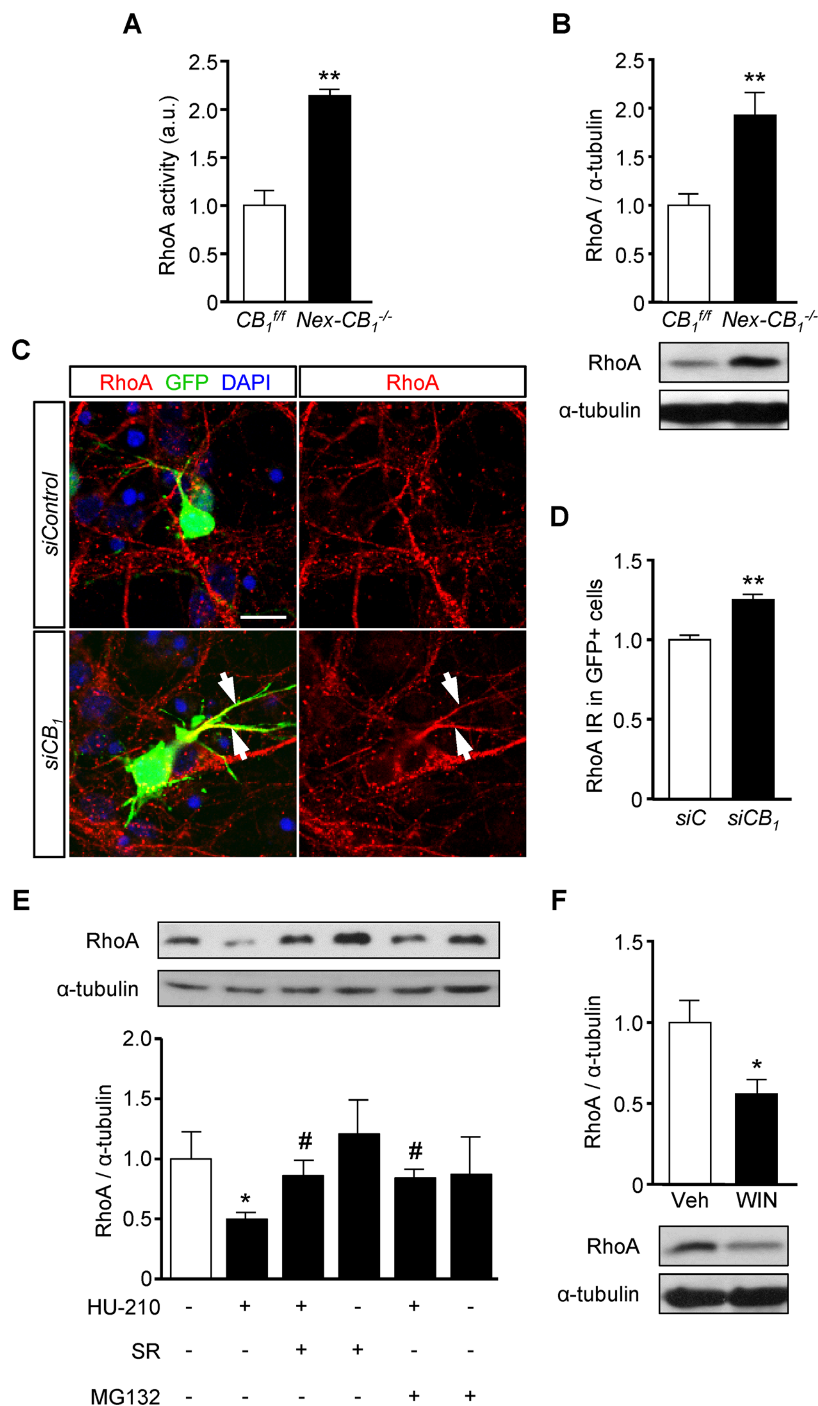

Figure R1.6. CB1R signaling controls RhoA protein levels in pyramidal neurons. (**A**) Analysis of RhoA activity levels in *CB1*$^{f/f}$ and *Nex-CB1*$^{-/-}$ E17.5 embryonic cortices. (**B**) RhoA protein levels were determined by Western blot in cortical tissue in E17.5 *Nex-CB1*$^{-/-}$ and *CB1*$^{f/f}$ littermates. Loading control was performed with anti α-tubulin antibody. (**C, D**) Analysis of RhoA levels in E14.5 primary cortical neurons electroporated with a si*CB1* or a control siRNA and maintained in differentiating conditions for 4 DIV. Mean RhoA immunoreactivity (IR) fluorescence intensity was calculated in the GFP^{+} area. Arrows point to RhoA-enriched processes in *CB1*-knockdown cells. *n*=3 independent experiments with at least 100 GFP^{+} cells from 6 imaging fields per experiment. (**E**) RhoA protein levels were determined by Western blot upon pharmacological manipulation with HU-210 (50 nM) for 6h in primary differentiating CPCs. In addition, CPCs were incubated in the presence of the CB1R receptor inverse agonist SR141716 (1 μM and with the proteasome inhibitor MG-132 (10 μM). (**F**) RhoA protein levels were determined by Western blot in cortical samples from E17.5 embryos exposed to the CB1R agonist WIN 55,212-2 (5 mg/Kg) or its vehicle for 6 hours. *n*= at least 3 different embryos per genotype or treatment. Graphs represent mean ± SEM. **P* < 0.05; ***P* < 0.01; #*P* < 0.05 *vs* HU-210-treated cells. Scale bar: 25 μm.

active RhoA levels were increased by 2-fold in E17.5 *Nex-CB1*$^{-/-}$ cortices compared to their *CB1*$^{f/f}$ littermates (Fig. R1.6A), suggesting that CB1R signaling dampens RhoA activity in migrating pyramidal neurons. This observation indicates that in *Nex-CB1*$^{-/-}$ cortical tissue either the active fraction of RhoA is increased and/or there is an accumulation of total RhoA protein. Hence, we examined total RhoA protein levels by Western blot and also found a 2-fold increase in *Nex-CB1*$^{-/-}$ tissue extracts compared with their *CB1*$^{f/f}$ littermates (Fig. R1.6B), thus indicating that *CB1* inactivation in postmitotic pyramidal neurons leads to an accumulation of RhoA protein rather than to an increase in the active fraction of RhoA. Subsequently, we analyzed RhoA expression in those samples by qPCR and found no significant differences (Fig. S1.4A), suggesting that CB1R

signaling likely controls RhoA at a posttranslational level rather than regulating its transcription. The *mRNA* expression levels of other Rho-family members involved in neuronal migration, *RhoB*, *Rac1* and *Cdc42*, were neither affected by *CB1* ablation (Fig. S1.4A).

To determine whether our acute *CB1* knockdown strategy also modifies RhoA protein levels, we performed *ex vivo* electroporation of si*CB1* or control siRNA together with GFP into E14.5 mouse embryonic dorsal telencephalon, and maintained dissociated cortical cells for 4 DIV. In agreement with *Nex-CB1*$^{-/-}$ cortical tissue, RhoA immunoreactivity was increased in si*CB1*-transfected compared to control-siRNA-transfected GFP^{+} cells (Figure R1.6C,D).

It has been recently reported that RhoA expression in pyramidal neurons is largely dispensable for their migration, and that its overactivation results in radial migration arrest (Pacary et al. 2011; Cappello et al. 2012). This is consistent with the expression pattern of RhoA in the developing cortex, which shows a clear downregulation of RhoA in migrating neurons compared to cortical progenitors (Azzarelli et al. 2015). Therefore, we designed an experimental approach aimed at recapitulating the cellular context of migrating pyramidal neurons to study how CB1R signaling affects RhoA levels at a mechanistic level. Thus, we obtained adherent cortical progenitor cell (CPC) cultures, which can be differentiated into cortical neurons by changing the medium and withdrawing growth factors (see Materials and Methods). Characterization of CPC cultures (Fig. S1.5A-E) revealed their appropriateness to model the cellular context of a migrating cortical neuron. As expected, neuronal differentiation reduced nestin and *Pax6* expression, while increasing the expression of the postmitotic neuron-enriched *Tuj1* and *CB1*. RhoA expression also decreased with differentiation, mirroring the abrupt downregulation that occurs during pyramidal neuron maturation *in vivo*. To evaluate whether CB1R manipulation during this process affects RhoA protein levels, we stimulated CB1R signaling in differentiating CPCs with the synthetic cannabinoid agonist HU-210 and observed that CB1R activation led to a decrease of total RhoA protein levels compared with vehicle-treated CPCs (Figure R1.6E). Noteworthy, HU-210-induced reduction of RhoA levels was prevented by SR141716. Differentiating CPCs were also coincubated with HU-210 and the proteasome inhibitor MG132, which abolished the HU-210-induced regulation of RhoA levels (Figure R1.6E). These results indicate that CB1R signaling promotes proteasomal degradation of RhoA in newborn pyramidal neurons.

Finally, we also evaluated whether pharmacological stimulation of the CB1R affects RhoA protein levels in developing cortical neurons *in vivo*. We administered the synthetic cannabinoid agonist WIN55,212-2 or its vehicle to pregnant females in gestational day 17 and embryos were collected 6 hours later. Cannabinoid administration resulted in a reduction of RhoA protein levels in embryonic cortical extracts (Figure R1.6F), without affecting the expression of *RhoA*, *RhoB*, *Cdc42* or *Rac1* (Fig. S1.4B). Considering that RhoB is highly expressed during cortical development, its homology and function redundancy with RhoA (Azzarelli et al. 2015), we also analyzed RhoB protein levels upon CB1R genetic and pharmacological manipulation. RhoB protein levels were

increased in *Nex-CB1*-deficient embryonic cortical extracts, whereas WIN-55,212-2 treatment *in vivo*, and HU-210 administration of CPCs *in vitro*, decreased RhoB levels (Fig. S1.6).

R1.6. RhoA downregulation is sufficient to rescue si*CB1*-induced migration arrest

The aforementioned findings indicate that *CB1* silencing leads to RhoA protein accumulation in newborn pyramidal neurons. To test whether this effect underlies the migration defects of si*CB1*-electroporated cells, we coelectroporated a *RhoA*-directed shRNA (sh*RhoA*) together with the si*CB1*. The efficacy of this sh*RhoA* to decrease *RhoA* expression has previously been demonstrated (Pacary et al. 2011). As shown in Figure R1.7, knocking-down *RhoA* fully rescued *CB1* silencing-induced migration arrest, and restored both the normal distribution of GFP^{+} cells along the different cortical compartments (Fig. R1.7B) as well as the adequate positioning of the cells within the CP (Fig. R1.7C). This observation provides evidence for the notion that RhoA accumulation upon *CB1* knockdown underlies the impairment of neuronal migration. Cofilin is a canonic downstream effector of RhoA that promotes actin filament disassembly. Since F-actin depolymerization is required for neuronal migration –as it allows the continuous dynamic recycling of actin cytoskeleton– we tested the ability of a non-phosphorylatable form of cofilin (cofilin^{S3A}) (Pacary et al. 2011), which constitutively depolymerizes F-actin, to rescue *CB1* knockdown-evoked migration arrest. *In utero* coelectroporation of *cofilin*^{S3A} at E14.5 rescued the si*CB1*-induced PN migration defects at E17 (Fig. R1.7A-C), and confirm that RhoA accumulation in *CB1*-deficient

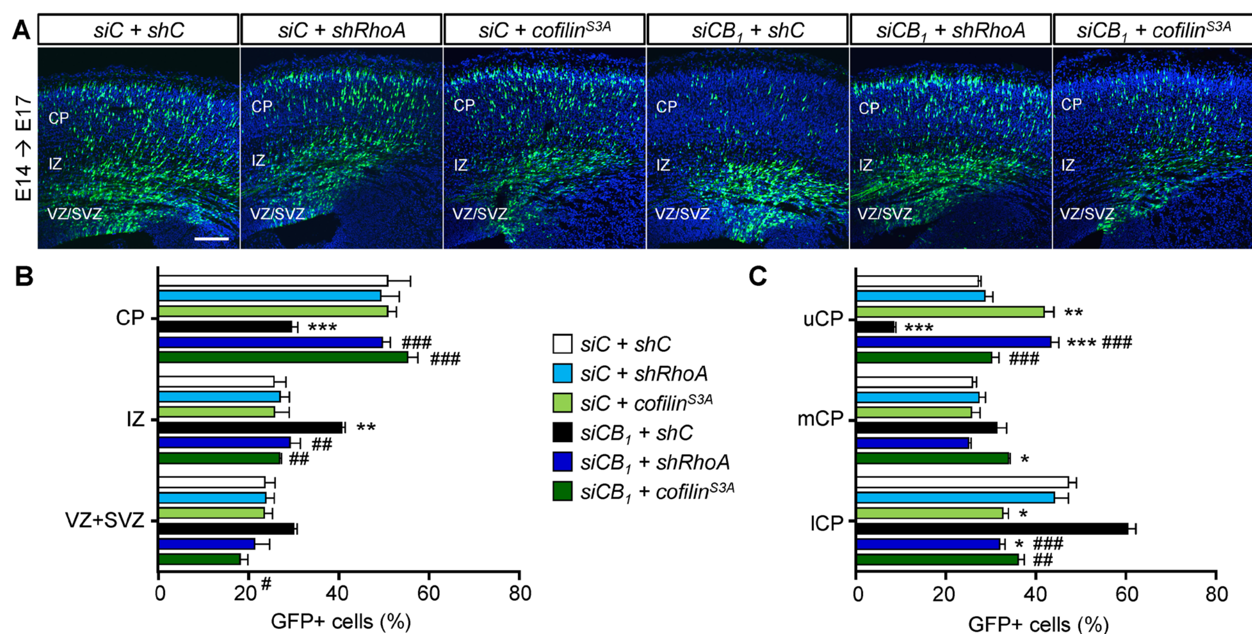

Figure R1.7. *RhoA* knockdown or expression of non-phosphorylatable cofilin rescues *CB1* silencing-induced migration arrest. (**A, B**) Migration analysis of cortical neurons electroporated *in utero* with a control siRNA or *siCB1* together or not with a *shRhoA* or a plasmid encoding a non-phosphorylatable form of cofilin [*cofilin*^{S3A}] and a GFP expression plasmid at E14.5 and analyzed 3 days later, at E17.5. Representative images are shown for the different conditions (**A**). GFP^{+} cell distribution in the indicated developing cortical areas and within the CP was quantified (**B,C**). *n*= at least 3 different embryos from different litters per condition. Graphs represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 vs siRNA control-electroporated brains; #*P* < 0.05; ##*P* < 0.01; ###*P* < 0.001 vs *siCB1*-electroporated brains. Scale bar: 100 µm.

cells compromises neuronal migration by altering actin cytoskeleton dynamics.

We next examined whether the rescue of radial migration by RhoA downregulation results in a correct neuronal positioning in the adult cerebral cortex. Electroporated embryos that developed until P60 were analyzed for the distribution of GFP+ cells. We found that, while the majority of si*CB1*-electroporated brains showed SBH, normal neuronal lamination –and absence of SBH– was observed in brains coelectroporated with sh*RhoA* (Fig. R1.8A). Taken together, these findings demonstrate that preventing RhoA accumulation rescues the migration defects caused by *CB1* silencing.

R1.7. Migration deficits induced by transient CB1 knockdown during development increases seizure susceptibility in adulthood

Similarly to human MCDs, experimentally-induced ectopic accumulations of cortical neurons are aberrantly wired to the cortical circuitry, thus leading to an overall increased susceptibility to seizures (Manent et al. 2009; Feliciano et al. 2011). In addition to the striking heterotopias, the functional contribution of the normocortex to the proepileptic phenotype has gained attention lately (Petit et al. 2014). We therefore studied whether migration deficits caused by *in utero CB1* knockdown sensitizes the adult offspring to seizures induced by the convulsant pentylenetetrazole (PTZ). We administered subconvulsive doses of PTZ intraperitoneally every 10 minutes to young adult (P60) mice, and measured the latency and cumulative dose of PTZ necessary for generalized

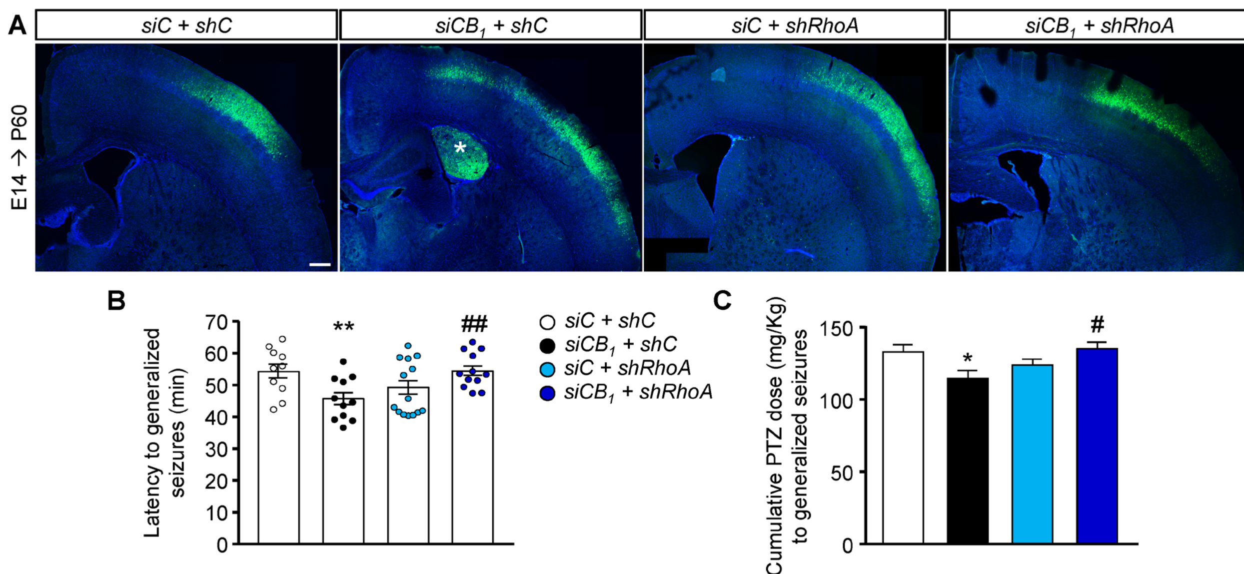

Figure R1.8. Transient *CB1* knockdown in migrating pyramidal neurons increases seizure susceptibility in adulthood. (**A**) Representative images of GFP+ cells in adult mice (P60) subjected to IUE at E14.5 with *siCB1* or control siRNA together with RhoA or control shRNA . GFP+ cell accumulation within the subcortical WM in *siCB1/shControl* condition is indicated (asterisk). (**B,C**) P60 mice subjected to IUE at E14.5 with *siCB1* or *siControl* combined with *shRhoA* or control shRNAs as above were injected i.p. with PTZ (22.5 mg/Kg) every 10 minutes until generalized seizures occurred. The mean latency to the occurrence of generalized seizures is represented (**B**) and the cumulative PTZ dose necessary for the onset of generalized seizures was calculated (**C**). *n*= 10-14 mice per group. Graphs represent mean ± SEM. **P* < 0.05; ***P* < 0.01 vs *siControl*-electroporated mice; #*P* < 0.05; ##*P* < 0.01; vs *siCB1*-electroporated brains. Scale bar: 200 μm.

seizures to occur. PTZ susceptibility was significantly increased in si*CB1*-electroporated mice, as shown by the reduced latency and PTZ cumulative dose necessary to evoke generalized seizures (Fig. R1.8B,C). Importantly, the rescue of si*CB1*-induced neuronal migration arrest achieved by sh*RhoA* coelectroporation also prevented the increase in seizure susceptibility (Fig. R1.8B,C). These observations indicate that developmental alterations generated by transient *CB1* loss of function in migrating pyramidal neurons decrease seizure threshold in adult offspring, and that concomitant RhoA downregulation rescues both neuronal migration deficits and seizure susceptibility.

AIM 2: SUMMARY

As presented in the Introduction, there is growing prevalence of cannabis-based therapies in numerous countries, with a wide and increasing array of usages. In addition, marijuana is the most commonly used recreational drug worldwide, and particularly, the most consumed during pregnancy in Western countries. However, cannabis consumption during pregnancy can exert deleterious consequences in the progeny, including anxiety, depression, psychosis risk, and cognitive or social impairments. A crucial role has been assigned to *CB1* in the development of long-range axonal connectivity by regulating corticofugal axon navigation and fasciculation, as well as in the CSMN cell-fate acquisition. Hence, in this project we modeled prenatal cannabis consumption in mice to identify the particular neurodevelopmental substrate responsible for cannabinoid-induced functional alterations that remain overt in adulthood. Administration of THC was conducted during a restricted embryonic time window, coinciding with the active period of glutamatergic neuron generation in the telencephalon. We found an impairment in CSMN generation that correlates with long-lasting skilled motor functional alterations and susceptibility to epilepsy. To unequivocally assess the role of CB1R signaling in THC-induced alterations, we employed constitutive *CB1*-deficient mice, which were resistant to THC-induced developmental alterations. Next, by using a Cre-mediated, lineage-specific, *CB1* expression-rescue strategy in a *CB1*-null background, we were able to selectively rescue the deficits in CSMN development characteristic of *CB1*-deficient mice and, in turn, fully restore the susceptibility to embryonic THC-induced cellular and functional deficits in adulthood.

Alternatively, we also found that prenatal THC exposure induced an increase in seizure susceptibility that was mediated by CB1Rs present in both developing dorsal telencephalic pyramidal neurons and forebrain GABAergic neurons. Hence, targeting CB1Rs with the most prominent marijuana-derived psychoactive compound in a particular neuronal population and time frame during embryonic development can evoke remarkable long-lasting neurological alterations in the offspring.

AIM2. IMPACT OF PRENATAL EXPOSURE TO THC ON BRAIN'S DEVELOPMENT AND FUNCTION

R2.1. Prenatal THC exposure interferes with cortical projection neuron development

To investigate the impact of prenatal THC exposure on cortical development, and to avoid the confounding influence that the cannabinoid could exert during very early gestational stages (Galve-Roperh et al. 2013; Wang & Dey 2006), we administered one daily intraperitoneal injection of THC or its vehicle to pregnant wild-type dams from E12.5 to E16.5. To minimize potential off-targets, a low dose of THC (3 mg/kg) was employed. Of note, maternal and neonate body weight was unaffected by THC treatment, thus indicating that the dose used did not induce deleterious effects on general physical status. As CB1R signaling is essential for CSMN specification (Díaz-Alonso et al. 2012), the effects of embryonic THC exposure on the developing cortex were first assessed by quantifying the generation of SCPNs. Confocal immunofluorescence analysis of ER81, a *bona fide* marker of SCPNs (Molyneaux et al. 2007), was performed in the treated offspring at P20. The number of $ER81^{+}$ neurons was decreased in THC-exposed animals when compared to their vehicle-treated controls (Fig. R2.1A,B). The impact of THC on CSMN development was also analyzed at the level of corticospinal axonal connectivity. For this purpose we carried out fluorescent retrograde labeling (Red RetroBeads) from the cervical spinal cord to unequivocally identify CSMNs (Arlotta et al. 2005) (Fig. R2.1C). Red-labeled *somata* in deep cortical layer V where shown to express ER81, thus confirming the validity of ER81 as an appropriate marker of CSMNs (Fig. R2.1D). More importantly, we also found a significant reduction in the number of labeled CSMN *somata* in THC-treated mice as compared to their controls, pointing to an alteration of CSMN development and subcerebral connectivity (Fig. R2.1E, F).

R2.2. Prenatal THC exposure induces long-lasting alterations in skilled motor function and seizure susceptibility

To examine the functional consequences of the impaired SCPN development induced by prenatal cannabinoid exposure, we first employed the skilled reaching test, which allows the dissection of CSMN-dependent motor function as reflected by the ability to retrieve a pellet of palatable food with a forelimb through a narrow slit (Tomassy et al. 2010). To rule out potential unspecific developmental alterations owing to maternal care deficits induced by THC administration (Calvigioni et al. 2014), we used $CB1^{-/-}$ females, devoid of the behavioral impact of THC, which were mated with heterozygous $CB1^{+/-}$ males. Therefore, we analyzed skilled motor function in the $CB1^{+/-}$ and $CB1^{-/-}$ offspring. $CB1^{+/-}$ mice have been shown to exhibit an increased efficacy of agonist-induced G protein-coupled receptor signaling, which becomes comparable to that of wild-

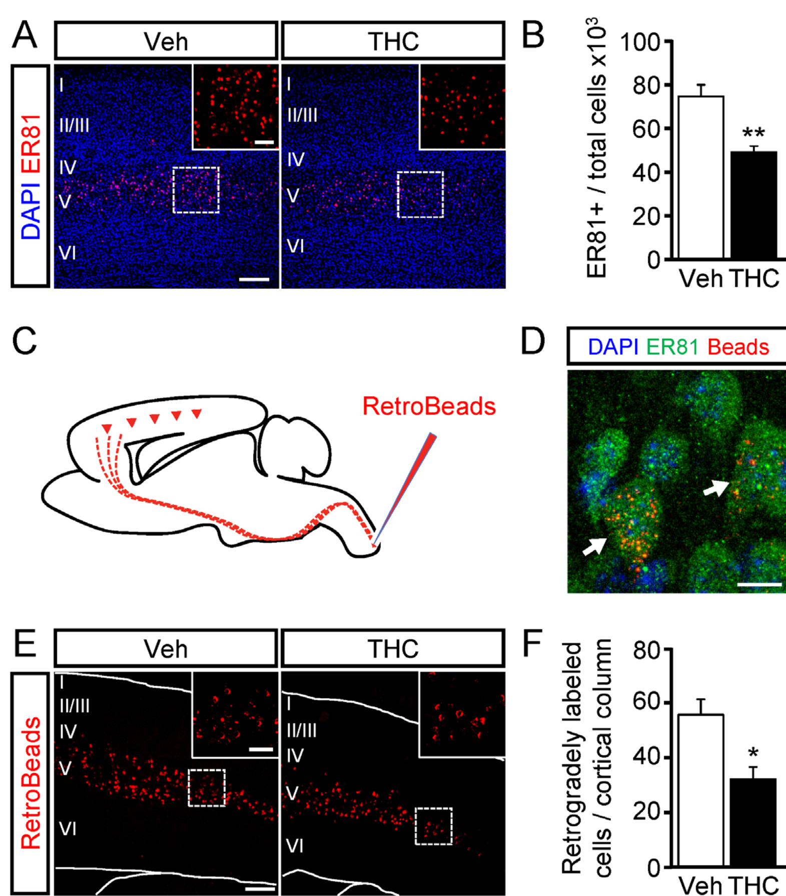

Figure R2.1. Embryonic THC exposure impairs subcerebral projection neuron development. (**A**) Subcerebral projection neurons in embryonically vehicle- and THC-administered mice at P15 were stained with an anti-ER81 antibody (*n* = 7 and 5, respectively). (**B**) ER81^{+} cell number was quantified and referred to the total cell number (DAPI) per cortical column. (**C**) CSMNs were labeled by injecting retrogradelly-transported red fluorescent beads (RetroBeads) in the cervical spinal cord at P10. (**D**) Representative image showing RetroBead colocalization with the subcerebral projection neuron marker ER81. (**E**) Representative images of retrogradelly-labeled somata in cortical layer V at P15. (**F**) RetroBead-labeled somata per cortical column were quantified in vehicle- and THC-exposed mice (*n* = 5 and 4, respectively). $^{*}P<0.05$, $^{**}P<0.01$ *vs.* vehicle-treated mice. Scale bars: (**A, E**) 200 µm (insets, 60 µm); (**D**) 10 µm.

type *CB1*$^{+/+}$ mice, thus supporting the validity of the experimental approach (Selley et al. 2001). THC-exposed *CB1*$^{+/-}$ animals showed a significant impairment in skilled motor function compared to their vehicle-treated counterparts (Fig. R2.2A). Remarkably, *CB1*$^{-/-}$ mice, which consistently with our previous report showed an impairment in this task compared to their vehicle-treated *CB1*$^{+/-}$ littermates (Díaz-Alonso et al. 2012), did not suffer from any worsening in their skilled motor performance upon THC exposure (Fig. R2.2A). Noteworthy, neither the number of trials (Fig. R2.2B) nor the success in unskilled conditions were changed among groups, ruling out generalized motivational or unspecific motor deficits. Corticospinal function was also assessed with the staircase test and, again, a decreased performance was evident in THC-exposed *CB1*$^{+/-}$ mice when compared to their vehicle-treated controls (Fig. R2.2C). In addition, control *CB1*$^{-/-}$ mice scored worse than their *CB1*$^{+/-}$ littermates, and THC treatment did not affect significantly their ability to reach the pellets. Control quantifications of unskilled reaching did not show significant differences among groups (Fig. R2.2D). Altogether, these data demonstrate the CB1R dependency of embryonic THC-evoked motor alterations.

Developmental THC administration induces alterations in synaptic connectivity and plasticity (Tortoriello et al. 2014; Mereu et al. 2003), but its long-lasting functional consequences and the contribution of specific neuronal subpopulations remain largely unknown. Therefore, we analyzed whether seizure susceptibility was affected in the adult offspring of THC-administered pregnant mice by using a PTZ administration paradigm. Noteworthy, latency to seizures was

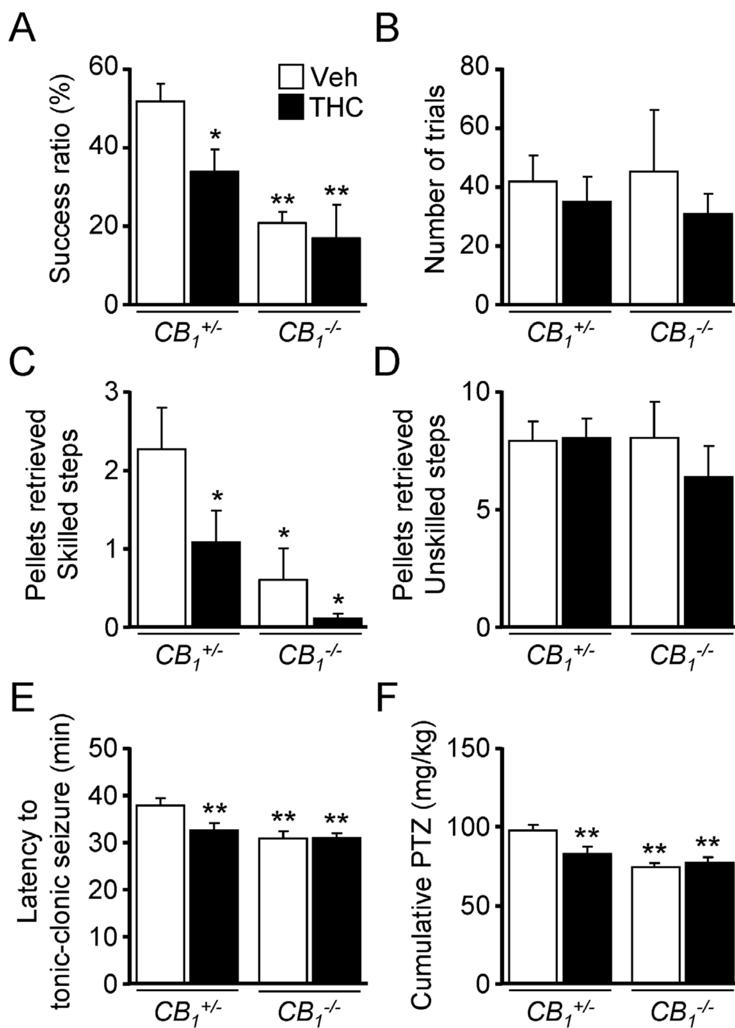

Figure R2.2. Embryonic THC exposure impairs subcerebral projection neuron development. (**A**) Subcerebral projection neurons in embryonically vehicle- and THC-administered mice at P15 were stained with an anti-ER81 antibody (*n* = 7 and 5, respectively). (**B**) ER81$^+$ cell number was quantified and referred to the total cell number (DAPI) per cortical column. (**C**) CSMNs were labeled by injecting retrogradelly-transported red fluorescent beads (RetroBeads) in the cervical spinal cord at P10. (**D**) Representative image showing RetroBead colocalization with the subcerebral projection neuron marker ER81. (**E**) Representative images of retrogradelly-labeled somata in cortical layer V at P15. (**F**) RetroBead-labeled somata per cortical column were quantified in vehicle- and THC-exposed mice (*n* = 5 and 4, respectively). **P*<0.05, ***P*<0.01 *vs.* vehicle-treated mice. Scale bars: (**A**, **E**) 200 μm (insets, 60 μm); (**D**) 10 μm.

significantly decreased in prenatally THC-exposed *CB1*$^{+/-}$ mice as compared to their vehicle-treated counterparts (Fig. R2.2E). Consequently, a reduced PTZ cumulative dose was required to induce generalized seizures in the THC-treated offspring (Fig. R2.2F). This effect of prenatal cannabinoid administration was reminiscent of the adult *CB1*$^{-/-}$ mice phenotype, which displays an increased seizure susceptibility in the kainic acid model (Monory et al. 2006; Ruehle et al. 2013). Importantly, we found no further enhancement of PTZ susceptibility in THC-treated *CB1*$^{-/-}$ mice with respect to their vehicle-treated counterparts, thus confirming the CB1R-specificity of THC action (Fig. R2.2F). Overall, the neuronal and functional analyses of prenatally THC-administered mice showed a similar phenotype to *CB1*-null mice, thus indicating that embryonic THC exposure interferes with the neurodevelopmental role of CB1R signaling.

R2.3. Prenatal THC exposure transiently impairs CB1R signaling

Given the functional similarities between embryonic THC exposure and *CB1* genetic inactivation, we sought to analyze the consequences of prenatal THC administration on *CB1* expression. CB1R protein levels, as determined by Western blot, were significantly downregulated in THC-treated embryonic brains at E17.5 when compared to controls (Fig. R2.3A,C). Notably, at a perinatal stage

(P2.5), CB1R levels returned to those of the vehicle condition (Fig. R2.3B,D), indicating that CB1Rs are altered only transiently in our embryonic THC exposure paradigm.

The presence of functional plasma membrane-exposed CB1Rs was next analyzed by the binding of the radioactively-labeled synthetic CB1R agonist [^{3}H]CP55,940. A clear reduction in cannabinoid binding was observed in the brains of THC-treated embryos at E17.5 (Fig. R2.3E,G), though its values recovered to the level of vehicle-treated animals by P2.5 (Fig. R2.3F,H). Overall, these findings support that embryonic THC administration transiently disrupts appropriate CB1R function in the developing brain.

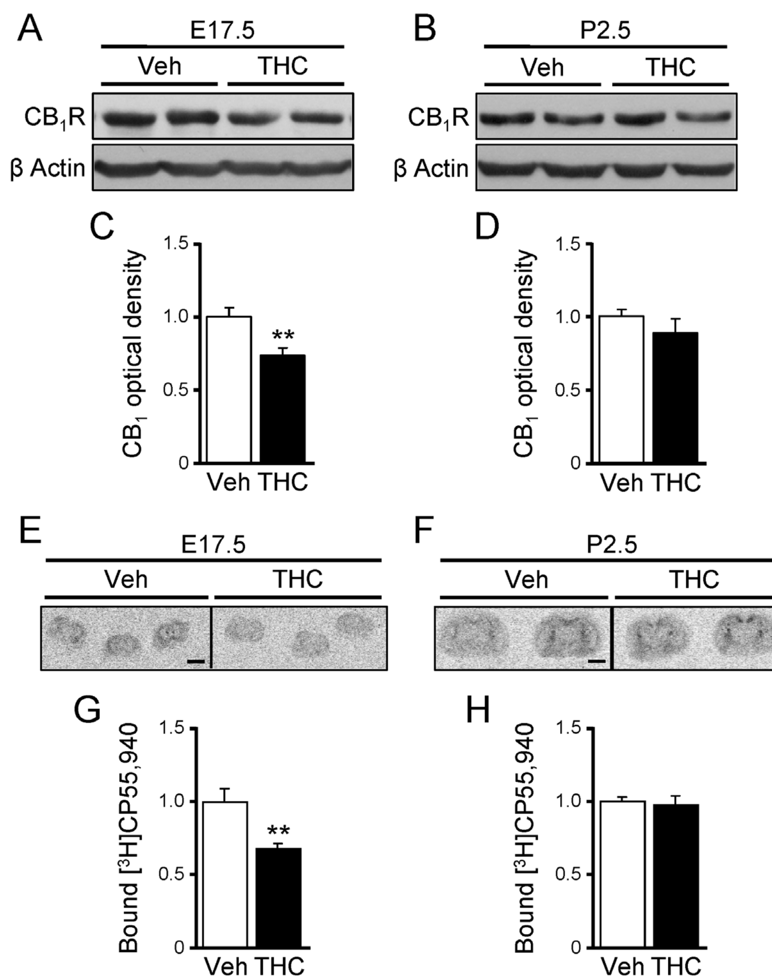

Figure R2.3. Embryonic THC exposure transiently downregulates CB1Rs. (**A-D**) CB1R protein levels were determined by Western blot in brain samples at E17.5 (**A**, **C**) and P2.5 (**B**, **D**) after THC or vehicle administration from E12.5 to E16.5. The optical density of CB1R band was quantified and normalized to β-actin [n = 9 and 6 (E17.5 vehicle- and THC-treated brain samples, respectively); n = 7 and 7 (P2.5 vehicle- and THC-treated brain samples, respectively)]. (**E-H**) Radiolabeled CP55,940 binding was quantified in coronal brain sections at E17.5 (**E**, **G**) and P2.5 (**F**, **H**) [n = 4 and 6 (E17.5 vehicle- and THC-treated brains, respectively); n = 8 and 8 (P2.5 vehicle- and THC-treated brains, respectively)]. **P<0.01 *vs*. vehicle-treated mice. Scale bars, 2 mm.

R2.4. Neuronal lineage-specific *CB1* reexpression selectively rescues the behavioral traits of embryonic THC exposure

To unequivocally determine the actions of embryonic THC administration upon specific neuronal populations, of we made use of a Cre-mediated, lineage-specific, embryonic *CB1* expression-rescue strategy in a *CB1*-null background (*Stop-CB1* mice) (Ruehle et al. 2013). The selective expression of CB1Rs in dorsal telencephalic glutamatergic neurons (*Glu-CB1-RS* mice) was achieved by expressing *Cre* under the regulatory elements of *Nex* locus (Ruehle et al. 2013).

In addition, we rescued *CB1* expression in forebrain GABAergic neurons (*GABA-CB1-RS* mice) by using the *Dlx5/6-Cre* mouse line (Monory et al. 2006). As control, a global rescue of *CB1* expression driven by *EIIa-Cre* (*CB1-RS*) was also used (Ruehle et al. 2013). Characterization of CB1R expression by immunofluorescence was performed in the different mouse lines (Fig. R2.4 and S2).

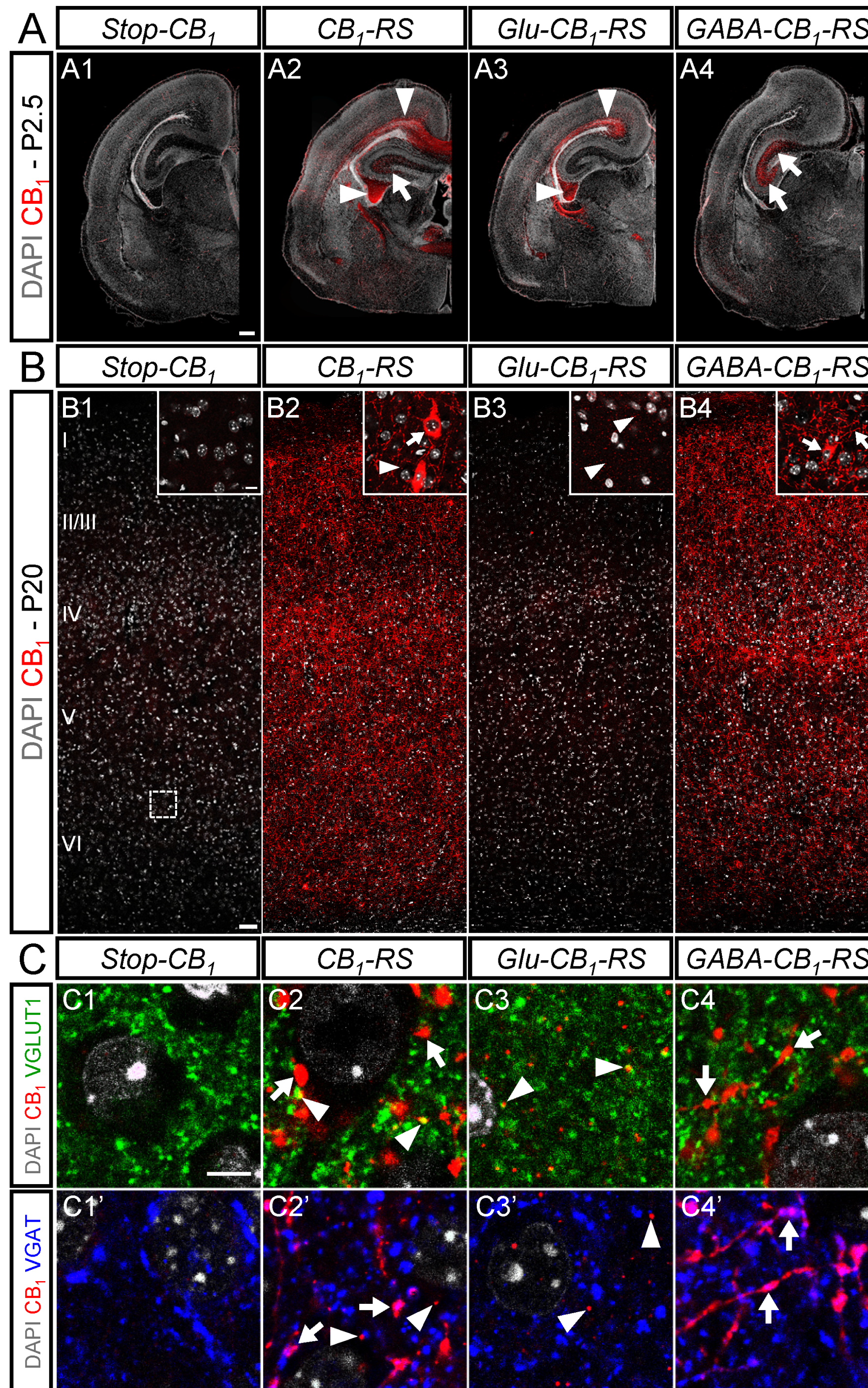

Figure R2.4. Characterization of selective *CB1* expression-rescue in dorsal glutamatergic neurons and forebrain GABAergic neurons. (**A**) Immunofluorescence analysis of CB1R reexpression driven by *Nex* (*Glu-CB1-RS*) (**A3**), *Dlx5/6* (*GABA-CB1-RS*) (**A4**), or *EIIa* (*CB1-RS*) (**A2**) promoters in *Stop-CB1* mice (**A1**) was performed in P2.5 coronal brain sections. (**B**) Cortical CB1R expression analysis in the same mouse strains was performed at P20 (**B1-B4**). (**C**) Double immunofluorescence was performed with anti-CB1R antibody combined with anti-VGLUT1 (**C1-C4**) or anti-VGAT (**C1'-C4'**) antibodies. Arrowheads point to glutamatergic CB1R signal, which is abundant in pyramidal neuron fibers at P2.5 (**A**), and to scarce immunopositive puncta corresponding to glutamatergic (VGLUT1-positive) terminals at P20 (**B** and **C**). Arrows point to the abundant CB1R signal corresponding to GABAergic (VGAT-positive) neurons within the hippocampal formation at P2.5 (**A**) and the mature cortex at P20 (**B** and **C**). Scale bars: (**A**) 200 µm; (**B**) 50 µm (inset, 10 µm); (**C**) 5 µm.

In *Stop-CB1* mice only background (non-specific signal) was detected, while in *Glu-CB1-RS* and *GABA-CB1-RS* animals CB1R immunoreactivity was observed with a distinctive pattern of expression (Fig. R2.4A-C). *Glu-CB1-RS* mice at P2.5 revealed a significant CB1R expression in descending corticofugal axons, while in *GABA-CB1-RS* mice a prominent CB1R expression was observed in the immature hippocampal formation (Fig. R2.4A1-A4). Cortical CB1R expression at P20 in *Glu-CB1-RS* mice appeared as scarce immunopositive *puncta*, in agreement with the low expression of the receptor in mature projection neurons (Soltesz et al. 2015), while most CB1R expression corresponded to GABAergic interneurons (Fig. R2.4B1-B4). Double immunofluorescence with VGLUT1 and VGAT, presynaptic markers of glutamatergic and GABAergic terminals, respectively, further confirmed the selectivity of the *CB1* expression-rescue strategy (Fig. 2.4C). Thus, in *Glu-CB1-RS* mice, the small *puncta* of CB1R did not colocalize with VGAT immunoreactivity, in agreement with their presynaptic location in glutamatergic neurons (Fig. R2.4C and Fig. S2).

To assess the reestablishment of the neurobiological substrate of THC-induced alterations, we quantified the number of $ER81^+$ cells in cortical layer V. Remarkably, in vehicle-treated *Glu-CB1-RS* mice the number of deep-layer $ER81^+$ cells per cortical column was significantly rescued when compared to *Stop-CB1* animals, and, in concert, *Glu-CB1-RS* mice gained susceptibility to THC-induced impairment of SCPN development (data not shown).

Once the lineage selectivity of the *CB1* expression-rescue strategy at the cellular level was proved, we investigated the functional impact of embryonic THC exposure in adulthood. THC or vehicle

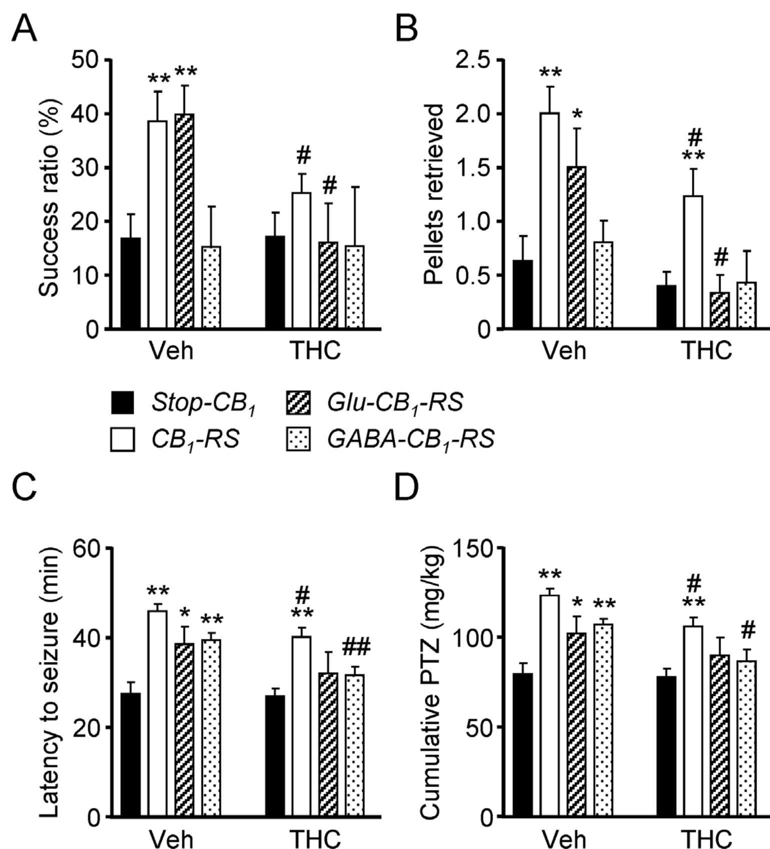

Figure R2.5. Selective CB1R expression-rescue restores functional alterations and reestablishes THC susceptibility in Stop-CB1 mice. (**A**, **B**) Skilled motor activity was assessed by the skilled pellet-reaching (**A**) and staircase (**B**) tests in adult CB1R-rescued mice [*n*= 15 and 15 (*Stop-CB1* vehicle- and THC-treated mice, respectively); *n* = 20 and 17 (*CB1-RS* vehicle- and THC-treated mice, respectively); *n* = 9 and 6 (*Glu-CB1-RS* vehicle- and THC-treated mice, respectively); *n* = 5 and 7 (*GABA-CB1-RS* vehicle- and THC-treated mice, respectively)]. (**C**, **D**) Seizure susceptibility to subconvulsive doses of PTZ was determined. Latency to seizures (**C**) and the cumulative dose of PTZ required (**D**) are shown [*n* = 13 and 19 (*Stop-CB1* vehicle- and THC-treated mice, respectively); *n* = 18 and 19 (*CB1-RS* vehicle- and THC-treated mice, respectively); *n* = 9 and 5 (*Glu-CB1-RS* vehicle- and THC-treated mice, respectively); *n* = 9 and 8 (*GABA-CB1-RS* vehicle- and THC-treated mice, respectively)]. $^*P<0.05$, $^{**}P<0.01$ *vs.* corresponding *Stop-CB1* mice; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* corresponding vehicle-treated group.

was administered to dams coming from matings of females *Stop-CB1* with *Nex-Cre;Stop-CB1* (*Glu-CB1-RS*) or *Dlx5/6-Cre;Stop-CB1* (*GABA-CB1-RS*) males, and their respective offsprings were analyzed at an adult age. *CB1* reexpression in *Glu-CB1-RS* mice, but not in *GABA-CB1-RS* mice, rescued the skilled motor deficits of *Stop-CB1* mice as assessed by both the skilled-reaching test (Fig. R2.5A) and the staircase test (Fig. R2.5B). Global rescue of *CB1* expression in *CB1-RS* mice also fully overcome the skilled motor deficits observed in *Stop-CB1* mice. Notably, as expected, prenatal THC-treatment elicit significant skilled motor deficits exclusively in *Nex-CB1-RS* and *CB1-RS* (Fig. R2.5A,B). Hence, *CB1* expression-rescue in dorsal telencephalic glutamatergic neurons is necessary and sufficient to confer THC susceptibility to corticospinal motor function.

Finally, we also analyzed PTZ-induced seizure susceptibility in the various *CB1* expression-rescued mice. The seizure-prone phenotype of *Stop-CB1* mice was partially restored in both *Glu-CB1-RS* and *GABA-CB1-RS*, and this effect was more remarkable when *CB1* was reexpressed systemically in *CB1-RS* mice (Fig. R2.5C,D). Remarkably, upon THC administration *Stop-CB1*, *Glu-CB1-RS* and *GABA-CB1-RS* mice were statistically indistinguishable and only *CB1-RS* mice retained a significant increase in seizure latency and PTZ cumulative dose compared to THC-exposed *Stop-CB1* mice. Notwithstanding, THC-exposed *CB1-RS* mice showed a significant increase in seizure susceptibility compared to vehicle-treated counterparts (Fig. R2.5C,D). These findings support the notion that prenatal CB1R signaling in both glutamatergic and GABAergic cell lineages is required for the appropriate balance of neuronal activity and overall brain excitability.

DISCUSSION

AIM1. ROLE OF THE ECS ON PYRAMIDAL NEURON RADIAL MIGRATION AND LONG-LASTING CONSEQUENCES OF DEVELOPMENTAL CB1R DYSFUNCTION

Overall, the findings corresponding to the first Aim of this Thesis reveal an unprecedented pivotal role of the cannabinoid CB1R signaling in the adequate migration and positioning of cortical pyramidal neurons, whose dysfunction can trigger profound and long-lasting alterations in brain function. It has been long assumed that adult *CB1*-deficient mice have increased seizure susceptibility owing to the loss of eCB-dependent retrograde suppression of glutamate release from excitatory terminals (Katona & Freund 2008; Soltesz et al. 2015). Of note, our evidence adds to this classical view by demonstrating that developmentally restricted loss of CB1R function also increases seizure susceptibility, likely by causing aberrant positioning of cortical neurons, thus conceivably affecting their wiring and sensitizing the resulting circuitry to epileptogenesis.

The classical idea sustains that the heterotopias are at the functional core of the characteristic hiperexcitability of MCDs, however, recent findings challenge this assumption and point to the involvement of the normotopic cortex as the major contributor to epilepsy (Cid et al. 2014; Petit et al. 2014). Another mechanism likely involved in the seizure-prone phenotype of si*CB1*-electroporated mice may be that mispositioned pyramidal neurons lead to an aberrant recruitment of GABAergic interneurons to the developing cortex (Lodato et al. 2011). It is plausible to assume that, upon developmental CB1R dysfunction, both the SBH and normotopic cells abnormally recruit interneuron subpopulations which, in turn, alter the excitatory/inhibitory balance or display altered wiring. Anyhow, the identification of the precise mechanisms responsible for the proepileptogenic phenotype require further investigation.

Neuronal migration is largely dependent on the dynamic regulation of the cytoskeleton (Nguyen & Hippenmeyer 2014). Actin cytoskeleton remodeling plays a fundamental role in this process, and the inhibition of RhoA function is a common feature of different promigratory pathways (Hand et al. 2005; Pacary et al. 2011; Tang et al. 2014; Azzarelli et al. 2014). The expression pattern of RhoA in the developing cortex, with high abundance in the VZ/SVZ and very low –if any– levels in the IZ and CP (Azzarelli et al. 2015), suggests that, from a cell-autonomous perspective, this protein is deleterious for pyramidal neuron migration, as demonstrated in a recent report (Cappello et al. 2012). RhoA ubiquitination and degradation plays an important role in the regulation of neuronal cell morphology (Bryan et al. 2005) and affects cancer cell migration (Nethe & Hordijk 2010). However, to the best of our knowledge, this is the first study that identifies a specific role of the proteasomal degradation of RhoA in the promotion of neuronal migration.

Taking into account the complementary expression pattern of both *CB1* and *RhoA* in the developing cortex, it is conceivable that CB1R-mediated RhoA degradation ensures a complete clearance of the remaining RhoA protein in newborn neurons to allow their migration. Our results are also in line with previous findings supporting that the actin cytoskeleton is a major

target of CB1R signaling in developing neurons (P. Berghuis et al. 2007; Oudin et al. 2011; Roland et al. 2014; Njoo et al. 2015). In addition, CB1R activation can reduce RhoB protein levels, and therefore we cannot exclude a potential contribution of this related GTPase in CB1R-dependent neuronal migration. Additionally, this observation raises the possibility that CB1R-dependent proteasomal degradation is a promiscuous mechanism affecting different Rho GTPases with interesting implications for a variety of cellular processes, but this remains to be explored.

We found that the migration defects caused by *CB1* silencing affect more dramatically a subset of the targeted GFP$^+$ cells. This might be related to the unequal knockdown efficacy among targeted cells and/or to the heterogeneity of a neuronal population in their capacity to compensate the loss of a given promigratory mechanism, as it occurs with other systems (Heng et al. 2008; Feliciano et al. 2011; Zheng et al. 2012; van den Berg et al. 2016). Similarly, in humans, MCDs frequently affect only relatively small neuronal populations, even when caused by germline mutations (Lim & Crino 2013; Barkovich et al. 2012). Interestingly, a recent study supports that the migration deficits caused by *Dcx* shRNA are due to off-target effects of such manipulation onto endogenous miRNAs (Baek et al. 2014). Nonetheless, the specificity of our CB1R genetic knockdown strategy and its consequences on radial migration are validated by the comparable phenotypes observed when using *CB1* siRNA, *CB1* shRNA, and Cre-mediated ablation of *CB1* in *CB1*$^{f/f}$ mice. Noteworthy, the severe migration defects observed upon acute *CB1* knockdown are notably subtler upon constitutive *CB1* mutation (Fig. S1.3A,B) and do not result in the formation of heterotopias. This is most likely due to compensatory mechanisms occurring in germline or lineage-specific knockout mice, as also occurs for other migration regulatory proteins such as Dcx (Bai et al. 2003), amyloid precursor protein (Young-Pearse et al. 2007), and EF-hand domain-containing protein 1 (de Nijs et al. 2009).

A wide variety of neurodevelopmental diseases are caused by the disruption of neuronal migration. Understanding the biological mechanisms responsible for a finely tuned corticogenesis emerges as a key requisite for the elaboration of rational therapeutic strategies for the consequences of MCDs, including epilepsy and neuropsychiatric disorders. Notably, a genetic origin has been identified for some human diseases caused by neuronal migration alterations, and the genes identified in such diseases correspond, in most cases, to cytoskeletal or cytoskeleton-regulatory proteins (Barkovich et al. 2012), however, mutation of most of the genes so far identified in human patients fail to mimic the phenotypes in mouse models (Cappello 2013).

Given this conceptual frame, the implications of the malfunctioning of the eCB system in the origin of neurodevelopmental disorders caused by cell migration defects are exciting perspectives for future research. Remarkably, evidences in the literature associate genetic alterations (*i.e.*, copy number variations) and gene polymorphisms of some of the eCB system elements as *CNR1*, *DAGLA*, *NAPEPLD*, *ABHD12*, or *CNRIP1*, with the occurrence of human diseases, such as autism spectrum disorders and intellectual disability, or the polyneuropathy, hearing loss, ataxia,

retinitis pigmentosa and cataract syndrome named as PHARC (Fiskerstrand et al. 2010; Bragin et al. 2014). Particularly, the CB1R-encoding *CNR1* gene has been put forward as a strong autism-related candidate gene, given the increased status of *de novo* mutations in this gene in samples from a cohort of 2588 autistic patients (Girirajan et al. 2013) or the strikingly high probability of *CNR1* haploinsufficiency (Huang et al. 2010). In agreement with these indications, CB1R signaling regulates neuronal identity by controlling the transcriptional factor axis *Ctip2-Satb2* (Díaz-Alonso et al. 2012), that together with *Tbr1*, is responsible for neuronal connectivity deficits that associate with mental retardation and autism (Carpentier et al. 2013; Deriziotis et al. 2014; Huang et al. 2014).

Alterations of multiple cellular mechanisms frequently converge in the pathogenesis of MCDs, and an unbalanced activity of CB1R signaling can affect cortical development by interfering with several processes in addition to neuronal migration (Diaz-Alonso et al. 2012). While SBH is most frequently originated from abnormal neuronal migration, some neocortical heterotopias are associated with ectopic progenitor cell divisions (Kielar et al. 2014). CB1R signaling controls the activity of the PI3K/mTORC1 pathway, both in neuronal precursors (Díaz-Alonso et al. 2014) and in mature neurons (Puighermanal et al. 2009), and the deregulation of this signaling route is at the origin of some focal MCDs, in particular tuberous sclerosis, type 2 focal cortical dysplasia, and megalencephaly (Lim & Crino 2013). Notably, mTORC2 signaling has been shown to control actin cytoskeleton by modulating Rho-GTPases and dysregulation of the complex is associated to a variety of neurodevelopmental pyschiatric disorders (Jacinto et al. 2004; Costa-Mattioli & Monteggia 2013). Despite there are no evidences to the date relating CB1R signaling with mTORC2 activity, a putative connection between both signaling platforms is an option that is worth tackling.

Beyond genetic alterations in elements of the cannabinoid signaling machinery, environmental insults can also affect CB1R-dependent neuronal migration. In particular, prenatal exposure to the cannabinoids present in marijuana has been shown to affect fetal development in both mice and humans (Hurd et al. 2005; Jutras-Aswad et al. 2009; Tortoriello et al. 2014). Detrimental consequences in executive function have been reported in studies following children from mothers that smoked marijuana during pregnancy (Fried & Smith 2001; Smith et al. 2004), although the neurobiological substrate of these changes remains largely unknown. Altered CB1R signaling induced by long half-life phytocannabinoids may eventually interfere with neuronal migration, as previously shown for synthetic cannabinoids (Saez et al. 2014). Likewise, given the capacity of sustained CB1R stimulation to induce transient CB1R downregulation –thus short-term *CB1* loss of function– gestational cannabinoid exposure may disturb cortical laminarization and cause changes in social behavior and cognition (Diaz-Alonso et al. 2012). Cannabinoids have a great potential as medicines owing to the broad distribution of their receptor targets throughout the body and pleiotropic functions, together with their high safety and fair tolerability (Mechoulam et

al. 2014; Pertwee 2012). In particular, CBD, the most relevant nonpsychoactive phytocannabinoid, has recently received a huge attention as a promising pharmacological tool for the management of refractory pediatric epilepsies, such as the Dravet and Gaston-Leroux syndromes (Devinsky et al. 2014). However, as our study and other pieces of evidence support (Maccarrone et al. 2014), cannabis intake, for both therapeutic and recreational use, must be exquisitely controlled if not absolutely discouraged during pregnancy or critical ontogenic periods in order to avoid interferences with the crucial role played by CB1Rs during brain development.

AIM2. IMPACT OF PRENATAL EXPOSURE TO THC ON BRAIN'S DEVELOPMENT AND FUNCTION

The evidence shed by the second Aim of this Thesis reveals that THC administration during pregnancy exerts long-lasting consequences in the offspring owing to a transient disruption of CB1R signaling that impedes the adequate temporally and spatially confined function of the receptor in neuronal development. Remarkably, the deleterious consequences of prenatal THC exposure in the progeny are independent of the classical neuromodulatory role of CB1R signaling in the adult brain and emerge as a consequence of the transient disruption of physiological CB1R signaling during prenatal development, before synaptic neuronal activity is established. These findings provide previously unidentified preclinical evidence for the risk of cannabis consumption during pregnancy. Cannabis is, by far, the most commonly consumed illicit drug during pregnancy in Western countries, and therefore its use constitutes a considerable public health issue. Over the last few decades, longitudinal studies on human cohorts (Volkow et al. 2014; Fried & Smith 2001), as well as research using animal models (Schneider 2009), have addressed the impact of early cannabinoid exposure in adulthood. The majority of these studies suggest that early cannabinoid exposure sensitizes the CNS to cognitive impairments, increases the risk of neuropsychiatric disorders such as schizophrenia and anxiety, induces cross-generational epigenetic modifications and enhances drug-addiction susceptibility (Szutorisz et al. 2014; Sonon et al. 2015; Rubino et al. 2015; Szutorisz & Hurd 2016). Noteworthy, there are confounding factors affecting the interpretation of these results as the outcome of maternal cannabis consumption on infant behavior or cognition are inconsistent among different studies, and some crucial variables are often excluded. Therefore, conclusions driven from them should be taken with caution (Rogeberg 2013; Huizink 2014; Volkow & Baler 2015). Our findings show that exposure to relatively low doses of THC in a narrow temporal window during prenatal development negatively impacts mouse cortical development, and this, in turn, has long-term functional consequences on the mature offspring. Specifically, we unequivocally identify the pool of CB1Rs located on developing cortical glutamatergic neurons as the sole reason for the deficits in corticospinal function induced by embryonic THC exposure.

The CB1R plays a pivotal neurodevelopmental role by transducing information from the endocannabinoid ligands present in the neurogenic niche into the coordination of the intrinsic developmental program of developing neurons (Diaz-Alonso et al. 2012; Galve-Roperh et al. 2013). In agreement, early developmental exposure of chicken embryos to a THC analog (Psychoyos et al. 2008) and genetic manipulation of the CB1R-interacting protein CRIP1 in *Xenopus laevis* development (Zheng et al. 2015) have been shown to disrupt the appropriate balance of transcription factors that intrinsically drive neural development. Thus, CB1R signaling refines the molecular, laminar, and hodology of projection neurons in the cerebral cortex (Maccarrone

et al. 2014; Diaz-Alonso et al. 2012). The present findings support that sustained exposure to THC may act as a functional suppressor of CB1R signaling, as THC interferes with developmental *CB1* function in a transient but functionally impacting manner. Therefore, prenatal cannabinoid exposure recapitulates the long-term structural and functional deficits in corticospinal connectivity previously demonstrated in *CB1* knockout mice (Díaz-Alonso et al. 2012). The susceptibility of axonal connectivity to *CB1* loss of function may persist at later developmental stages in susceptible areas and critical periods, as it has been demonstrated that THC consumption in adolescents can also result in axonal connectivity deficits (Zalesky et al. 2012). Nevertheless, in another study, daily marijuana consumption did not induce volumetric changes in white matter nor several brain areas (Weiland et al. 2015).

Our findings are in partial agreement with a previous study reporting that chronic prenatal THC administration alters neuronal connectivity by disrupting cytoskeletal dynamics in motile axons. Specifically, prolonged THC administration (from E5.5 to E17.5) was shown to disrupt cannabinoid signaling, interfering with MAGL and DAGLα –enzymes responsible for 2-AG metabolism– and also induced CB1R downregulation (Tortoriello et al. 2014). However, in that study, the functional consequences of chronic prenatal THC administration on axonal projections were not determined, instead they focused on characterizing axon fasciculation abnormalities. Manipulation of endocannabinoid levels in perinatal stages by chronic pharmacological inhibition of FAAH, the main anandamide-degrading enzyme, induced depressive and cognitive impairment traits, despite the fact that no specific neuronal development alterations could be demonstrated (Wu et al. 2014). On the other hand, pharmacological inhibition of MAGL increased 2-AG levels that, acting on CB2Rs present on oligodendrocytes, induced axon fasciculation alterations by interfering with Slit2/Robo1 signaling, although the functional consequences of these actions remain unknown (Alpár et al. 2014). Other studies have also shown that perinatal cannabinoid administration induces cognitive deficits that can be linked to neuronal transmission adaptations, particularly plasticity of glutamatergic neuron activity and aberrant synaptic organization (Mereu et al. 2003; Antonelli et al. 2005; Bernard et al. 2005; Rubino et al. 2015). Thus, whereas it is already known that postnatal cannabinoid exposure may have negative consequences on some neurological functions in the adult brain (Volkow et al. 2014), our findings unveil long-lasting functional brain alterations induced by restricted prenatal cannabinoid administration that can be unequivocally ascribed to specific developing neuronal lineages. Notably, the results shown above reveal a direct impact of THC administration on the developing embryo that does not rely on indirect consequences of maternal programming and that is evident without the requirement of a second hit, as proposed for cannabis-induced risk of psychosis (Calvigioni et al. 2014). In any case, translating the long-term implications of developmental cannabis exposure into humans requires a very stringent control of confounding factors (Rogeberg 2013; Volkow & Baler 2015), and this is more important when analyzing cognitive and psychiatric traits than merely determining motor

performance or general brain excitability.

In addition to corticospinal motor function alterations, embryonic THC exposure increased seizure susceptibility in adult mice, even when CB1Rs return to normal levels shortly after cessation of THC exposure. The neuromodulatory role of CB1Rs in the mature brain act as a synaptic circuit breaker (Katona & Freund 2008; Soltesz et al. 2015), crucial for the control of brain excitability. Accordingly, acute treatment with THC exerts anticonvulsant effect in various models (Karler et al. 1974; Turkanis et al. 1979). In addition, great interest has recently emerged in the potential application of cannabis preparations enriched in CBD for the management of pediatric epilepsy disorders such as Dravet and Gaston-Leroux syndromes (Devinsky et al. 2014). However, results presented herein demonstrate that THC interferes with the developmental role of *CB1* and induces a proepileptogenic neural circuitry configuration independently of its neuromodulatory role in the adult brain.

Our *CB1* expression-rescue experiments show that the THC-induced increase in seizure susceptibility relies on alterations not only of PNs but also of GABAergic neurons, supporting a specific role of *CB1* in the development of forebrain GABAergic neurons. In one (not very convincing) study, the authors claimed that prenatal THC increases CCK^{+} interneuron density in the hippocampus (Berghuis et al. 2005), whereas in a posterior study from the same group they describe a contrasting effect of diminished hippocampal $CB1R^{+}$ basket cell innervation (Tortoriello et al. 2014). Notably, our results are more easily reconcilable with the second case, whereby prenatal cannabinoid exposure leads to an inhibitory deficit that likely accounts for the proepileptogenic phenotype of THC-exposed *GABA-RS* mice.

Further investigations are required to underscore the potential contribution of these, and perhaps other, neuronal lineages targeted by prenatal cannabinoid exposure. Anyhow, our preclinical observations support that although cannabis preparations can exert anticonvulsive actions in children and adults, they could also enhance the risk of seizures –by suppressing CB1R function– when administered before birth, thus raising a note of caution that might be considered when the potential therapeutic uses of cannabinoid-based medicines are defined and regulated for pregnant women.

CONCLUSIONS & PERSPECTIVES

The accumulated evidences harvested during the process of this Doctoral Thesis allow us to delineate the main conclusions of this work.

I) The CB1 cannabinoid receptor tightly controls the process of radial migration of newborn pyramidal neurons during mouse embryonic development.

II) Engagement of CB1Rs by endocannabinoids in newborn pyramidal neurons ensure the required cell-autonomous withdrawal of RhoA to endure radial migration and final positioning within the cerebral cortex.

III) Subchronic administration of THC (for 5 consecutive days) at a dose of 3 mg/Kg consistently induces a substantial downregulation of CB1Rs that mimics the phenotypic traits of *CB1* genetic deficiency. Therefore, this developmental, temporally-restricted protocol constitutes a useful tool to interrogate the developmental *CB1*-specific functions and the functional outcome of their disruption.

IV) Prental exposure to THC interferes with the developmental role of CB1 and induces a proepileptogenic neural circuitry independently of its neuromodulatory role in the adult brain.

V) Developmental CB1R signaling safeguards proper corticogenesis, since genetic or pharmacological manipulations lead to profound and long-lasting cellular and functional alterations in the progeny, with particular relevance for the skilled motor performance and overall brain excitability.

VI) Alterations of the embryonic ECS function might be responsible, or contribute to some Malformations of Cortical Development (MCDs) with increased epileptogenesis.

Uncountable questions rise in view of these results that deserve proper research, and hopefully, an answer.

For instance, it would be quite interesting to evaluate whether embryonic THC-dependent CB1R downregulation may have an impact on the process of pyramidal neuron radial migration, as we observed with genetic downregulacion.

Additionally, go further into the cellular and molecular mechanisms responsible of the increased seizure susceptibility in the case of both genetic and pharmacological manipulation, as well as to address whether there is a real contribution of SBH to the phenotype, is a noteworthy issue.

To finish, finely dissect the functions of the different CB1R pools (i.e. CB1Rs expressed by different neuronal subpopulations) and their precise contribution to the observed –and other– phenotypic manifestations of prenatal ECS dysfunction, ensure a long way in the exciting field of research on the role of the Endocannabinoid System during mammalian brain development.

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ADDENDA

SUPPLEMENTARY FIGURES

Aim 1: Role of the ECS on pyramidal neuron radial migration and long-lasting consequences of developmental CB1R dysfunction.

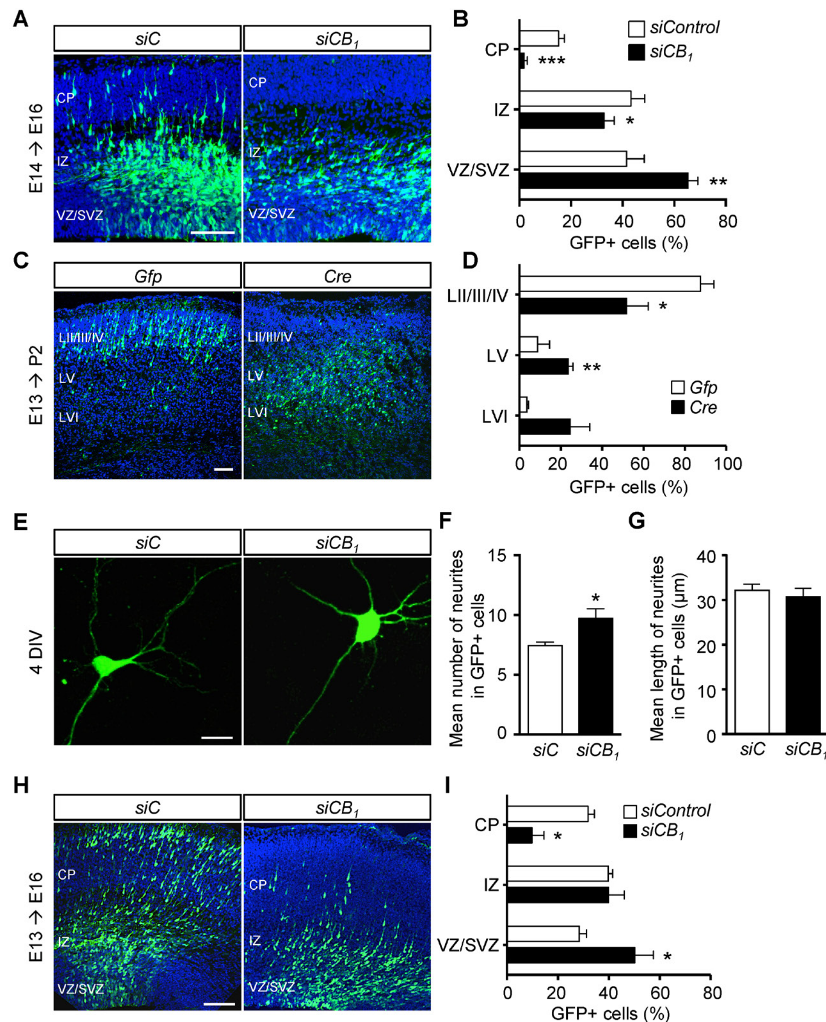

Figure S1.1. CB1 siRNA-mediated neuronal migration arrest operates in different pyramidal neuron lineages. (**A**, **B**) Migration analysis of cortical neurons electroporated *in utero* with *siCB1* or a control siRNA together with a *Gfp* expression plasmid at E14.5 and analyzed at E16.5. Representative images and neuronal quantification in the indicated cortical compartments are shown. (**C**, **D**) Migration analysis of *CB1$^{f/f}$* cortical neurons electroporated in utero with *Cre* or a control *Gfp* at E13.5 and analyzed at P2. (**E-G**) Morphological assessment of primary cortical neurons electroporated with *siCB1* or control siRNA at E14.5 and maintained in differentiation conditions for 4 DIV. The number of primary neurites and the mean neurite length were calculated. (**H**, **I**) Migration analysis of cortical neurons electroporated in utero with *siCB1* or *siControl* together with a *Gfp* expression plasmid at E13.5 and analyzed at E16.5. *n*= at least 3 different embryos from different litters per condition and 3 independent experiments with at least 100 GFP^{+} cells from 6 imaging fields for morphological experiments in vitro. Graphs represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scale bars: **A**, **C**, **H**, 50 µm; **E**, 15 µm.

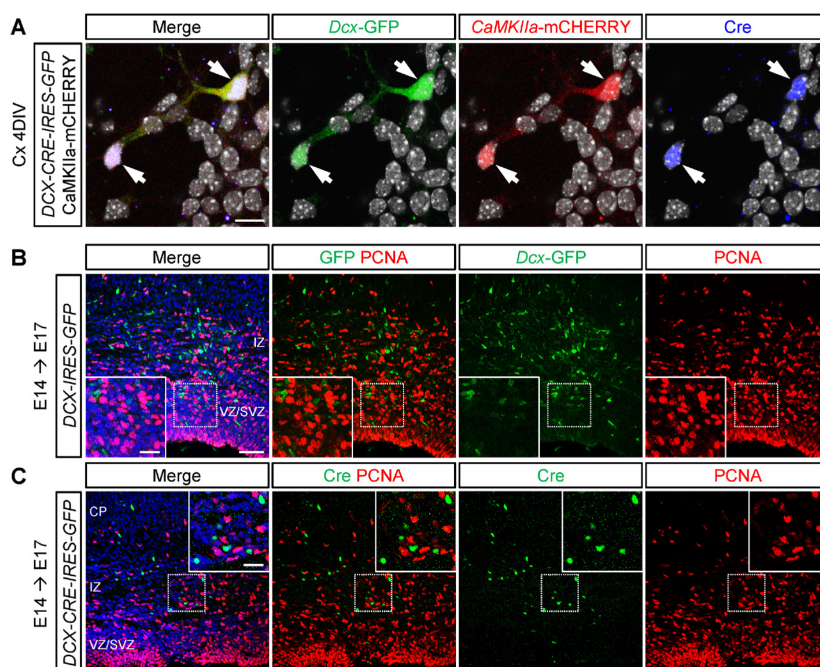

Figure S1.2. Selectivity of conditional Dcx-driven Cre expression plasmid. (**A**) E14.5 cortical neurons were electroporated *ex utero* with a *Dcx*-driven *Cre* expression plasmid with intrinsic GFP, together with a *CaMKIIa*-driven *mCherry* expression plasmid, in order to speciffically address GFP, mCHERRY and Cre expression restricted to postmitotic principal neurons. (**B-C**) A *Dcx*-driven *Cre* expression plasmid with intrinsic GFP was electroporated in utero and embryos analized at E17.5. Codistribution analysis of the proliferative marker PCNA along with intrinsic *Dcx*-GFP expression (**B**) or with *Dcx*-driven Cre expression (**C**) were performed. Representative images are shown. Scale bars: A, 10 µm; B, C, 40 µm (insets 20 µm).

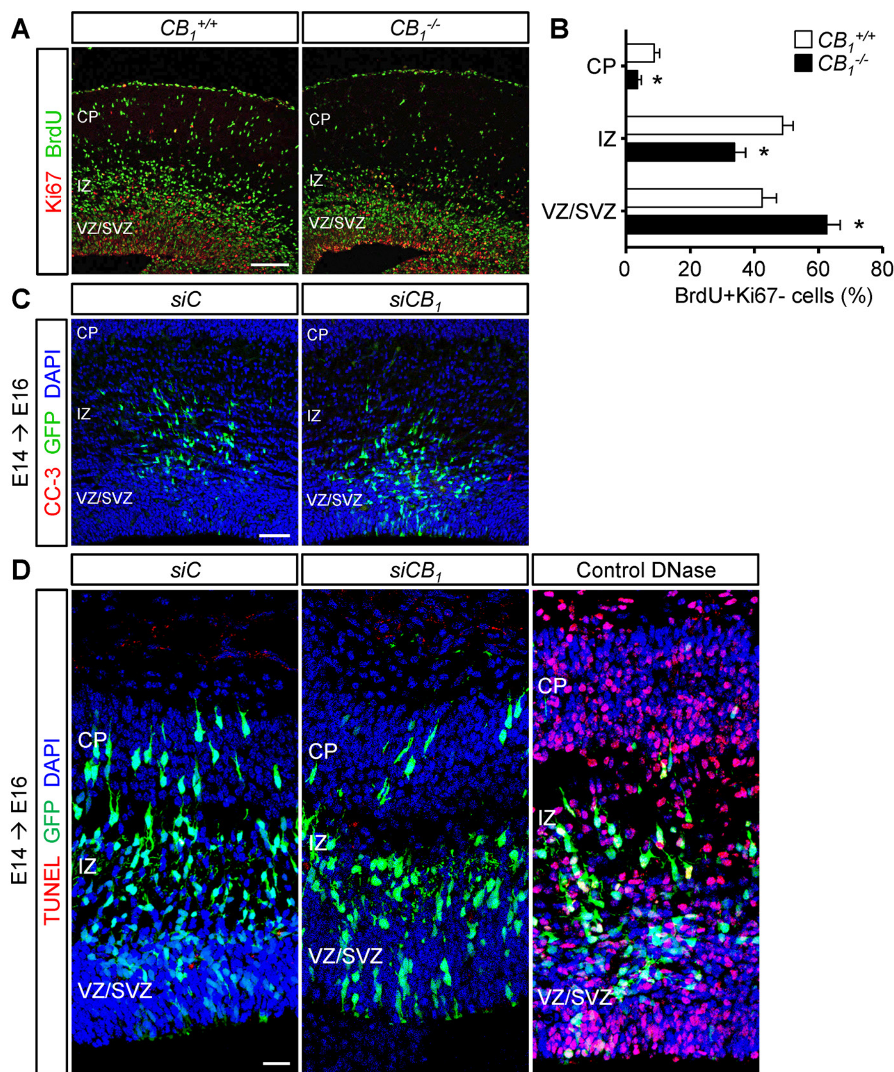

Figure S1.3. *CB1* regulation of radial migration is postmitotic and does not involve changes in cell survival. (**A**, **B**) Analysis of the migration of BrdU+Ki67- cells at E16.5 in $CB1^{-/-}$ embryos and their $CB1^{+/+}$ littermates injected with BrdU at E14.5. (**C**, **D**) Immunofluorescence analysis of cleaved caspase-3 (CC-3) and TUNEL staining in *siCB1* and control siRNA-electroporated brains. DNase treated sections were employed as positive control in TUNEL analysis. *n*= 4 embryos per genotype for each experiment. Graphs represent mean ± SEM. *P < 0.05. Scale bars: **A**, **C** 100 µm; **D**, 20 µm.

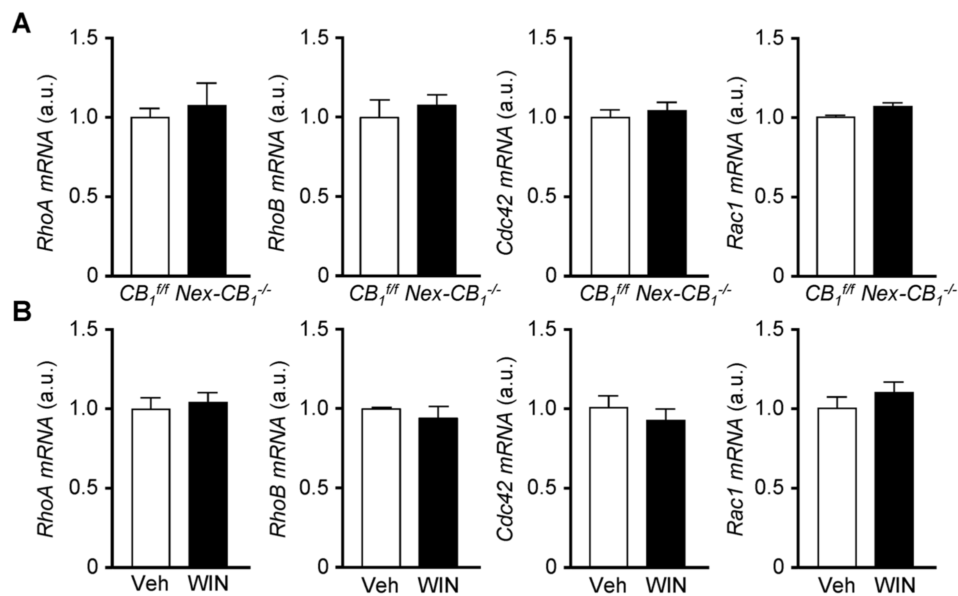

Figure S1.4. CB1 receptor manipulation does not affect RhoA, RhoB, Cdc42 or Rac1 mRNA expression. (**A**) qPCR analysis of the expression levels of *RhoA*, *RhoB*, *Cdc42* and *Rac1* in E17.5 $Nex\text{-}CB1^{-/-}$ and $CB1^{f/f}$ embryonic cortices. (**B**) qPCR analysis of the expression levels of *RhoA*, *RhoB*, *Cdc42* and *Rac1* in E17.5 WIN 55,212-2-treated embryonic cortices. *n*= at least 3 different embryos per group. Graphs represent mean ± SEM.

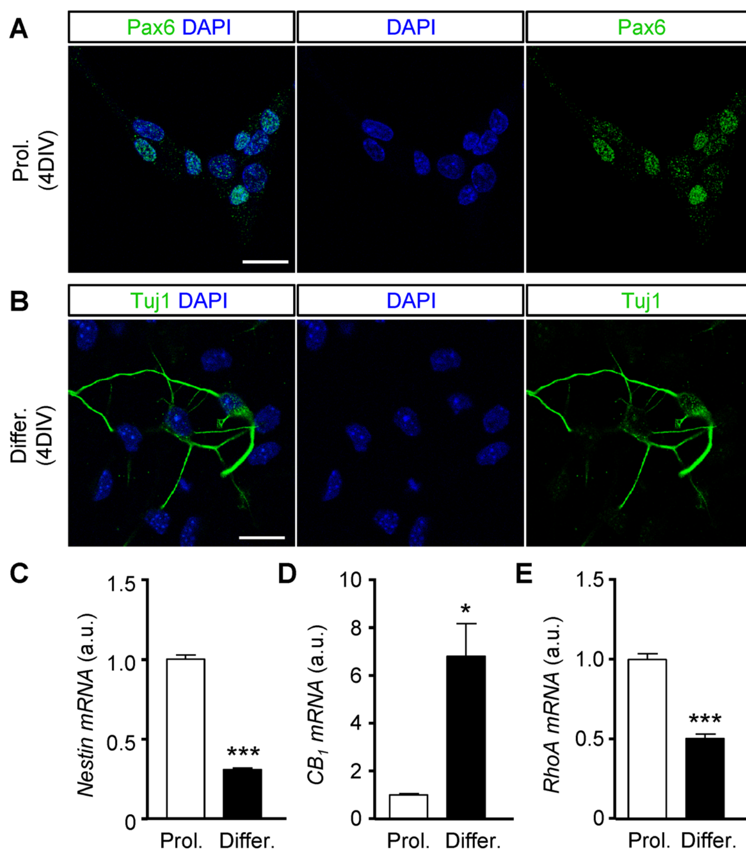

Figure S1.5. Differentiation of adherent cortical progenitor cell (CPC) cultures can model the cellular context of migrating neurons in the developing cerebral cortex. (**A**, **B**) Characterization of the CPC cultures in proliferation and differentiation conditions. The expression of Pax6 and Tuj1 was analyzed in order to identify their radial glial-like dorsal telencephalic progenitor identity and their ability to undergo neuronal differentiation, respectively. (**C-E**) qPCR analysis of the expression levels of *nestin*, *CB1* and *RhoA* in proliferating vs differentiating CPCs. *n*= 3 independent experiments. Graphs represent mean ± SEM. *P < 0.05; ***P < 0.001. Scale bars: 25 μm.

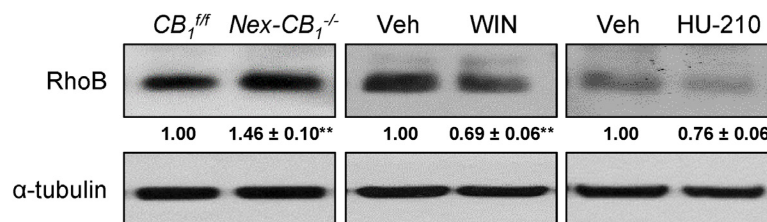

Figure S1.6. CB1 signaling controls RhoB protein levels in pyramidal neurons. (**A**) RhoB protein levels were determined by Western blot in cortical tissue derived from E17.5 *Nex-CB1*$^{-/-}$ and *CB1*$^{f/f}$ littermates; E17.5 embryos exposed to the CB1R agonist WIN 55,212-2 (5 mg/Kg) or its vehicle for 6 hours and HU-210 (50 nM) or its vehicle for 6h in primary differentiating CPCs. Loading control was performed with anti α-tubulin antibody. *n*= at least 3 different embryos per genotype or treatment, and 3 independent CPC cultures.

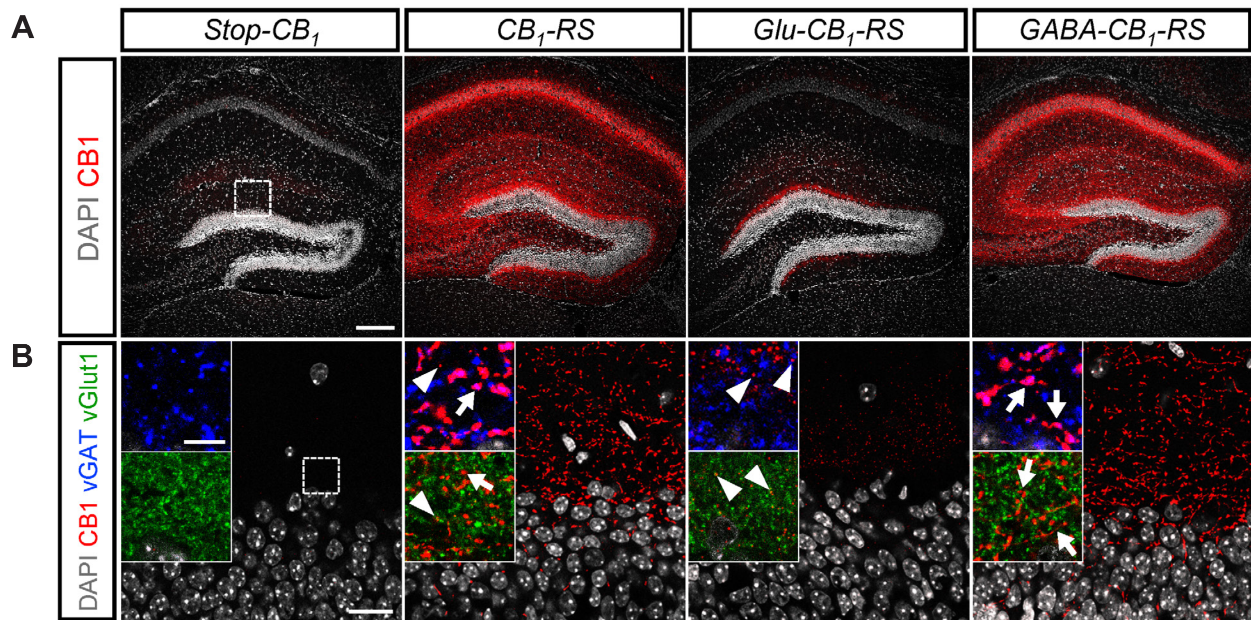

Figure S2. Characterization of selective *CB1* expression-rescue in dorsal glutamatergic neurons and forebrain GABAergic neurons of adult mouse hippocampus. (**A**) Immunofluorescence analysis of CB1R reexpression driven by *Nex* (*Glu-CB1-RS*), *Dlx5/6* (*GABA-CB1-RS*), or *EIIa* (*CB1-RS*) promoters in *Stop-CB1* mice was performed in P20 coronal brain sections. (**B**) Double immunofluorescence was performed with anti-CB1R antibody (red) combined with anti-VGLUT1 (green) or anti-VGAT (blue) antibodies. Arrowheads point to scarce immunopositive puncta corresponding to glutamatergic (VGLUT1-positive) terminals in CA1 at P20. Arrows point to the abundant CB1R signal corresponding to GABAergic (VGAT-positive) neurons within the hippocampal CA1 at P20. Scale bars: (**A**) 200 µm; (**B**) 20 µm (inset, 5 µm)

PAPERS RESULT OF THIS THESIS

1. Díaz-Alonso, J., Aguado, T., **de Salas-Quiroga, A**., Ortega, Z., Guzmán, M., & Galve-Roperh, I. (2014). 'CB1 Cannabinoid Receptor-Dependent Activation of mTORC1/Pax6 Signaling Drives Tbr2 Expression and Basal Progenitor Expansion in the Developing Mouse Cortex'. *Cereb. Cortex*, 25(9), 2395-2408.

2. **de Salas-Quiroga, A.***, Díaz-Alonso, J.*, García-Rincón, D., Remmers, F., Vega, D., Gómez-Cañas, M., Lutz, B., Guzmán, M., and Galve-Roperh, I. (2015) 'Prenatal exposure to cannabinoids evokes long-lasting functional alterations by targeting CB1 receptors on developing cortical neurons'. *Proc. Natl. Acad. Sci. USA*, 112(44), 13693–13698.

3. Díaz-Alonso, J.*, **de Salas-Quiroga, A.***, Paraíso-Luna, J., García-Rincón, D., Garcez, P., Parsons, M., Andradas, C., Sánchez, C., Guillemot, F., Guzmán, M. and Galve-Roperh, I. (2016) 'Loss of Cannabinoid CB1 Receptors Induces Cortical Migration Malformations and Increases Seizure Susceptibility'. *Cereb. Cortex* [Epub ahead of print].