Molecular mechanisms regulating the intracortical migration of interneurons

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ABBREVIATIONS

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Ac	Anterior commissure
AEP	Anterior entopeduncular domain
Alcam	Activated leukocyte cell adhesion molecule
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Anterior-posterior
ARIA	Acetyl choline receptor inducing activity
ARX	Aristaless-Related Homeobox
BACE	β-site of amyloid precursor protein cleaving enzyme
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
Ca2+	Calcium
CB	Calbindin
Cbln4	Cerebellin 4
CCK	Cholecystokinin
Cdh	Cadherin
CfuPN	Corticofugal projection neurons
CGE	Caudal ganglionic eminence
Citp2	COUP-TF-interacting protein 2
CNS	Central nervous system
COS	CV-1 origin SV40
Coup-tf	Chicken ovalbumin upstream promoter-transcription factor
CP	Cortical plate
CpU	Caudate Putamen
CPNs	Callosal projection neurons
CR	Calretinin
CRD	Cystein-rich domain
CRs	Cajal-Retzius Cells
CSMNs	Corticospinal motor neurons
CthPN	Corticothalamic projection neurons
Cux	Cut like
CXCL/R	Cystein X cystein ligand/receptor
Cx3cl/r1	Chemokine (C-X3-C Motif) Ligand 1
DAPI	4',6-diamidino-2-phenylindole
Dbx1	Developing brain homeobox protein 1
Dgcr8	DiGeorge syndrome critical region gene 8
Dlx	Distal-less homeobox
DP	Dorsal pallium
DsRed	Discosoma sp. Red
DV	Dorso-ventral
E	Embryonic
EGF	Epidermal growth factor
Emx	Empty spiracles homologue

Eph R	Ephrin receptor
ErbB	Erythroblastic leukemia viral oncogene
ERK	Extracellular regulated kinase
FACS	Fluorescent activated cells sorting
FDR	False Discovery Rate
Fezf2	Fez family zinc finger 2
Fgf	Fibroblast growth factor
Fgfr	Fibroblast growth factor receptor
Foxg1	Forkhead box G1
GABA	Gamma aminobutiric acid
GDNF	Glial cell-derived neurotrophic factor
GE	Ganglionic eminences
GGF	Glial growth factor
GFP	Green fluorescence protein
GFAP	Glial fibrillary acidic protein
Gi	Inhibitory G protein
GP	Globus pallidus
GPCR	G-protein coupled receptor
GRK	G protein-coupled receptor kinases
Gsh	Genomic screened homeobox
GTP	Guanosine 5'-triphosphate
H	Hippocampus
HER	Receptor tyrosine-protein kinase erbB
HGF	Hepatocyte growth factor
Htr3a	5-Hydroxytryptamine (Serotonin) Receptor 3A, Ionotropic
ICD	Intracellular domain
IG	Infragranular
Ig	Immunoglobulin
IN-Cxcr7	Dlx5,6-Cre-IRES-GFP; Cxcr7flox/flox
IP3	Inositol triphosphate
IRES	Internal ribosome entry site
IZ	Intermediate zone
JAK	Janus kinase
JMa	Juxtamembrane
KCC	Potassium chloride co-transporter
Kcnd	Potassium voltage-gated channel shal-related subfamily D
Kcnh	Potassium voltage-gated eag-related subfamily H
LDL	Low density lipoprotein
LGE	Lateral ganglionic eminence
Lhx	LIM homeobox
LP	Lateral pallium
MafB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MAPK	Mitogen activated protein kinase
Mash	Mammalian achaete-schute homolog

MGE	Medial ganglionic eminence
MP	Medial pallium
mRNA	Messenger ribonucleic acid
Mek	Mitogen-activated protein kinase-1
Mme	Membrane metallo-endopeptidase
MZ	Marginal zone
NCx	Neocortex
NDF	Neu differentiation factor
Neo	Neomycin
NeuroD	Neurogenic differentiation
Nrg	Neuregulin
Ngn	Neurogenin
Nkx	Nirenberg kin homeobox
NMR	Nuclear magnetic resonance
nNOS	Nitric oxide synthase
NPY	Neurpeptide Y
Nrg	Neuregulin
Npn1/2	Neuropilins 1 and 2
NT4	Neurotrophin 4
OPC	Olig2-dependant oligodendrocyte precursor cell
P	Postnatal
Pax	Paired box
Pcdh	Protocadherin
PBS	Phosphate buffered saline
pERK	Phosphorylated extracellular regulated kinase
PI3K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PLLP	Posterior lateral line primordium
PN	Projection neurons
POA	Preoptic area
PP	Preplate
Ppp2r2c	Protein Phosphatase 2, Regulatory Subunit B, Gamma
Prox1	Prospero homeobox 1
Ptprr	Protein Tyrosine Phosphatase, Receptor Type, R
PV	Parvalbumin
Raf	Raf Proto-Oncogene, Serine/Threonine Kinase
RGCs	Radial glia cells
RIN	RNA integrity number
RNA	Ribonucleic acid
Robo	Roundabout
S	Septum
Satb2	Special AT-rich sequence-binding protein 2
SCPN	Subcerebral projection neurons
Sdf1	Stromal derived factor 1

SEM	Stardard error of the mean
Sema	Semaphorin
Sfrp	Secreted frizzled-related protein
SG	Supragranular
Shh	Sonic hedgehog
Sip1	Smad interacting protein-1
Slc24a4	Solute Carrier Family24, Sodium/Potassium/Calcium Exchanger, Member 4
Slit 1/2	Slit homolog protein ½
SMDF	Sensory and motor neuron derived factor
Sox	SRY-box
Sp8	Trans-acting transcription factor 8
Spock3	Sparc/Osteonectin, Cwcv & Kazal-Like Domains Proteoglycan (Testican) 3
Src	V-SRC Avian Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene
SS	Somatosensorial
SST	Somatostatin
Sstr	Somatostatin receptor
STAT	Signal transducer and activator of transcription
Str	Striatum
SVZ	Subventricular zone
Syt	Synaptotagmin
TACE	Tumour necrosis factor-α converting enzyme
TFR	Transferrir receptor
TAG	Transient axonal glycoprotein
Tbr1	T-box brain gene 1
Timp2	TIMP Metallopeptidase Inhibitor 2
Tmeff	Transmembrane Protein EGF-Like And Two Follistatin-Like Domains
TrkB	Tyrosine Kinase B
TrkBR	Tyrosine Kinase B Receptor
uPAR	Urokinase-type plasminogen activator receptor
VIP	Vasoactive intestinal peptide
VP	Ventral pallium
VZ	Ventricular zone
χ^2-test	Chi-square test
Wnt3a	Wingrless-type MMTV integration site families

SUMMARY

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The cerebral cortex is composed of two main types of neurons, inhibitory GABAergic interneurons and excitatory glutamatergic pyramidal cells. These two major classes of cortical neurons are generated in different and distant proliferative regions in the developing brain and follow diverse strategies to reach their final position. While pyramidal cells are born in the ventricular zone of the dorsal telencephalon, interneurons originate in the ganglionic eminences and migrate longer distances to populate the cortex. Since disruption in the migration of GABAergic interneurons leads to defects in the organization of the adult cortex, understanding the mechanisms that control the guided migration of cortical interneurons from their origin to their final location is fundamental to improve our knowledge of the cerebral cortex in health and disease.

The mechanisms regulating the tangential migration of interneurons from their subpallial origin to the developing cortex have been extensively elucidated. In contrast, the processes and molecules controlling their distribution and final integration within the cerebral cortex remain unidentified. Here, we have investigated the mechanisms regulating the entry of interneurons into the developing cortical plate, in which pyramidal cells are being organized into specific layers. We have used a candidate approach to unravel the mechanisms that regulate the switch in the mode of migration of interneurons from tangential to radial. We searched for significant differences in a set of genes that play a role in cell migration, adhesion, and axon guidance and that are expressed in the developing cortical plate at relevant stages. We found that Neuregulin-3 (Nrg3), a member of the neuregulin family of genes, is highly expressed in pyramidal cells in the developing cortical plate since its inception, and is maintained in pyramidal cells as they mature. Our experiments revealed that Nrg3 is a potent short-range chemoattractant for MGE-derived interneurons, which therefore contribute to their normal allocation within the cortex. Gain and loss of function studies are consistent with this notion, reinforcing the idea that the timed entry of interneurons in the developing cortical plate is required for their normal lamination.

To shed some light into the mechanisms controlling the final laminar position of MGE-derived interneurons, we took an unbiased approach through gene profiling analyses in whole genome Affimetrix® arrays. We identify a set of genes that are differentially expressed before and after interneurons allocate into their final position in the cortex. Functional analysis of one of these candidates, the chemokine Cx3cl1, revealed that this factor does not seem to be fundamental for the regulation of this process.

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RESUMEN

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La corteza cerebral se compone de dos tipos principales de neuronas, las interneuronas GABAérgicas y las células piramidales glutamatérgicas. Estas dos clases de neuronas corticales se generan en regiones proliferativas distantes durante el desarrollo del cerebro y siguen diversas estrategias para alcanzar su posición final. Así, mientras que las células piramidales nacen en la zona ventricular del telencéfalo dorsal, el pallium, las interneuronas se originan en las eminencias ganglionares del subpallium y migran largas distancias hasta llegar a la corteza. Dado que la alteración en la migración de las interneuronas GABAérgicas causa defectos en la organización de la corteza cerebral adulta, la comprensión de los mecanismos que controlan la migración guiada de las interneuronas corticales desde su origen hasta su destino final es fundamental para mejorar nuestro conocimiento de la corteza cerebral en condiciones normales y patológicas.

Los mecanismos que regulan la migración tangencial de las interneuronas desde sus origen en el subpallium hasta la corteza han sido ampliamente investigados. Por el contrario, los procesos y las moléculas que controlan sus distribución e integración final en la corteza cerebral permanecen sin identificar. En esta Tesis hemos investigado los mecanismos que regulan la entrada de las interneuronas en la placa cortical, donde las células piramidales se organizan en capas específicas. En primer lugar, hemos explorado la función de genes que pudieran controlar la migración de las interneuronas cuando cambian su migración de tangencial a radial. Para ello hemos buscado diferencias significativas en genes cuya expresión aumenta en la placa cortical durante el desarrollo. Hemos descubierto que neuregulina-3 (NRG3), un miembro de la familia de las neuregulinas, se expresa a muy altos niveles en las células piramidales en la placa cortical desde su formación, y su expresión se mantiene e incluso aumenta en las células piramidales durante sus maturación. Nuestros experimentos sugieren que NRG3 contribuye a la atracción de las interneuronas que se originan en la eminencia ganglionar medial y contribuye a sus distribución en la corteza cerebral. Nuestros experimentos de ganancia y pérdida de función de *Nrg3* son consistentes con esta hipótesis, lo que refuerza el concepto de que la entrada controlada de las interneuronas en la placa cortical es necesaria para su correcta laminación.

Para entender los mecanismos que controlan la posición final de las interneuronas originadas en la eminencia ganglionar medial en las capas corticales, hemos utilizado el análisis de perfiles de genes a través de *microarrays*. Hemos identificado un conjunto de genes que son expresados diferencialmente en las interneuronas antes y después de que

establecen su posición final en la corteza. El análisis funcional de uno de estos candidatos, la chemoquina CX3CL1, sugiere que esta proteína no es fundamental para la regulación de este proceso.

INTRODUCTION

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1. The cerebral cortex: general overview of organization

1.1 Cortical development

The cerebral cortex is an extremely complex biological structure responsible for high order processes such as cognition, sensory perception and consciousness and it plays a fundamental role in integrating information derived from multiple sensory modalities. At the anatomical level, the cerebral cortex is subdivided into several major regions: *archicortex* (hippocampal formation), *paleocortex* (also known as piriform cortex or olfactory cortex) and *isocortex* (also called neocortex).

The neocortex constitutes the largest and newest part of the cerebral cortex and it is probably the most complex structure of the mammalian brain. As result of evolutionary processes, the neocortex has undergone an evident expansion and corresponds to the most recent acquisition. Thus, the neocortex represents the largest fraction of the total increase in brain size and the highest degree of specialization in more phylogenetic recent species (Krubitzer and Kaas 2005). While the neocortex is smooth in rats, mice and other mammals, it includes deep sulci (grooves) and gyri (wrinkles) in primates and several mammals; these folds serve to increase considerably the area of the neocortex. In this Introduction, I will describe the organization, development and specification of the cerebral cortex in the mouse, with a particular focus on the neocortex.

1.1.1 Structural organization of the neocortex: layers and columns

The neocortex is organized into areas, specialized cortical regions that establish specific connections and are characterized by different patterns of gene expression. The main areas include the primary somatosensory (S1), motor (M1), visual (V1) and auditory (A1) cortices. In addition, the mouse neocortex has association cortices that integrate information derived from primary sensory cortical areas. The processing that occurs in the sensory association areas is the basis of complex mental processes associated with each sense. The sensory association areas receive information about simple contours, boundaries and sensory qualities like, for instance, color or pitch. Each of these sensory, motor or association area connects with other brain structures or with other cortical areas, and serves different functions related to specific sensory modalities (Sur and Rubenstein 2005; O'Leary et al. 2007).

Despite the functional and anatomical differences that distinguish each cortical area, they share a similar cytoarchitecture. In fact, we can distinguish two main structural organizations in the neocortex, one on the tangential plane, constituted by cortical layers, and one on the radial plane, represented by columns. On the tangential plane, the neocortex is subdivided in up to six different layers whose characteristics vary slightly depending on the cortical region. Cortical layers are functionally classified into supragranular (layers I and II/III), granular (layer IV) and infragranular (layers V and VI). The most superficial layer (layer I) is largely devoid in cell somas and is particularly abundant in neural projections. Layer II/III is characterized by a high cellular density and represents the main source of inter-hemispheric connections. Layer IV is the primary target of thalamocortical connections, and it is particularly prominent in primary sensory cortices. Layers V and VI are the principal output stations of subcortical connections. In particular, layer V gives rise mainly to efferent projections toward the basal ganglia, brain stem and spinal cord. In contrast, layer VI projects to the thalamus. As a general principle, the laminar identity of cortical neurons predicts their main pattern of connectivity in the cortex and their contribution to circuitry formation.

The other main feature of cortical organization is the existence of columns, functional units arranged along the entire radial dimension of the neocortex. Mountcastle (1957) proposed that vertical columns of neurons in the cerebral cortex are the fundamental processing units of the neocortex (Jones and Rakic 2010), a theory inherited by Hubel and Wiesel (1968-1969) in their concept of cortical modules and receptive fields. Although electrical recordings have revealed functional clustering and neuronal interactions along the tangential dimension of the cortex, showing a link between lineage-dependent transient electrical coupling and the assembly of precise excitatory neuron microcircuits in the neocortex (Yu et al., 2012), whether such modules could be defined by their anatomical, molecular, physiological characters is still unclear. However, it has been recently proposed that multiple molecules play a role in regulating the phases of cortical columns assembly together with the tangential dispersion of neocortical projection neurons. For example, the functional analysis of Ephrin mutants has demonstrated that Eph receptor A (EphA) and ephrin A (Efna) signaling are essential for the assembly of cortical columns through the lateral dispersion of clonally related neurons (Torii et al., 2009). Moreover, it has been shown that ephrin-B1 knockouts display a wider lateral dispersion, resulting in the enlargement of ontogenic columns (Dimidschstein et al., 2013). Together,

these observations predict the existence of a molecular basis for columnar organisation, but additional evidences are needed to understand whether each columnar module can be defined simply based on its structural, anatomical, molecular and physiological characteristics.

1.2 Cytoarchitecture of the neocortex

The cerebral cortex contains hundreds of different types of neurons. Cortical neurons are classified into two main categories: excitatory pyramidal neurons and GABAergic inhibitory interneurons. Excitatory pyramidal neurons, also known as projection neurons, are distinguished by their characteristic pyramidal shape and are specialized in transmitting information between different cortical areas and from cortical areas to other regions of the brain. Interneurons are inhibitory neurons, characterized by aspiny dendrites and locally projecting axons that typically contribute to local neural assemblies, where they provide inhibitory inputs and shape synchronized oscillations (Klausberger and Somogyi, 2008).

1.2.1 Pyramidal neurons

Pyramidal cells are the most abundant class of cortical neurons, roughly representing 80% of the total neuronal population. Projection neurons located in different neocortical layers are generated in a tightly controlled sequence of events by cortical ventricular zone (VZ) and subventricular zone (SVZ) progenitors between embryonic day (E) 10.5 and E17.5 in the mouse (Angevine and Sidman, 1961; Caviness and Takahashi, 1995) (Figure 1D). Pyramidal cells follow an inside-out pattern of migration that they use to shape the cortical layers and to populate the developing neocortex.

Pyramidal cells send their axons to many distant regions, establishing connections with cortical, subcortical and subcerebral targets. They use glutamate as neurotransmitter and, therefore, they are excitatory cells. Different classes of projection neuron populate the neocortex and have specific functions. They have different laminar and areal allocations, dendritic morphologies and physiological features, and they express unique combinations of molecular markers (Molyneaux et al., 2007). One important feature of pyramidal cells is their pattern of axonal projections that is commonly used as the main criteria for their classification. Briefly, we can divide pyramidal cells in two groups, commissural and corticofugal neurons.

Commissural pyramidal cells are also known as callosal projection neurons (CPNs). They are small to medium pyramidal size, primarily located in layers II/III, V and VI, and send their axon across the corpus callosum. They can send single projections to the contralateral cortex; dual projections to the contralateral cortex and ipsilateral or contralateral striatum; and dual projections to the contralateral cortex and ipsilateral frontal cortex. They never project axons to targets outside the telencephalon (Figure 1A-1C).

Corticofugal (CfuPNs) pyramidal cells are subcortical projection neurons and can be further classified in two groups, corticothalamic neurons (CthPNs) and subcerebral projection neurons (SCPNs). CthPN are located in cortical layer VI, with a smaller population in layer V, and project subcortically to different nuclei of the thalamus (Figure 2B-2C). SCPN are also referred to as type I layer V projection neurons. This group includes pyramidal neurons of the largest size, which are located in deep-layer V and extend projections to the brainstem and spinal cord. They can be even further subdivided into several distinct projection neuron subtypes, based on their targets: corticotectal neurons are located in the visual area of the cortex and send their main projections to the superior colliculus and collateral projections to the rostral pons; corticopontine neurons send their primary projections to the pons, and corticospinal motor neurons (CSMNs) are located in the sensorimotor area of the cortex and send their primary projections to the spinal cord, with secondary collaterals to the striatum, red nucleus, caudal pons and medulla (Molyneaux et al., 2007).

Recent studies have identified a number of molecular markers that are specific to distinct classes of pyramidal neurons (Figure 1 C). However some of these markers are not only expressed in mature neurons but also in progenitor cells, so they are not ideal for their classification. For example, the transcription factor Fez family zinc finger 2 (Fezf2) is crucial for the specification of SCPNs. It is expressed at high levels by SCPNs and at low levels by CthPNs (Inoue et al. 2004; Arlotta et al. 2005; Chen et al. 2005b; Molyneaux et al. 2005). Citp2 (COUP-TF-interacting protein 2) acts downstream of Fezf2 to specify SCPN identities (Arlotta et al. 2005). Tbr1 (T-box brain proteins 1) instead represses Fezf2 and Ctip2 and specifies CthPNs (Bedogni et al. 2010; McKenna et al. 2011). In contrast, Satb2 is necessary for CPN specification and is highly expressed by commissural and associative pyramidal cells (Alcamo et al., 2008; Britanova et al., 2008).

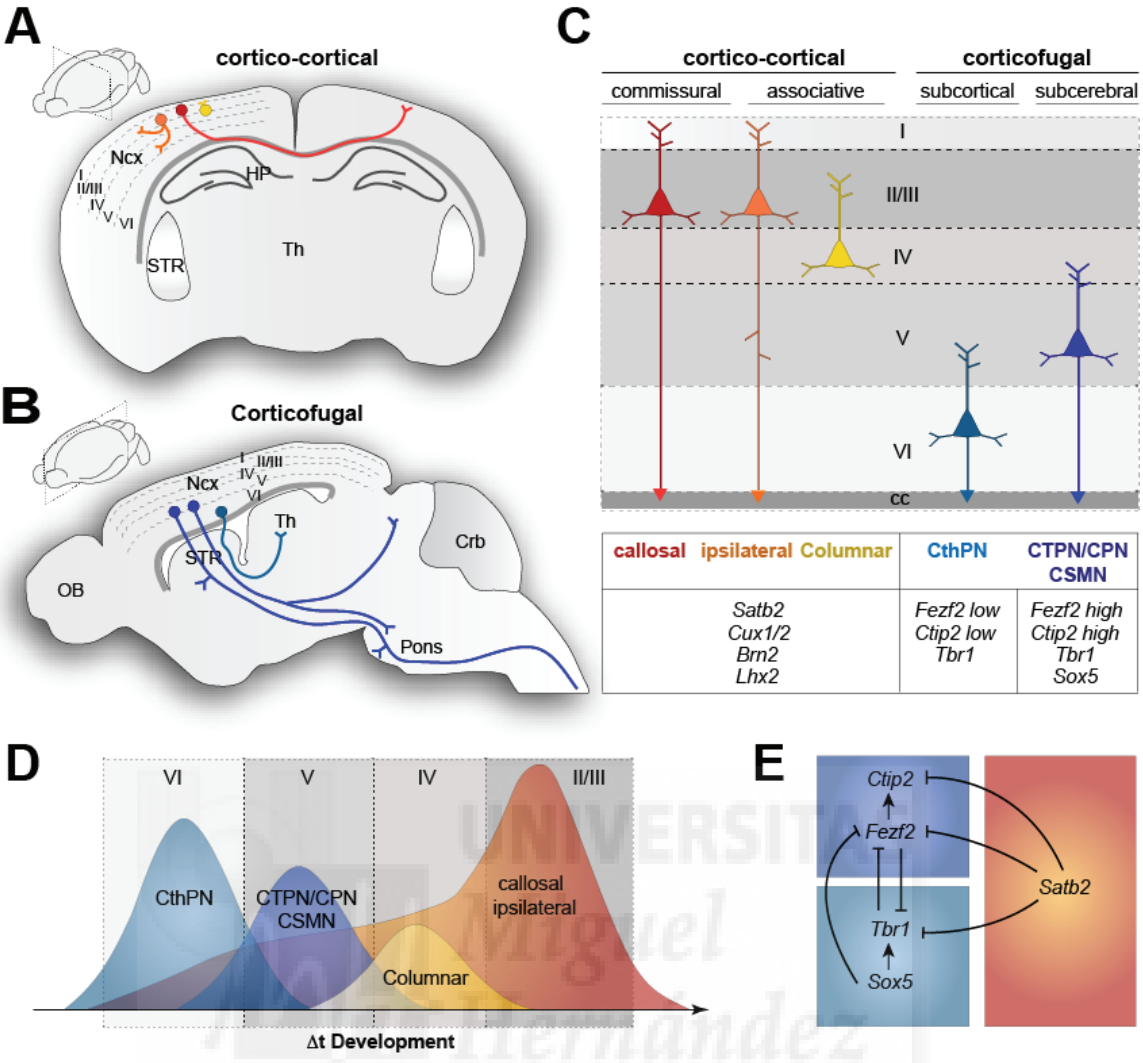

Figure 1. Classification and molecular specification of cortical projection neuron subtypes. A, B) Schematic of a coronal and sagittal sections through the adult brain showing the pattern of connectivity of cortico-cortical and corticofugal PN subtypes respectively, classified according to their axonal projections. C) Magnified view of the laminar localization and molecular characterization of PN subtypes shown in A) and B). PN are broadly classified into cortico-cortical and corticofugal subtypes depending if they project to cortical of subcortical structures respectively. Cortico-cortical PN are further subdivided into commissural (neurons that connect with the contralateral hemisphere via the corpus callosum or anterior commissure) and associative (neurons that connect ipsilaterally) subtypes. Cortico-cortical PN typically express Satb2, Cux1 and other molecular markers and localize mostly in superficial cortical layers. CfuPN comprehend subcortical and subcerebral subtypes that project to the thalamus and to other subcerebral structure (e.g. pons and spinal cord) respectively. They are characterized primarily by the expression of Fezf2, Ctip2 and Tbr1 transcription factors and localize in deep cortical layers. **D**) Temporal specification of PN subtypes during embryonic development. PN subtypes are produce in partially overlapping sequential waves. CThPN are the first followed by subcerebral, columnar and commissural types. **E**) Main molecular pathways involved in the specification of PNs during development. Arrows indicate transcriptional activation or repression. Cc, corpus callosum; Crb, cerebellum; HP, hippocampal formation; Ncx, neocortex; OB, olfactory bulbs; STR, striatum; Th, thalamus; I-VI, cortical layers I to VI. CTPN, cortico-tectal projection neuron; CPN, cortico-pontine projection neuron; CSMN, cortico-spinal motor neurons. Adapted from Greig et al. 2013.

1.2.2 Cortical interneurons

Cortical GABAergic interneurons, first referred as "short-axon" neurons by Ramón y Cajal (1899), are key regulators of activity in the cerebral cortex, and are considered to be the main cellular elements that control hyperexcitability in the brain (Dichter et al., 1987). They represent about 20% of the total neuronal population in the cortex and use the γ-aminobutyric acid (GABA) as their main neurotransmitter.

More than 20 different classes of interneurons have been identified in the hippocampus and neocortex, each of them with different functions (Fishell and Rudy, 2011; Klausberger and Somogyi, 2008). To be able to unambiguously classify the interneurons despite their enormous diversity, we have to consider their morphological, neurochemical, and electrophysiological properties (Ascoli et al., 2008; DeFelipe et al., 2013). Each of these properties influences the specific role of different classes of interneurons within the cortical circuitry. Interneurons can be classified in five major groups (see Figure 2C):

1) Fast-spiking interneurons that typically express the calcium binding protein parvalbumin (PV) and are morphologically represented by basket and chandelier cells (Markram et al., 2004; Taniguchi et al., 2013). They represent roughly 40% of the total population of cortical interneurons.

2) Interneurons with intrinsic-burst-spiking or adapting non-fast-spiking electrophysiological profiles characterized by the expression of the neuropeptide somatostatin (SST). At least two different classes of interneurons belongs to this group: Martinotti cells, with a characteristic axon extending into layer I (Ma et al., 2006; Xu et al., 2013), interneurons that branch abundantly around the cell soma and primarily synapse onto PV+ expressing interneurons (Xu et al., 2013). This second group of interneurons constitutes approximately 30% of the entire population of interneurons.

3) Rapidly adapting interneurons with bipolar or double-bouquet morphologies, which typically express the vasointestinal peptide (VIP) and may also contain the calcium binding protein calretinin (CR) (Rudy et al., 2011).

4) Neurogliaform cells, which have a very characteristic morphology, with highly branched short dendrites and a defining dense local axonal plexus (Armstrong et al., 2012).

They have a late-spiking firing pattern, and many express Reelin and the ionotropic serotonin receptor 3a.

5) Multipolar interneurons with irregular or rapidly adapting electrophysiological properties that often contain neuropeptide Y (NPY) (Lee et al., 2010). The last three groups account for the remaining 30% of interneurons.

It is worth noting that additional proteins such as Kv3.1, cholecystokinin (CCK), and neuronal nitric oxide synthase (nNOS) are good markers of subtype identity, while others such as calbindin (CB) and Kv3.2 are expressed in many different cell types (De Felipe et al., 1993; Kubota and Kawaguchi, 1994; Cauli et al., 1997; Gonchar and Burkhalter, 1997; Kubota and Kawaguchi, 1997; Chow et al., 1999; Garaschuk et al., 2000; Gupta et al., 2000; Monyer and Markram, 2004).

While this classification system is largely accepted in the field, many researchers believe that is not definitive. This is because distinct interneuron subtypes often have one or more overlapping characteristics with other subtypes. Efforts are currently constant to further classify interneurons subtypes based on their transcriptome profile and additional protein markers, to led to a more homogeneous, complete and satisfactory classification.

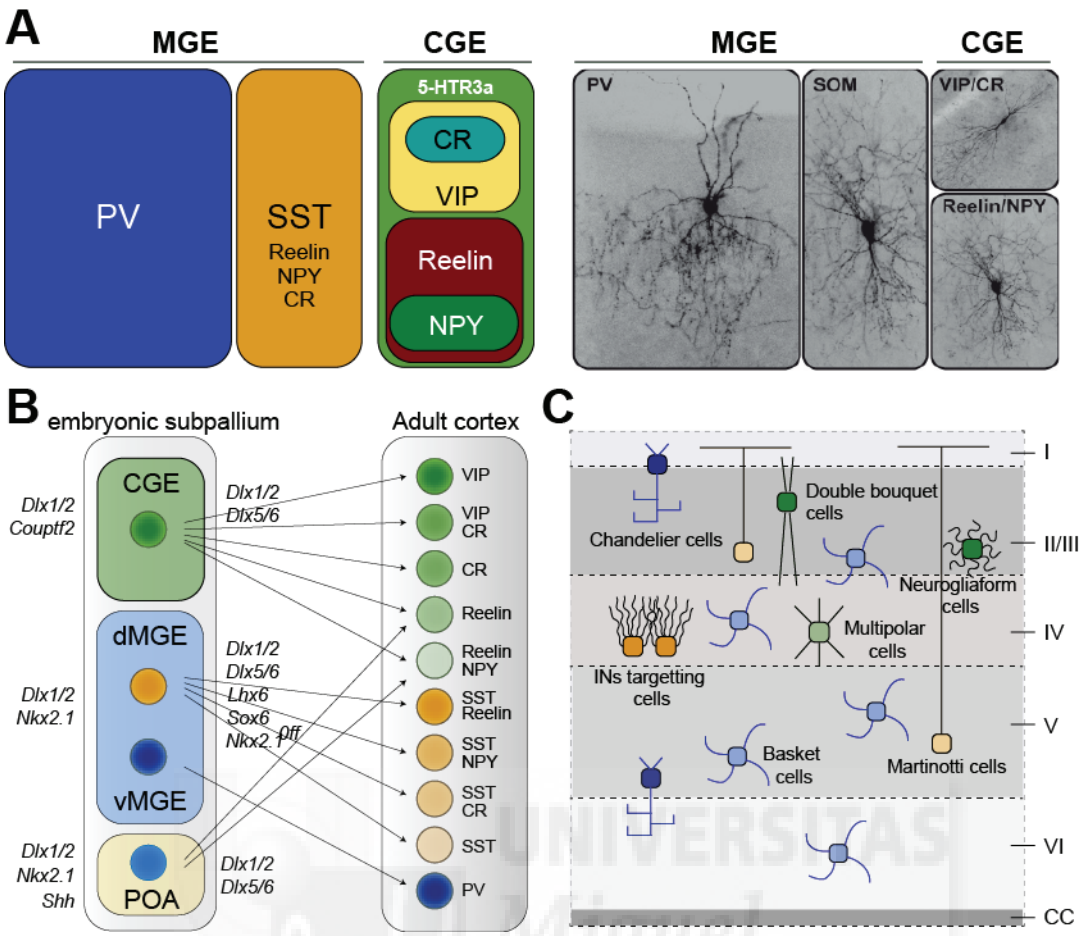

Figure 2. Classification and molecular specification of cortical inhibitory interneuron subtypes. A) Characterization of cortical interneuron subtypes according to the expression of neurochemical markers and morphology. Cortical GABAergic interneurons belong to three main non-overlapping groups. PV and SST – expressing interneurons are embryonically originated in the medial ganglionic eminences while the 5HTR3a – expressing ones derived from the CGE. Each of these classes comprehends different subtypes that are specified through a complex network of transcription factors. The main molecules involved in their developmental specification are shown in B) while the laminar distribution of the different subtypes in the adult cortex is depicted in C). Cc, corpus callosum; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; POA, preoptic area; I-VI, cortical layers I to VI. Adapted from Gelman and Marín 2010 and Bartolini et al. 2013.

2. Molecular specification of the telencephalon

The cerebral cortex, despite being a highly complex structure, derives from a simple sheet of neuroepithelium in the anterior lateral part of the neural plate (Fishell, 1997; Rubenstein et al., 1998). The neural tube patterns along the anterior-posterior (AP) and the dorso-ventral (DV) axis to give rise to all the telencephalic and subpallial structures.

The neural tube is composed of different vesicles along the AP axis: prosencephalon or forebrain, mesencephalon or midbrain, and romboencephalon or hindbrain. The prosencephalon consists of the diencephalon and the telencephalic vesicles, which constitute the primordium for the cerebral cortex and basal ganglia in the adult brain. According to the prosomeric model (Rubenstein et al. 1994; Rubenstein et al. 1998; Puelles and Rubenstein 2003) (Figure 3 A), the forebrain is subdivided into six different segments called prosomeres, and the telencephalon arises from the alar domain of the secondary prosencephalon (prosomeres 1-4). Besides an AP axis, the telencephalon becomes further subdivided dorsoventrally (DV) in several areas and progenitor domains through the action of morphogens (Rallu et al., 2002). These factors are secreted by signalling centers generally called organizers, in a temporal and spatial regulated sequence of events. Morphogens are present in a concentration gradient and they specify the fate of cells along this gradient. In the DV axis, the telencephalon is divided into the pallium (i.e., the roof of the telencephalon, also called dorsal telencephalon) and the subpallium (also called ventral telencephalon) (Campbell 2003) (Figure 3B-C).

There are several molecules that have been involved in the early DV patterning of the telencephalon, including bone morphogenic proteins (such as *Bmp4*), members of the fibroblast growth factor (FGF, among which *Fgf8* and *Fgf17* are the most studied), wingless-type MMTV integration site family factors (*Wnt3a*) and Sonic hedgedog (*Shh*), (Hébert and Fishell 2008) (Rallu et al. 2002). Shh has been shown to play a major role in the development of the ventromedial telencephalon. In *Shh* mutant mice, the MGE virtually disappears, although the subpallium still expresses some ventral genes, such as Genomic screened homeobox 2 *(Gsh2)* or Distal-less homeobox 2 *(Dlx2)* (Chiang et al., 1996, Rallu et al., 2002).

Fgf8 is essential for the specification of the neuronal ventral identities, acting in a dose dependent manner. FGF receptor mutants lack expression of the transcription factors Lhx6 and Lhx7, two LIM-domain transcription factors involved in the specification of the MGE (Gutin et al., 2006, Liodis et al., 2007). In *Fgfr1* and *Fgfr2* double mutants the defects are more severe than in single mutants. In addition to a profound misspecification of the MGE, transcription factors that are also expressed in the LGE, such as Gsh2, are abolished (Campbell et al., 2003, Hébert & Fishell 2008).

The early subdivision of the telencephalon in pallial and subpallial territories along the DV axis is linked to the generation of glutamatergic and GABAergic neurons,

respectively. Below I will describe in more detail the specification steps followed by these two territories and the genetic programs that regulate the production of the different neuronal subtypes of the neocortex.

2.1 Dorsal forebrain patterning and arealization

Several transcription factors are known to play a role in the acquisition of areal identities in the developing pallium. Among them, paired box gene 6 (*Pax6)*, empty spiracle homeobox 2 (*Emx2*), *Sp8,* and *Couptf1* are the most studied. *Pax6* is expressed at the neural plate stage throughout the telencephalic vesicle, and it interacts with *Nkx2.1* and *Ghs2* transcription factors to define the pallial-subpallial boundary (Corbin et al., 2003). The boundary between *Pax6* and *Nkx2.1* expression initially demarcates this boundary. Slightly later in development (at E9.5 in mice), the *Pax6*- and *Nkx2.1*-expressing regions become separated by a domain of *Gsh2* expression. As a result, the pallial-subpallial boundary becomes defined by the limit of *Pax6* and *Gsh2* expression (Figure 3 B).

Emx2 is important for cortical arealization. In the pallium, *Pax6* and *Emx2* are expressed in complementary gradients of gene expression: *Pax6* is expressed in a rostro-caudal and ventro-dorsal high to low gradient while *Emx2* shows high expression at more caudal levels. These opposite gradients contribute to the establishment of cortical area identities, with *Pax6* and *Emx2* being implicated in specifying frontal/motor and caudal sensory/visual areas, respectively (Bishop et al., 2000; Mallamaci et al., 2000; Muzio et al., 2002).

Gain and loss of function studies have shown that *Sp8* is involved in the specification of rostral cortical areas (Sahara et al., 2007; Zembrzycki et al., 2007). By contrary, *Couptf1* is involved in the specification of caudal cortical regions, where it functionally represses the specification of frontal/motor cortices in favor of somatosensory and visual area identities (Armentano et al., 2007; Figure 3C). Thus, these transcription factors are expressed in gradients through specific areas of the cortex. How these gradients are translated into the formation of discrete domains that reflect areas boundaries is still under debate. Recent studies are trying to shed light on these mechanisms. For instance, a recent study identified many enhancer sequences that show spatial restricted patterns of activity in pallial territories (Visel et al., 2013).

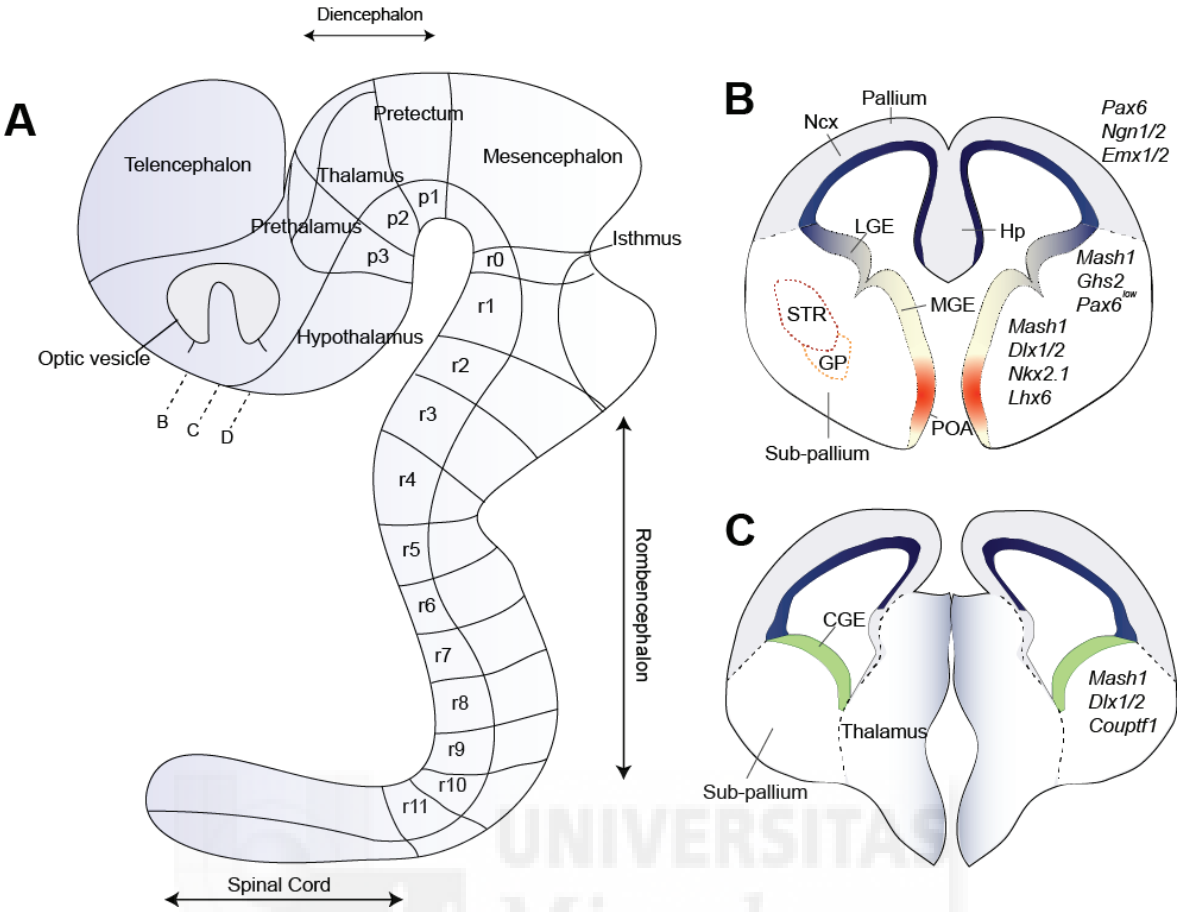

Figure 3. Patterning of the embryonic telencephalon. (A) Schematic of the prosomeric model. (B, C) Schematics of coronal sections through the telencephalon showing the main subdivisions along the dorso-ventral axis at two representative levels. The embryonic telencephalon is broadly patterned into dorsal (pallium) and ventral (subpallium) forebrain through the action of morphogenes. Subpallial territories are further divided into LGE, MGE, CGE and POA according to the differential expression of transcription factors. The main transciprion factors involved in the patterning of the different forebrain subdivision are listed at the side of each developing structure. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; POA, preoptic area; Hp, hippocampal formation. (A) Adapted from Puelles et al. 2008.

2.1.1 Origins and molecular specification of projection neurons

Corticofugal projection neurons (CthPNs and SCPNs) are sequentially generated during early neurogenesis, with peaks of neuronal production around E12.5 and E13.5, respectively. One of the main factors controlling the specification of CfuPNs is the transcription factor *Fezf2*. In particular, *Fezf2* promotes the specification of the SCPN subtypes (e.g. CSMNs) by activating the expression of the transcription factor *Ctip2* (Arlotta et al., 2005; Chen et al., 2005b; Molyneaux et al., 2005). Conversely, *Tbr1*

regulates the development of CthPNs by directly repressing the transcription of *Fezf2* and indirectly *Ctip2* (McKenna et al., 2011) (Figure 1A).

Callosal PNs are late-born (between E14.5 and E16.5 in mouse) commissural neurons. The transcription factor *Satb2* molecularly defines CPN identities for all cortical layers by directly repressing *Ctip2* expression (Alcamo et al., 2008; Britanova et al., 2008). *Satb2*-deficient neurons upregulate Ctip2 expression, fail to extend axons through the corpus callosum and instead project subcortically. Conversely, the expression of typical CPNs markers (e.g., cut-like homeodomain transcription factor, Cux1) is affected in *Satb2* mutant mice. CPNs represent a heterogeneous population, and gene expression studies have revealed that superficial and deep-layer CPNs are molecularly distinct (Molyneaux et al., 2009).

2.1.2 Origins and molecular specification of cortical GABAergic interneurons

Several fate-mapping and transplantation studies in rodents have identified the ventral telencephalon (subpallium) as the sole source of cortical interneurons (Xu et al., 2004; Butt et al., 2005; Flames et al., 2007; Fogarty et al., 2007; Miyoshi et al., 2007; Wonders et al., 2008; Xu et al., 2008) (Figure 3B-3C). The subpallium consists of the ganglionic eminences (GEs), and the preoptic area (POA) and anterior entopeduncular (AEP) domains. The GEs can be further subdivided into three anatomically distinct regions, the medial (MGE), lateral (LGE), and caudal (CGE) ganglionic eminences. In the mouse embryo, the MGE is the first to develop morphologically around E9 followed by the LGE one day later and the CGE around E11 (Smart, 1976; Sousa and Fishell, 2010). As the name suggests, the CGE is a structure positioned caudal to the MGE and LGE. It remains controversial whether the CGE is a distinct entity or only a fusion of the more caudal parts of the MGE and LGE. While the LGE and MGE are clearly morphologically separated by a sulcus, there is no clear anatomical boundary between these two regions and the CGE. Furthermore, a unique CGE-specific molecular signature or even identity, if any, remains to be identified. Apart from the eminences, the ventral telencephalon includes also a large part of the septum (Puelles et al., 2000) and the telencephalic stalk (i.e., the non-evaginated telencephalon).

Interneurons are generated at different times depending on their origin. For instance, while MGE-derived interneurons are mostly born between E11.5 and E17.5, the majority of CGE-derived interneurons are produced between E12.5 and E18.5, with a peak at E16.5

(Nery et al., 2002; Butt et al., 2005; Miyoshi et al., 2007; Miyoshi et al., 2010; Taniguchi et al., 2012). Both in vitro culture experiments and fate mapping of temporal cohorts have revealed the capability of MGE progenitors to produce different interneuron subtypes depending on the time of neurogenesis (Xu et al., 2004; Miyoshi et al., 2007). Specifically, a high proportion of SST+ cells are born at relatively early developmental stages, while PV+ cells are generated at a consistent rate throughout MGE-derived interneuron production.

At the end of the embryonic life, the morphological boundaries between the GEs regions disappear and are no longer identifiable in the postnatal brain. Thus, regional differences within the embryonic subpallium are mainly based on the differential expression of transcription factors that define territories specialized in the production of different types of GABAergic interneurons (Figure 3B- 3C). Moreover, GEs histogenesis requires a complex interplay between morphogens and transcription factors to ventralize the structure and promote interneuron production. Sonic hedgehog (SHH) and fibroblast growth factors (FGFs) contribute not only to the dorso-ventral patterning but also to subpallium development (Jessell, 2000; Briscoe and Ericson, 2001; Ingham and McMahon, 2001).

Expressed throughout the subpallial subventricular zone (SVZ), the *Dlx* family of homeobox transcription factors is of particular importance for GABAergic interneuron differentiation, migration, and process formation. Specifically, *Dlx1* and *Dlx2* are functionally redundant genes required for GABAergic interneuron production and specification (Anderson et al., 1997; Pleasure et al., 2000; Petryniak et al., 2007). *Dlx1* and *Dlx2* strongly promote neurogenesis versus oligodendrogenesis, as evidenced by a dramatic increase in the expression of "*Olig2*-dependent oligodendrocyte precursor cell" (OPCs) in *Dlx1/2*-null mutants mice (Petryniak et al., 2007).

Dlx1/2-null mutants have a severe deficit in survival and migration of interneurons, with a 70% reduction of these cells in the neocortex (Anderson et al., 1997; Sussel et al., 1999). Working in concert with *Dlx1/2*, the proneural gene *Mash1* (also known as *Ascl1*) is expressed in the subpallial SVZ and is required for the production and differentiation of GABAergic interneurons (Casarosa et al., 1999; Petryniak et al., 2007; Long et al., 2009). Similar to *Dlx1/2,* elimination of *Mash1* expression results in a substantial decrease in GABAergic neocortical interneurons (Casarosa et al., 1999). *Mash1* is widely expressed in the subpallium and is essential for maintaining subpallial identity through a cross-

repressive mechanism with *Neurogenin1/2* that instead controls neuronal production within pallial territories (Fode et al. 2000; Schuurmans and Guillemot 2002). Moreover, it is now well established that virtually all cortical GABAergic interneurons derive from cells expressing *Dlx5/6* (Stühmer et al., 2002). While *Dlx1/2* and Mammalian achaete-schute homolog *(Mash1)* are expressed throughout the subpallium, transcription factors that are intimately involved in interneuron fate-specification exhibit a more restricted expression pattern (Flames et al., 2007), raising the possibility that the developing ventral telencephalon contains multiple progenitor pools, each with a distinct progeny fate potential. Below, we will describe the specific pattern of transcription factors expression in each of the GE areas.

Medial ganglionic eminence

The MGE generates the vast majority (~70%) of cortical interneurons. In mice, interneuron production in the MGE takes place between E9.5 and E16.5, with a peak around E12.5-E13.5 (Miyoshi et al., 2007). In addition to cortical interneurons, the MGE also generates interneurons destined for the striatum and hippocampus, oligodendrocytes and (inhibitory) projection neurons for the basal forebrain (Kessaris et al., 2006; Xu et al., 2008). The homeobox transcription factor *Nkx2.1* is specifically expressed by MGE and POA proliferative zones (Sussel et al., 1999) and is rapidly downregulated in cortical interneurons as they migrate toward the cortex, while it remains expressed in a subset of striatal interneurons (Marín et al., 2000). It has been shown that the Nkx2.1 levels are controlled by Smad interacting protein-1 (*Sip1*), a zinc finger homeobox gene, also known as *Zfhx1b* or *Zeb2* (Van de Berghe, 2013; McKinsey et al., 2013). Within the ventral telencephalon, Sip1 is expressed at progressively increased levels within postmitotic interneurons as they migrate toward the cortex. Interestingly, molecular analysis demonstrates that expression of Nkx2.1 remains elevated in most tangentially migrating interneurons upon loss of Sip1, thus suggesting that in the absence of this transcription factor cortical interneurons are unable to downregulate *Nkx2.1*, a necessary requirement to reach the cortex (Nobrega-Pereira et al., 2008).

In vivo loss of function experiments have shown that Nkx2.1 plays a key role in the maintenance and establishment of MGE progenitors as well as the specification of MGE-derived interneurons (Anderson et al., 2001; Butt et al., 2008). In *Nkx2.1* mutant mice, MGE/POA progenitor cells are re-specified to more dorsal fates, and there is a dramatic

reduction (~60%) in the total number of cortical GABAergic cells (Sussel et al., 1999). In particular, early removal of *Nkx2.1* from MGE progenitors re-specifies interneurons into early LGE medium spiny neuron identity, while its late removal leads to acquisition of CGE interneuron profiles (Butt et al., 2008). Furthermore, both in vitro cultures experiments (Xu et al. 2004) and transplantation studies in vivo (Butt et al. 2005; Cobos et al. 2007; Butt et al. 2008; Wonders et al. 2008) have shown that the MGE gives rise to two main non-overlapping classes of cortical interneurons: PV-expressing and SST-expressing interneurons (Figure 2A, 2B). These results have also been confirmed by genetic fate-mapping studies (Fogarty et al., 2007; Xu et al., 2008).

Shh signaling acts upstream of *Nkx2.1* in the specification of the MGE territory (Xu et al. 2005) and modulates the production of MGE-derived interneuron subtypes in a dose-dependent manner (Xu et al. 2010). In fact, it was suggested that the *Shh* gradient determines the final fate of interneurons, with high levels of *Shh* favouring the generation of SST-expressing interneurons over PV-expressing interneurons (Xu et al., 2010). Moreover, *Nkx2-1* can maintain *Shh* expression within the early MGE, a process depending on the FoxA2/HNF-3b transcription factor (Sussel et al., 1999).

Nkx2.1 specifies PV+ and SST+ cortical interneuron subtypes by directly activating the LIM-homeobox transcription factor *Lhx6* (Du et al. 2008), which is maintained during migration of interneurons and in the adult cortex (Lavdas et al. 1999). In the absence of Lhx6, NPY+ fates are promoted at the expense of PV and SST expression, and cortical interneurons show an abnormal allocation in the neocortex (Liodis et al. 2007, Zhao et al., 2008). Other genes act downstream of or in concert with *Nkx2.1*. For example, high levels of *Lhx7* expression shift the fate of interneurons toward globus pallidus GABAergic neurons and into cholinergic interneurons from the striatum (Zhao et al., 2003; Fragkouli et al., 2005). Recently, the Sry-related HMG-box-containing transcription factor *Sox6* has been shown to act downstream of *Lhx6* (Batista-Brito et al. 2009) and to be required for the generation of the appropriate number of PV and SST interneurons (Azim et al., 2009; Batista-Brito et al., 2009). In these mice models, a concomitant increase of NPY interneurons was also observed (Azim et al., 2009; Batista- Brito et al., 2009).

These results predict that a basic molecular pathway involving *Nkx2.1*, *Lhx6* and *Sox6* transcription factors acts sequentially in the specification of the MGE-derived PV- and SST-expressing interneurons. However, it is still unclear whether both interneuron subtypes share a common progenitor and/or derive from segregated pools of progenitor

cells. Gene expression studies have revealed a substantial molecular diversity of MGE progenitor cells based on a differential expression of transcription factors that in combination define putative proliferative sub-domains (Flames et al., 2007; Tucker et al., 2008). In particular, the analysis of the expression of several transcription factors within the ventricular zone (VZ) of the MGE has led to the proposal that this region can be compartmentalized into five different progenitor domains (Flames et al., 2007). For instance, the dorsal region of the MGE (dMGE) preferentially gives rise to SST-expressing interneurons. In contrast, the ventral part of the MGE (vMGE) was shown to generate mostly PV-expressing interneurons.

Caudal ganglionic eminence

CGE contributes to the generation of 30–40% of all cortical interneurons (Rudy et al., 2011). The identification of the CGE as a separate area from the other two GEs was initially based only on morphological indications, and the lack of clear anatomical boundaries with the LGE complicated its recognition and understanding. The CGE appears relatively late during development compared for example to the MGE and, as a matter of fact, the peak of CGE interneuron production occurs around E15.5-E16.5 (Miyoshi et al., 2010). The molecular and migratory properties of CGE-derived cells are not altered in *Nkx2.1* and *Gsh2* mutant mice, in which MGE and LGE development is affected, respectively (Nery et al., 2002). Moreover, transcriptome-wide comparison of the three GEs revealed the existence of unique molecular profiles within the CGE (Willi-Monnerat et al. 2008).

The first direct evidence of a substantial contribution of CGE progenitor cells to specific populations of interneurons derives from gene expression and cell transplantation studies (Nery et al. 2002). These early observations were subsequently confirmed and expanded by other *in vitro* and *in vivo* studies and it is now well established that the CGE generates bipolar, double-bouquet and neurogliaform interneurons that express the ionotropic serotonin receptor 3a (5-HT3a) (Lee et al. 2010). Several studies have shown that CGE derived interneurons express CR, VIP or Reelin (Pleasure et al., 2000; Butt et al., 2005; Miyoshi et al., 2010). *Gsh* or *Gsx* homeobox transcription factors act at the top of the genetic network involved in CGE cell specification. *Gsh2* is particularly relevant for the generation of CR bipolar interneurons (Xu et al., 2010). Interestingly, *Gsh1* and *Gsh2* are co-expressed but have antagonist functions within the CGE: *Gsh2* promotes progenitor states while *Gsh1* induces neuronal differentiation (Pei et al., 2011). The control of the

choice between proliferation and differentiation by *Gsh* genes seems to involve the downstream target *Mash1* (Fode et al., 2000) (Figure 3C). In *Mash1* mutants there is a premature differentiation of progenitors located in the SVZ and a precocious expression of *Dlx* genes (Casarosa et al., 1999; Yun et al., 2002), downstream effectors. On the other hand, overexpression of *Mash1* contributes to neuronal differentiation (Fode et al., 2000). *Dlx1* and *Dlx2* are co-expressed in subsets of progenitor cells and contribute to cell maturation by repressing *Gsh2* and *Mash1* (Yun et al., 2002). Other CGE transcription factors include *Nrf2f1*, *Nrf2f2*, *Couptf1* and *Couptf2,* as well as *Sp8*. These genes are however not exclusive of the CGE, as they have been also observed in the dorsal MGE and in the POA (Lodato et al., 2011).

The family of Coup-tf transcription factors represents one of the main players in the specification of CGE interneurons. Conditional *Coup-tf1* loss of function results in the respecification of CGE interneurons subtypes to MGE fates (Lodato et al. 2011), while transplantation studies have revealed a role for *Coup-tf2* in directing the migration of CGE-derived interneurons (Kanatani et al. 2008). *Nrf2f2* is important for directing interneurons through a caudal migratory path (Cai et al., 2013). *Sp8* function in the hierarchy of CGE specification/maturation is yet unknown (Ma et al., 2012).

Additional CGE markers have been recently discovered, such as Prospero homeobox 1 (*Prox1)* (Ma et al. 2012; Rubin and Kessaris 2013, Miyoshi et al., 2015). The expression of the homeodomain transcription factor *Prox1* is selectively maintained in postmitotic CGE-derived GABAergic cortical interneurons during embryonic and postnatal development, where it directs migration and maturation programs of each CGE-derived cortical interneuron subtype (Miyoshi et al., 2015). The molecular partners that work both in concert and in parallel with Prox1 to confer distinct CGE-derived interneuron properties are still unknown.

Preoptic area

The POA is the most ventral region of the developing subpallium and it has been shown to generate around 10% of GABAergic interneurons population (Gelman et al. 2009). A clear anatomical boundary between POA and MGE is not visible at rostral levels but the molecular profile of this region reveals a unique identity. Progenitor cells in the POA shares with the MGE the expression of *Nkx2.1* (Gelman et al., 2009). In addition, *Shh* but not *Lhx6* are expressed in the POA (Flames et al., 2007). Developing brain homeobox

protein 1 (*Dbx1)* and *Nkx6-2* are markers of the dorsal and ventral POA, respectively (Figure 2B). The function of these genes remains, however, unclear. Fate mapping and *in utero* transplantation demonstrated that POA generates a wide range of interneurons subtypes (Gelman et al., 2011), including PV+, SST+, Reelin+ and NPY+ cortical interneurons with heterogeneous electrophysiological properties (Gelman et al., 2009; Gelman et al., 2011) (Figure 2 B).

2.2. Neuronal migration in the developing cerebral cortex

Pyramidal neurons and interneurons are born in different regions of the developing telencephalon: pyramidal cells are born in the pallium, while cortical interneurons are originated in the subpallium. Consequently, both cell types follow different strategies to reach the neocortex. Pyramidal neurons migrate radially forming the cortical layers, while interneurons migrate first tangentially, from the subpallium to the cortex, then radially, starting to allocate into the developing cortex (Marín and Rubenstein 2003).

2.2.1 Pyramidal cell migration

The first cohort of postmitotic neurons migrating radially from the pallial VZ form a transient layer called preplate (PP), roughly at E10.5 in mice. The PP consists of the first cohort of pyramidal neurons, but is also rapidly colonized by Cajal-Retzius cells (CRs). CRs constitute a transient population generated by discrete pallial structures and that disperse throughout the surface of the cortex where they play an important role in the regulation of the migration of pyramidal cells (Bielle et al., 2005; Yoshida et al., 2006; Villar-Cerviño and Marín 2012). After the first pyramidal neurons are born, multiple waves of neurons are generated from progenitor cells in the ventricular (VZ) and subventricular zone (SVZ). Newborn pyramidal cells migrate radially splitting the PP into the marginal zone (MZ) superficially and the subplate (SP) deeply, thereby forming the cortical plate (CP) in which the remaining cortical layers will form. During development, consecutive waves of post-mitotic PNs migrate radially toward the CP, passing over previously generated neurons and forming in this way the six layers of the neocortex following an inside-out pattern. Birthdating studies have in fact shown that, as result of this migration pattern, early-born pyramidal cells primarily occupy deep cortical layers, while late-born neurons reside in progressively more superficial layers (Angevine and Sidman 1961; Fairén et al., 1986). So, the laminar allocation of pyramidal cells, at least at the population level, strongly correlates with PNs birthdate.

Two modes of PN radial migration have been described, somal translocation and glial-guided locomotion. Somal translocation is used by PNs during early stages of development. During this type of movement migrating neurons first extend a radially oriented and long leading process that is attached to the pial surface (or MZ), and subsequently translocate the nucleus within the leading process until they reach the target position. A series of functional studies of Cajal-Retzius cells have largely focused on their regulation of radial migration by Reelin (Supèr et al., 2000; Bielle et al., 2005).

However, recent reports have also revealed their roles in instructing radial migration via contact-mediated signaling (Gil-Sanz et al., 2013). Heterophilic cell adhesions mediated by nectin1-expressing Cajal-Retzius cells stabilize the leading processes of nectin3-expressing migrating projection neurons to anchor to the MZ, facilitating their somal translocations toward the cortical surface.

As the cortical thickness increases, PNs migrate mostly using locomotion. This process refers to the migration of newborn neurons in close proximity to the basal processes of radial glia cells (RGCs). These cells, which are the progenitors of pyramidal cells (Noctor et al. 2001), have their cell bodies in the VZ and extend their long processes spanning the entire thickness of the developing cortex. Thus, RGCs are both the progenitors of PNs and also serve as a physical scaffold that is used by migrating neurons to move radially toward the CP (Noctor et al., 2001; Noctor et al., 2004).

Many secreted molecules and intracellular proteins have been shown to regulate pyramidal neuron migration and the formation of cortical layers (Marín et al. 2010). Among them, the signaling pathway elicited by *Reelin* is one of the best characterized. *Reelin* is a glycoprotein secreted by Cajal-Retzius cells. The study of *reeler* mice (carrying an spontaneous mutation of *Reelin)* provided the opportunity to appreciate the central role of this molecule in the migration of PNs. In fact these mice showed severe defects in cortical cytoarchitecture, characterized by inverted lamination pattern (Caviness 1982; Franco et al., 2011). Interestingly, the Reelin signaling pathway interacts with other molecules, such as ephrins, to regulate the migration and the position of cortical PNs (Sentürk et al. 2011).

2.2.2 Interneuron migration

Interneurons generated in the subpallium follow complex migratory routes to reach their final destination in the neocortex (Corbin et al., 2001; Marín and Rubenstein, 2001, 2003).

As their pyramidal cells counterparts, MGE derived cortical interneurons migrate in an "inside-out" manner depending on their birthday (Cavanagh and Parnavelas, 1989; Anderson et al., 2002; Miyoshi et al., 2007), occupying first the deep layers and then the upper layers of the cortex. Neocortical interneurons migrate first tangentially from the subpallial regions to reach the neocortex, where they initially disperse through the marginal zone (MZ) and the SVZ, before migrating radially and start occupying their final location in the cortical layers. Consequently, the process of interneuron migration can be divided in three different phases: 1) Tangential migration to the pallium; 2) intracortical dispersion and formation of stereotyped migratory stream; and 3) CP invasion and laminar allocation (Marín 2013). During these phases, interneurons follow highly stereotyped routes of migration (Ayala et al 2007; Lavdas et al 1999; Marín & Rubenstein 2003; Nery et al 2002), which suggests that the entire process is tightly controlled by genetic factors. In contrast to this idea, some groups have proposed the long-distance tangential migration of interneurons is a largely random process (Ang et al., 2003; Tanaka et al., 2009). In fact, it has been proposed that the tangential dispersion of interneurons in the MZ happens through a "random-walk" behavior (Tanaka et al., 2009). However, it is unclear how random tangential migration of individual interneurons could lead to an organized distribution in the neocortex leading the construction of functional circuits.

Tangential migration of interneurons to the pallium

Tangential migration is mediated by the coordination of several guidance cues that function to both selectively repel and attract cortical interneuron populations (Marín and Rubenstein, 2003) (Figure 4 B). For instance, it has been shown that Ephrin-A5/EphA4R signaling mediates cortical interneuron repulsion. In particular, it has been shown that Ephrin-A5 is expressed in the VZ of the MGE and LGE at embryonic stages, during the time of tangential migration of interneurons. By contrast, EphA4 (the receptor of ephrinA5) is expressed by interneurons and exhibits a complementary expression pattern respect to Ephrin-A5 in the SVZ at these ages (Zimmer et al., 2008). In the absence of Ephrin-A5, cortical interneurons invade the VZ, a phenotype that is rescued when the slices were treated with recombinant Ephrin-A5 (Zimmer et al., 2008). EphA4R-mediated forward signaling is also used by interneurons to avoid migrating towards the ventral-most region of the subpallium (Zimmer et al., 2011), as it also binds Ephrin-B3 present in the

ventral MGE and POA. It is also important to note that EphA4R promotes interneuron migration through EphrinA2 reverse signaling (Steinecke et al., 2014).

It has been proposed that Slit/Robo could be another signaling pathway mediating chemorepulsion. In particular, Zhu et al. (1999) suggested that Slit proteins might repel interneurons from the subpallium to the cerebral cortex. The ligands Slit homolog 1 and 2 (Slit1 and 2) are expressed in the VZ of GEs (Marín et al., 2003), and in turn interneurons express their receptor, Roundabout homolog 1 (Robo1) (Bagri et al., 2002; Marillat et al., 2002) in a complementary expression pattern in the VZ. In support of the chemorepellent function of Slit/Robo signaling, it has been shown that secreted Slits from the VZ of the LGE repel ganglionic eminence cells away from the SVZ (Zhu et al., 1999). However, mice deficient for both *Slit1/2* do not have obvious interneuron migration defects (Marín et al., 2003). On the other hand, recent work suggests that Slits also regulate neurogenesis in the MGE (Borrell et al., 2012), and so it is conceivable that the early steps in the migration of interneurons might also depend on the contribution of these factors to their initial polarization. In addition, loss of Slit ligands or removal of *Robo1* leads to aberrant striatal invasion by cortical interneurons (Andrews et al., 2006; Hernandez-Miranda et al., 2011), which suggests that Robo/Slit might be involved in regulating the migration of cortical interneurons around the striatum (Andrews et al. 2007; Marín et al. 2003).

Several other lines of evidence suggest that the striatum is a hostile territory for the migration of cortical interneurons (Figure 4B). Interneurons destined for the cortex express the receptors Neuropilin 1 and Neuropilin 2 (*Npn1/2*), which are responsive to their repulsive ligands Semaphorin (*Sema3A/3F*) expressed in the striatum (Marín and Rubenstein, 2001). In addition, it has been shown that chondroitin 4-sulfate-carrying proteoglycans expressed in the striatum restricts the diffusion of Sema3A away from this region (Zimmer et al., 2010), which may allow interneurons to migrate towards the cortex, traversing territories that are immediately adjacent to the developing striatum (Marín et al., 2001; Nobrega-Pereira et al., 2008). Furthermore, in vitro experiments indicate that interactions between ephrinA molecules and their EphA receptors may also contribute to the repulsion of cortical interneurons away from the striatum (Rudolph et al., 2010).

During their transit through the subpallium, cortical interneurons actively avoid entering not only the striatum but also the POA (Figure 4B). The molecular nature of this chemorepulsive activity has not been identified so far. It was originally proposed that Slits could mediate the repellent effect of POA in the migration of MGE-derived interneurons

(Zhu et al., 1999), but both experimental manipulations and genetic analyses indicate that these factors do not contribute to the chemorepulsive activity found in the POA (Marín et al., 2003).

There are a number of factors that promote interneurons migration towards the cortex. Newborn interneurons seem to respond to several motogenic cues that promote their tangential migration, including trophic factors and neurotransmitters (Heng et al., 2007) (Figure 4). For instance, the migration of MGE-derived interneurons is strongly stimulated by brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4) (Polleux et al., 2002). This has been shown in experiments in which recombinant proteins were applied to organotypic slice cultures. The Tyrosine Kinase B (TrkB) receptor mediates the effect of these neurotrophins (Polleux et al., 2002). In addition, glial-derived neurotrophic factor (GDNF) and hepatocyte growth factor both stimulate the migration of interneurons in vitro (Powell et al., 2001; Pozas & Ibañez, 2005). The direct involvement of all these molecules in the regulation of the migration of MGE-derived interneurons in vivo is controversial. For example, the *in vitro* effect of BDNF and NT4 on migrating interneurons, is not supported by the analysis of mouse mutants for *TrkB*, in which the number and position of cortical interneurons are unchanged (Carmona et al., 2006; Sanchez- Huertas and Rico, 2011). The function of GDNF in the migration of cortical interneurons seems to be mediated by its GFRa1 receptor, and the heparan sulfate proteoglycan syndecan-3, independently of the RET tyrosine kinase, and genetic evidence supports a role for these molecules in vivo (Canty et al., 2009; Bespalov et al., 2011). Nevertheless, the complex distribution abnormalities observed in GFRa1 receptor mutants suggest that GDNF may play a role in the organization of MGE-derived cortical interneurons that extends beyond modulating cortical interneurons motility (Pozas and Ibanez, 2005, Canty et al., 2009). Finally, while mutant mouse for the urokinase-type plasminogen activator receptor (*uPAR*) that cleaves and releases the active form of HGF/SF (Powell et al., 2001) or mutant mice for *MET* (Eagleson et al., 2011) shows a decreased number of interneurons in the cortex, the cell-autonomous effect of HGF/SF-mediated signaling in this process has been questioned since MET is not found to be expressed in cortical interneurons in vivo (Eagleson et al., 2011).

In vitro experiments have shown that both GABA and glutamate enhance the migration of MGE-derived interneurons (Cuzon et al., 2006; Manent et al., 2006; Bortone & Polleux, 2009; Inada et al., 2011). This function is mediated through the tonic activation

of GABAA and AMPA receptors, respectively, which are expressed in interneurons soon after these cells start their migration (Soria et al., 1999; Metin et al., 2000; Cuzon et al., 2006; Cuzon & Yeh, 2011). The mechanism through which GABA and glutamate promotes the migration of interneurons remains unclear, but it seems to depend on the ability of these neurotransmitters to depolarize the plasma membrane of embryonic interneurons, thereby increasing their levels of intracellular calcium (Owens et al., 1999, Soria et al., 1999, Metin et al., 2000, Bortone & Polleux, 2009). Cortical interneurons express GABAA and GABAB receptors and as a result of an inverted chloride gradient, they respond to GABA by membrane depolarization that triggers opening of L-type voltage-sensitive Ca2+ channels and induces Ca2+ transients (Bortone and Polleux, 2009). Thus, ambient GABA and glutamate contribute to regulate the motility of cortical interneurons by setting the appropriate calcium "tone" in migrating neurons.

In addition to motogenic factors, other cues direct migration of cortical interneurons via a chemoattractive effect. MGE-derived interneurons follow a gradient of increasing permissivity towards the cortex, created by the diffusion of long-range chemoattractive cues from the pallium (Marín et al., 2003; Wichterle et al., 2003). Of note, the only chemattractive molecule that has been described to date is Neuregulin-1 (Nrg1), which plays a major role in the guiding interneurons via two different isoforms, soluble Ig-Nrg1 and membrane bound CRD-Nrg1 (Flames et al., 2004).

Interneurons fated to occupy different telencephalic structures (e.g., striatum or cortex) navigate a very similar environment but respond to different guidance cues. In fact, intrinsic genetic programs regulate in different ways the expression of the molecules that play a role in these processes (Nóbrega-Pereira et al., 2008; Nóbrega-Pereira and Marín 2009; Van den Berghe et al., 2013). For instance, the Nkx2.1 transcription factor represses the expression of Neuropilin1 and Neuropilin2, receptors for the repulsive molecules Sema3A and Sema3F that are expressed in the developing striatum. MGE-derived striatal interneurons continue to express Nkx2.1 during their tangential migration, and so they downregulate neuropilins and are allowed to colonize the striatum (Nóbrega-Pereira et al. 2008). Conversely, MGE-derived cortical interneurons downregulate Nkx2.1 expression as they begin their migration. This leads to the expression of neuropilins in cortical interneurons, which renders them sensitive to the semaphorins expressed in the striatum and so they avoid entering this territory in their way to the cortex (Marín et al., 2001). Nkx2.1 levels are regulated by Sip1 (Van den Berghe et al., 2013), although the exact

molecular mechanisms remain unclear. McKinsey and colleagues (2012) have shown that Sip1 functions downstream of Dlx2, which binds directly to two conserved enhancers necessary for Sip1 expression. The data support a possible model by which Dlx2 positively regulates expression of Sip1, which in turn negatively regulates (directly or indirectly) Nkx2.1 levels to control the migration of interneurons to the cortex. They have also showed that Sip1 is required in the MGE to generate cortical interneurons that express Cxcr7, MafB, and cMaf. In its absence, Nkx2.1 expression is not repressed, and cells that ordinarily would become cortical interneurons appear to transform toward a subtype of GABAergic striatal interneurons (McKinsey et al., 2012). Sip1 also seems to influence interneuron migration through the regulation of guidance receptors. Van den Berghe and colleagues (2013) have found that Sip1 activates the Netrin receptor Unc5b, and that expression of this receptor is necessary for interneuron migration to the cortex.

It has also been recently shown that striatal interneurons use similar mechanisms than cortical interneurons to migrate towards the striatum avoiding the cortex (Villar-Cerviño et al., 2015). In particular, striatal interneurons express ErbB4 to migrate towards the developing striatum and they are actively repelled by the cerebral cortex, through Eph/ephrin signaling. These results reveal that, similar to cortical interneurons, MGE-derived striatal interneurons depend on both target chemoattraction and off-target chemorepulsion to reach their final destination (Villar-Cerviño et al., 2015).

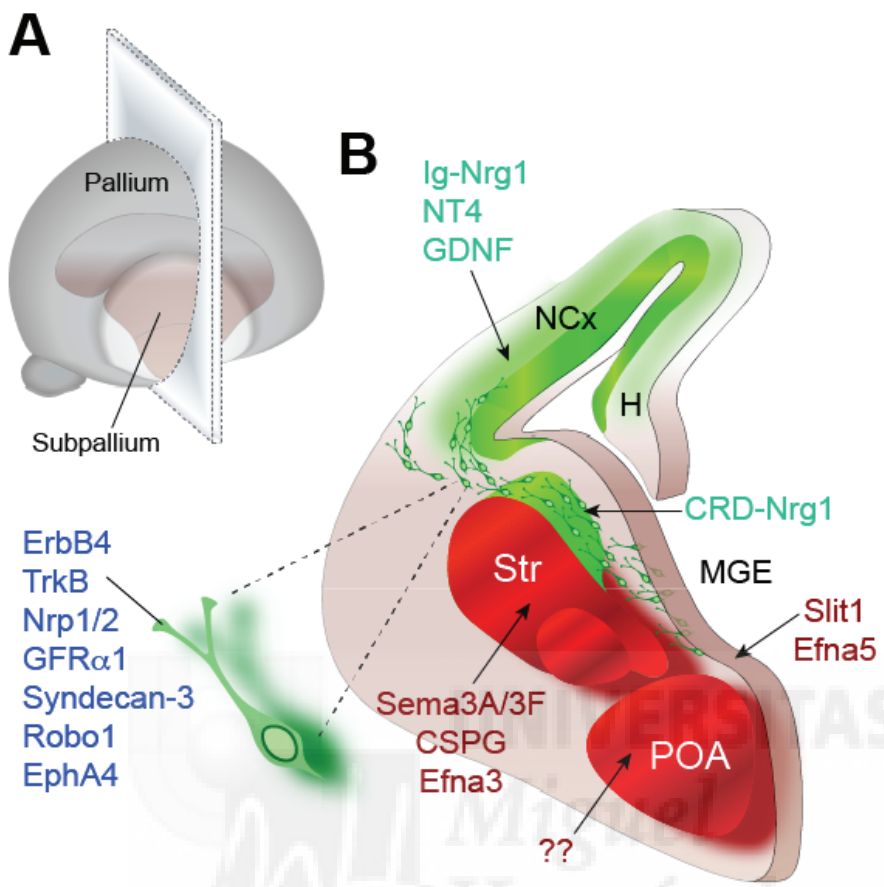

Figure 4. Molecular mechanisms controlling the migration of MGE-derived interneurons to the cortex. (A) Schematic representation of a mouse embryonic day 13.5 telencephalic hemisphere, showing the location of the pallium and subpallium structures. (B) Schematic representation of a transversal hemi-section through the telencephalon, with the illustration of the migration of MGE-derived interneurons. Interneurons respond to to chemorepulsive (red) and chemoattractive (green) factors, many of which have been identified, in the basal ganglia and cortex. Migrating interneurons, in turn, express a complex set of receptors to interact with these molecules. CSPG, chondroitin sulfate proteoglycans; H, hippocampus; NCx, neocortex; Str, striatum. Adapted from Marín, 2013.

Intracortical dispersion and formation of stereotyped migratory streams

Cortical invasion does not occur in an unsystematic way, as cortical interneurons organize and move in migratory streams (Marín & Rubenstein, 2001). Most interneurons choose between two large migratory streams within the developing cortex, a superficial route, the marginal zone (MZ), and a deeper route that principally overlaps with the subventricular zone (SVZ) of the pallium (Lavdas et al., 1999; Wichterle et al., 2001) (Figure 5A). Between E15 and E16, a smaller third stream courses through the subplate (SP), deep to the developing cortical plate. The choice of the migratory route by cortical interneurons is unlikely to be random, but it does not seem to depend on the origin of interneurons (i.e., MGE, CGE or POA) (Miyoshi and Fishell, 2011). This suggests that specific classes of interneurons might have a preference to choose one of the migration routes. This idea

remains to be experimentally tested, but transcriptomic analyses of interneurons isolated from both streams suggests that different classes of interneurons migrate through each of these streams (Antypa et al., 2011), and some functional studies support this idea. For example, interneurons lacking integrin a3 receptors fail to migrate via the MZ in the absence of Netrin 1, whereas migration through the SVZ seems to occur normally (Stanco et al., 2009). Moreover, GABAB receptor blockage in vitro alters the proportions of interneurons migrating through the MZ and SVZ (Lopez-Bendito et al., 2003). Furthermore, it was found that mutations in the cell cycle regulatory protein *Rb* prevent the migration of cortical interneurons through the MZ stream (Ferguson et al., 2005).

During their tangential dispersion, interneurons do not invade the CP, suggesting that the migration of cortical interneurons throughout the cortex requires initially the active avoidance of this area. Avoidance of the CP does not seem to involve repulsive cues expressed by projection neurons, but rather the formation of a permissive corridor in the MZ and SVZ through the expression of the chemokine Cxcl12 in these areas (Lopez-Bendito et al., 2008). Cxcl12 (also named Sdf1) is strongly expressed by the meninges and by intermediate progenitor cells transitorily present in the SVZ (Tham et al., 2001; Stumm et al., 2003; Daniel et al., 2005; Tiveron et al., 2006), and is also expressed by cells in the SP (Stumm et al., 2007). Cxcl12 has been shown to be a potent long-range chemoattractant for MGE derived interneurons in vitro (Li et al., 2008; Lopez-Bendito et al., 2008), but its limited diffusion properties in vivo would explain the relative confinement of interneurons to the migratory streams found in the cortex. Consistent with this idea, mouse mutants with altered expression of Cxcl12 in the meninges or in the SVZ have defects in the intracortical migration of interneurons that are specific to the affected migratory route (Tiveron et al., 2006; Sessa et al., 2010; Zarbalis et al., 2012, Abe et. al., 2015).

Two Cxcl12 receptors have been identified in migrating interneurons, Cxcr4 and Cxcr7 (Tiveron et al., 2006; Lopez-Bendito et al., 2008; Wang et al., 2011). Cxcr4 signals through Gα (i/o) while Cxcr7 transduces independently on heterodymeric G proteins (Wang et al., 2011). In immature MGE neurons, Cxcr7 acts as potent activator of MAP kinase signaling required for ERK1/2 phosphorylation (Wang et al., 2011). Although the two receptors may elicit different signaling pathways in response to Cxcl12 (Wang et al., 2011), Cxcr7 seems to primarily regulate the levels of Cxcr4 present in the plasma membrane of migrating cells (Sanchez-Alcaniz et al., 2011). In the absence of Cxcr7, Cxcr4 is rapidly degraded in migrating interneurons, owing to accumulation of Cxcl12. In

the absence of Cxcr4 or Cxcr7, many interneurons fail to confine their migration to the MZ and SVZ, as observed in normal embryos, and instead invade the CP prematurely (Tiveron et al., 2006; Li et al., 2008; Lopez-Bendito et al., 2008; Sanchez-Alcañiz et al., 2011; Wang et al., 2011). (Figure 5 B). The complicated fine-tuning mechanism that regulate Cxcr4 and Cxcr7 receptors dynamically adapts chemokine responsiveness in migrating neurons, thereby preventing their desensitization as they migrate through these tangential routes for a protracted period of time (Sanchez-Alcaniz et al., 2011).

It is worth noting that, despite the prominent defects observed in the intracortical dispersion of interneurons in the absence of Cxcl12 signaling (Li et al., 2008; Lopez-Bendito et al., 2008; Tanaka et al., 2010), interneurons reach the cortex in normal numbers in the absence of chemokine signaling (Tiveron et al., 2006; Li et al., 2008; Lopez-Bendito et al., 2008; Sanchez-Alcaniz et al., 2011; Wang et al., 2011). This observation reinforces the idea that the mechanisms driving the migration of interneurons from the subpallium to the cortex and those controlling their intracortical migration are different.

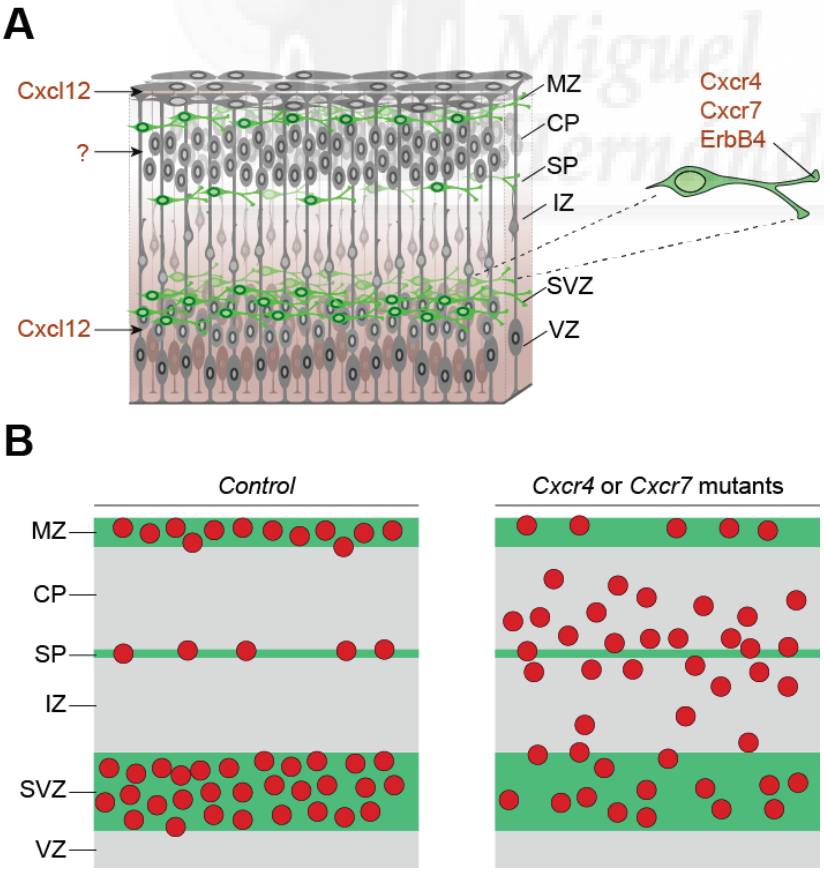

Figure 5. Migratory streams and intracortical dispersion of interneurons. (A) Schematic representation of a coronal section through the embryonic pallium, in which migrating interneurons and radial glia cells are shown. Interneurons migrate preferentially through the MZ and the SVZ, which contain high levels of the chemoattractant Cxcl12. Some interneurons also migrate through the SP. Most MGE-derived interneurons express ErbB4, Cxcr4 and Cxcr7 receptors. (B) Schematic diagrams showing the distribution of migrating interneurons in normal embryos and in mouse mutants lacking the Cxcl12 receptors Cxcr4 or

Cxcr7. CP, cortical plate; IZ, intermediate zone; SP, Subplate; SVZ, Subventricular zone; VZ, ventricular zone. Adapted from Marín, 2013.

Cortical plate invasion and laminar allocation

The molecular mechanisms regulating the tangential to radial switch in the migration of cortical interneurons and the subsequent CP invasion are largely unknown. It has been shown that the exit of interneurons from the migratory streams is coordinated with the loss of responsiveness to Cxcl12 (Li et al., 2008), but it is unclear how this process is regulated. Moreover, the analysis of Cxcr4 and Cxcr7 mutants, in which interneurons accumulate prematurely in the CP (Tiveron et al., 2006; Li et al., 2008; Lopez-Bendito et al., 2008; Tanaka et al., 2010; Sanchez-Alcaniz et al., 2011; Wang et al., 2011), suggests that pyramidal cells in the CP express a chemoattractive activity for migrating interneurons.

From a cellular perspective, interneurons seem to rely on radial glial cells to enter the CP during their tangential to radial switch. Time-lapse analyses have revealed that interactions with the basal processes of radial glial cells can influence the migration of interneurons into the CP (Yokota et al., 2007). Moreover, in vitro experiments indicate that this interaction might be mediated by connexins. For example, Connexin-43 seems to play a role in guiding interneurons radially towards the CP (Elias et al., 2010), similarly to the glial dependent migration of pyramidal cells (Elias et al., 2007; Valiente et al., 2011).

Interneuron layering

Studies over the last decade have revealed some important aspects on the regulation of layer acquisition by cortical interneurons. Most notably, several studies have suggested that the laminar distribution of cortical interneurons is regulated by projection neurons (Hevner et al., 2004; Pla et al., 2006; Yabut et al., 2007; Lodato et al., 2011b). Experiments using the *Reeler* mouse model, in which the cortical layers are inverted, showed that cortical interneurons distribute abnormally within the cortex (Hevner et al., 2004, Pla et al., 2006, Yabut et al., 2007), in a process that seems to be independent of Reelin (Pla et al., 2006). The subsequent work from Lodato and colleagues (2011) further supported the involvement of pyramidal cells in the regulation of this process. Using *Fezf2* mutant mice that lack SCPNs, they showed that the distribution of MGE interneurons was impaired. In addition, they generated ectopic of clusters of SCPN under the white matter and showed that these cells attract many interneurons in a sub-type specific mode (Lodato et al., 2011). Altogether, these results suggest that interneurons adopt their final position in the cortex

through interactions with distinct classes of pyramidal cells, rather than just based on their birthdate.

Another recent finding suggests that microglia may also regulate the laminar positioning of cortical interneurons (Squarzoni et al., 2014). Microglia invade the cortex following a gradient similar to interneurons (Cunningham et al., 2013; Swinnen et al., 2013; Squarzoni et al., 2014). In the absence of microglia, or when microglia is abnormally activated, MGE-derived interneurons enter the CP prematurely, which leads to their abnormal laminar distribution (Squarzoni et al., 2014).

The allocation of interneurons in their final position in the cortex depends on the interaction with other cells, but these interactions seem largely programmed for each cohort of interneurons. Thus, interneurons generated at different developmental stages exit the migratory streams at different times, even if the signaling that regulate the exit from the streams is the same, which indicates that this process is regulated by an intrinsic mechanism. For instance, interneurons born early invade the CP before late-born interneurons (Lopez-Bendito et al., 2008). Further evidence supports this concept of intrinsic regulation. For example, it was found that the motility of interneurons in cortical slices gradually decreases as development proceeds and is almost abolished by the end of the first postnatal week (Inamura et al., 2012). Consistent with this notion, late-born interneurons transplanted in younger embryos settle in deep layers instead of occupying the expected superficial layers (Pla et al., 2006). In addition, pharmacological disruption of the synthesis of serotonin leads to alterations in the laminar organization of CGE-derived interneurons (Vitalis et al., 2007), which suggests that other brain regions may also influence the layering of interneurons. In this latter case, however, it is not entirely clear whether the effect of serotonin on interneurons might be indirectly mediated by the role that this neurotransmitter plays in the maturation of pyramidal cells.

In contrast to the MGE, interneurons generated within the CGE do not appear to follow an inside-out pattern of layer allocation. CGE-derived cells typically occupy the superficial layers of the neocortex, without clear correlation between their temporal origin and their specific layer destination (Miyoshi et al., 2010). This suggests that the time of origin plays a role in the laminar positioning and specification of interneurons generated in the MGE, but not CGE.

Stop signals for migrating interneurons

Some factors have been proposed to have a role as stop signal for migrating interneurons. For instance, it was proposed that the frequency of Ca2+ transients is reduced as the neurons complete their migratory course (Kumada and Komuro, 2004). Other studies proposed that the intrinsic regulation of motility of interneurons might be linked to the expression of the potassium-chloride transporter KCC2. In particular, it has been suggested that it could modulate the motility of interneurons by reverting the chloride potential and thus reducing membrane depolarization upon GABAA receptor activation to serve as a stop signal for migration (Bortone and Polleux, 2009; Inamura et al., 2012). This is in agreement with the observation that cortical interneurons up-regulate the expression of the KCC2 chloride transporter as soon as they exit the tangential mode of migration and start their radial sorting in the cortex (Miyoshi and Fishell, 2011).

Local excitatory and inhibitory signals may also influence the final positioning of interneurons (De Marco Garcia et al., 2011; McKinsey et al., 2013). For instance, some studies have suggested that early patterns of activity may control this process (de Lima et al., 2008). Migrating interneurons, for example, sense GABA and glutamate during their migration to the cortex using GABAA and AMPA/NMDA receptors (Lujan et al., 2005). Moreover, it was shown that attenuating the activity of specific interneuron populations affects the migration and morphological development of interneurons (De Marco Garcia et al., 2011). A number of activity-dependent genes specifically expressed by cortical interneurons have been identified. These include *Dlx1, Elmo1*, and *Mef2c*. Moreover the observation that voltage-gated Ca2+ influx may induce *de novo* gene expression suggests that local activity might regulate direct region-specific differentiation and maturation of interneurons (De Marco Garcia et al., 2011; West and Greenberg, 2011).

3. Neuregulins in neuronal development

Neuregulins constitute a complex family of widely expressed epidermal growth factor (EGF)-like proteins that perform many functions during neural development. Neuregulins interact with and activate receptor tyrosine kinases of the ErbB family, each of which initiates specific intracellular signaling pathways, including classical canonical and non-canonical mechanisms. Neuregulin signaling has been implicated in many processes including neuronal migration (Rio et al., 1997; Anton et. al, 1997; Flames et al., 2004),

axon guidance (Lopez-Bendito et al., 2006), myelinization (Taveggia et al., 2005), synapse formation and plasticity (Schmucker, J. et al., 2003), and neurotransmission (Bjarnadottir et al., 2007). Mutations and SNPs in genes encoding neuregulins have been linked to the etiology of several neurological disorders, including bipolar and depression disorders, but mainly schizophrenia.

3.1 Neuregulin structure

Neuregulin 1 (Nrg1) was the first member of the family to be discovered (Holmes et al.; Peles et al., Wen et al., 1992). Initially it was linked to the stimulation of Schwann cell growth and induction of acetylcholine receptor expression (Falls, 2003; Mei and Xiong, 2008). Presumably through the use of distinct 5′ flanking regulatory elements and alternative splicing, *Nrg1* generates six types of protein (I–VI) (Carraway et al., 1997; Chang et al., 1997; Harari et al., 1999; Howard et al., 2005; Kinugasa et al., 2004; Uchida et al., 1999; Watanabe et al., 1995; Zhang et al., 1997). This is common to all neuregulin genes, which give rise to several splice isoforms (>30 for Nrg1 and >15 for Nrg3, for example) (Kao et al., 2010; Mei and Xiong, 2008) involved in different functions.

Immature neuregulins are transmembrane proteins, which release, upon proteolytic cleavage, the soluble N-terminal that contain the EGF-like signaling domain (Schroering et al., 1998; Wang et al. 2001). This region is located in the membrane-proximal region of the extracellular domain that is necessary and sufficient for the activation of the ErbB receptor tyrosine kinases, leading to their dimerization, tyrosine phosphorylation and the activation of downstream signaling pathways. The EGF-like domain contains roughly 50 amino acids and is characterized by three pairs of cysteins that are important for its tertiary structure and biological function. The neuregulin family of proteins shares high sequence homology in their EGF-like domain that distinguishes them from other EGF ligands (Buonanno and Fischbach, 2010).

Nrg1 is perhaps the most studied of all neuregulins. More than 30 Nrg1 isoforms have been described. Type 1 Nrg1 was originally named heregulin, neu differentiation factor (NDF), and ARIA (acetyl choline receptor inducing activity) (Holmes et al., 1992; Peles et al., 1992). Type II and III Nrg1 were identified as GGF (for ‘glial growth factor’) (Lemke and Brockes, 1984) and SMDF (for ‘sensory and motor neuron derived factor’), respectively (Ho et al., 1995). Each type of Nrg1 has a distinct N-terminus, Ig domain and/or cysteine-rich domain. Nrg1 isoforms differ in their expression levels and patterns

of expression in various tissues, including the brain (Meyer et al., 1997; Carraway et al., 1997). It is well established that different isoforms have different functions, as deduced from the analysis of mice carrying mutations that inactivate specific isoforms (Meyer et al., 1997; Fischbach et al., 1997; Kramer et al., 1996).

Most Nrg1 isoforms are synthesized as membrane-bound precursors (pro-Nrg1), with the EGF domain positioned outside of the cell (Figure 6). Pro-Nrg1 undergoes proteolytic cleavage at the juxtamembrane region that lies on the C-terminal side of the EGF-like domain. This leads to the release of a diffusible, mature form of Nrg1, except in the case of type III Nrg1, which remains anchored to the membrane. The cleavage is catalyzed by three type I transmembrane proteases: tumor necrosis factor-α converting enzyme (TACE, also known as ADAM17) (Loeb et al., 1998; Montero et al., 2007), β-site of Amyloid precursor protein cleaving enzyme (BACE, also known as Memapsin 2) (Hu et al., 2006; Willem et al., 2006) and Meltrin beta (also known as ADAM19) (Yokozeki et al., 2007). Some Nrg1 isoforms are synthesized without a transmembrane domain and are thus directly released into the extracellular space (Falls et al., 2003). The expression and processing of pro-Nrg1 are under tight temporal and spatial regulation, mostly by neural activity (Bao et al., 2003; Eilam et al., 1998; Han et a., 1999; Ozaki et al., 2004).

The majority of Nrg1 isoforms produce paracrine signaling, while the type III (CRD) seems to serve as a juxtacrine signal. The two isoforms differ only in their N-terminal region. Specifically, type III Nrg1 has two-pass transmembrane proteins, with a hydrophobic segment within the cystein rich domain (CRD) serving as a second transmembrane domain. Thus, the CRD domain is mostly intramembrane and intracellular (Figure 6). When type III and type I Nrgs are expressed in parallel cultures, the amount of type III Nrg1 released into the medium is much less than the amount of type I Nrg1, but the amount of type III Nrg1 exposed at the cell surface, most of which is the transmembrane N-terminal fragment, is much more than the amount of type I Nrg1 (Wang et al., 2001). The juxacrine function of the type III Nrg1 was also proposed in co-culture experiment of Schwann cells and sensory neurons. Several studies suggest that type III Nrg1 is an essential component of this contact-dependent signal (Salzer et al., 1980; Morrissey et al., 1995; Wolpowitz et al., 2000).

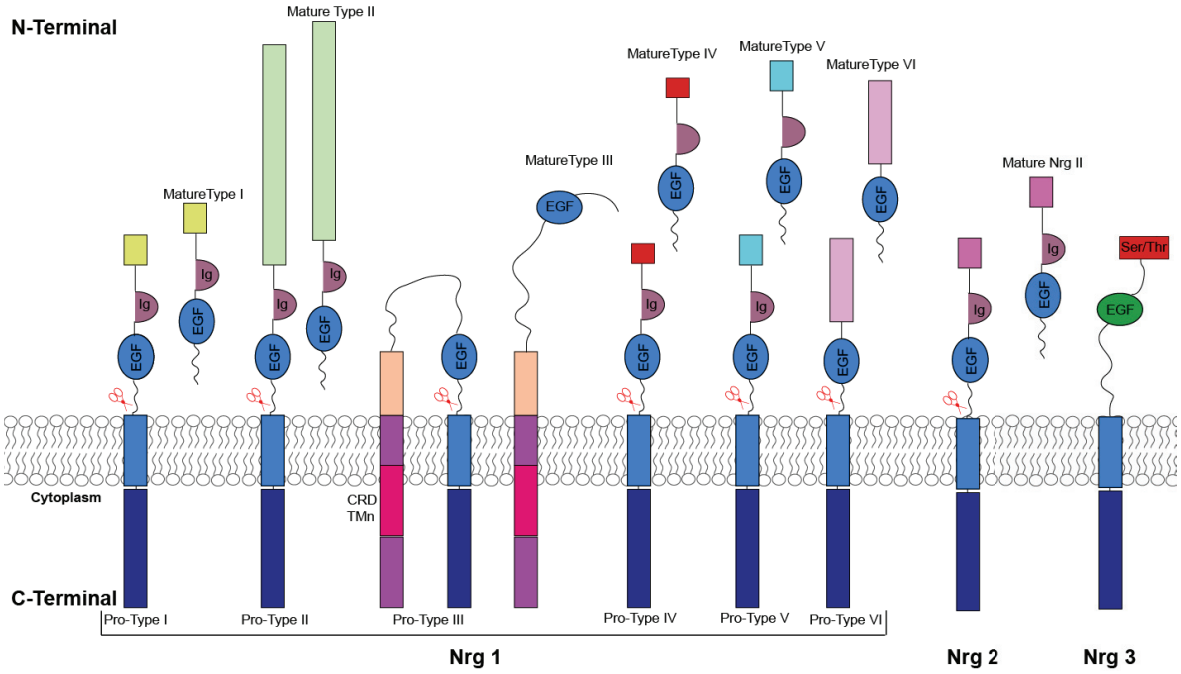

Figure 6. Structure of Nrgs. *Nrg1*: six types of *Nrg1* isoforms characterized by distinct N-terminal sequences. In the type III isoforms, this sequence contains a CRD that has a transmembrane domain (TMn) and both the N- and the C-terminal regions are located inside the cell. All six types of *Nrg*1 isoforms have an EGF-like domain. Types I, II, IV and V have an Ig-like domain between the N-terminal sequence and the EGF domain. Most *Nrg*1 isoforms are synthesized as transmembrane pro-*Nrg*1s with the EGF domain located in the extracellular region, with exception of *Nrg3* TypeIII. Cleavage by tumour necrosis factor-α converting enzyme, β-site of amyloid precursor protein cleaving enzyme or meltrin β (indicated by the scissors) generates mature *Nrg*1s that are soluble, except in the case of Type III, which is thought to function in a juxtacrine manner. *Nrg2* is most closely related to *Nrg*1 TypeI. *Nrg3*: the extracellular domain of Nrg3 lacks Ig-like domains. It contains a unique Ala/Gly rich segment at the N-terminus, a mucin-like Ser/Thr rich region containing abundant sites for O-linked glycosylation, and an EGF motif. The last motif is distinct from those encoded by the Nrg1 and Nrg2. CRD, cysteine-rich domain; EGF, Epidermal Growth Factor; Ig, Immunoglobulin; pro-*Nrg*1s, precursor polypeptides. Adapted from Mei et al., 2008.

3.2 Nrg1 signaling

Nrg1 mediates intracellular signaling through three main mechanisms: (1) canonical forward signaling, (2) non-canonical forward signaling, and (3) backward signaling (Mei et al., 2003). In canonical forward signaling, Nrg1 induces the dimerization of ErbB receptors and activates their kinase domain, which leads to both auto- and trans-phosphorylation of the intracellular domains. This process seems to involve ErbB endocytosis (Gu et al., 2005; Liu et al., 2007; Yang et al., 2005), and is followed by the activation of the Raf–MEK–ERK and PI3K–Akt–S6K pathways. This largely depends on the ErbB receptor involved, as determined by the formation of heterodimers (ErbB2–ErbB3, ErbB2–ErbB4 and ErbB3–ErbB4) or homodimer (ErbB4–ErbB4) receptor pairs.

In addition, some of the receptors may exist in different forms. For example, *ErbB4* is transcribed into four alternatively spliced isoforms.

In non-canonical forward signaling, the juxtamembrane-a (JMa) isoform of ErbB4 is cleaved to release both the extracellular and intracellular domain of the receptor. To release the soluble extracellular domain that bind to Nrg1, the Jma isoform is cleaved by TACE. To release the ErbB4 intracellular domain (ErbB4-ICD) that translocates to the nucleus and initiate transcription (Sardi et al., 2006), the cleavage is mediated by Presenilin-dependent γ-secretase (Ni et al., 2001; Lee et al., 2002). Finally, in backward signaling the nature of ligand and receptor is inverted in a way that pro-Nrg1 serves as a receptor for the ligand ErbB4. Pro-Nrg1 undergoes proteolytic processing cleavage similarly to ErbB4, and Nrg1-ICD can be transported into the nucleus (Bao et al., 2003). The cleavage activity responsible for Nrg1-ICD translocation into the nucleus remains to be determined (Bao et al., 2004).

3.3 Nrgs functions in GABAergic circuitry assembly

Neuregulins and ErbB kinases, in particular Erbb4, are critical for the assembly of the GABAergic circuitry including interneuron migration, axon and dendrite development, synapse formation and plasticity. It has been shown that the receptor ErbB4 is expressed at embryonic stages in the MGE and later in the migratory streams of interneurons migrating to the embryonic cortex (Yau et al., 2003). It seems that this receptor is not expressed in pyramidal cells (Vullhorst et al., 2009; Fazzari et al., 2010), but only in several classes of interneurons subsets (Abe et al., 2011; Fazzari et al., 2010; Fox and Kornblum, 2005; Neddens and Buonanno, 2011; Vullhorst et al., 2009; Woo et al., 2007; Yau et al., 2003), with a predominant expression in PV+ cells (Fazzari et al., 2010). In the postnatal cortex, Erbb4 was shown to be located in axonal terminals of interneurons (Fazzari et al., 2010; Woo et al., 2007) and on the postsynaptic site of excitatory and inhibitory synapses in GABAergic interneurons (Fazzari et al., 2010; Huang et al., 2001; Krivosheya et al., 2008; Vullhorst et al., 2009; Woo et al., 2007). The expression of ErbB4 in interneurons at very different stages suggest that neuregulin signaling plays important roles in the development of cortical interneurons, from migration to synapse formation.

3.3.1 Nrg1 signaling in interneuron migration

Nrg1 plays a prominent role as a chemoattractive molecule guiding the tangential migration of interneurons from the subpallium toward the cortex. MGE-derived interneurons respond to two Nrg1 isoforms, the membrane bound CRD-Nrg1 (type III) and soluble Ig-Nrg1 (type I) (Flames et al., 2004). These isoforms act as short- and long-range chemoattractants for tangential migration, respectively. CRD-Nrg1 is highly expressed in the LGE, where it creates a permissive corridor for the migration of interneurons through this region. Ig-Nrg1 is a soluble isoform expressed in the VZ of the pallium, and is involved in attracting tangentially migrating interneurons towards the cortex. Mutations in Nrg1 or ErbB4 result not only in the failure of interneurons to enter the LGE but also in a prominent reduction of interneurons reaching the cortex (Flames et al., 2004).

Nrg1 also provides a link between tangential neuronal migration and axon guidance. Thus, it has been shown that the tangential migration within the ventral telencephalon of a specific neuronal population referred as "corridor cells" is essential for the normal guidance of thalamocortical projections (López-Bendito et al., 2006). The molecular basis of this interaction relies on signaling between different Nrg1 isoforms and ErbB4 (López-Bendito et al., 2006). Nrg1-Erbb4 signaling has also been implicated in the migration of GABAergic interneurons in the rostral migratory stream (RMS) (Antón et al., 2004). In ErbB4 mutant mice, neuroblasts migrating through the RMS are disorganized, which leads to defects in the differentiation of mature interneurons in the olfactory bulb (Antón et al., 2004; Ghashghaei et al., 2005).

3.3.2 Nrg1 signaling in interneuron wiring

Nrg1-ErbB4 signaling contributes to synapse formation in cortical interneurons. Nrg1 primarily promotes the formation and maturation of excitatory synapses on GABAergic interneurons (Abe et al., 2011; Del Pino et al., 2013; Ting et al., 2011). This effect might be mediated by stabilizing PSD-95 (Ting et al., 2011), which is known to promote the maturation of glutamatergic synapses (El-Husseini et al., 2000). In addition, Nrg1-ErbB4 signaling promotes the formation and maintenance of GABAergic synapses onto pyramidal neurons. In particular, Chandelier cells lacking Erbb4 make fewer synapses onto the axon initial segments of pyramidal neurons in vivo (Del Pino et al., 2013; Fazzari et al., 2010). On the other hand, the role of Nrg1/ErbB4 signaling in synaptogenesis between interneurons is largely unknown. It seems that ErbB4 could be dispensable for the

formation and maturation of GABAergic synapses onto PV+ fast-spiking basket cells (Yang et al., 2013).

3.3.3 Nrg3 structure and functions

Very little is known about the function of Nrg3 in the developing brain. When it was first discovered, Nrg3 was shown to bind to and activate the receptor ErbB4, which seems to be its only receptor (Zhang et al., 1997). Although Nrg3 binds exclusively to Erbb4, the other ErbB receptors that heterodimerize with ErbB4 can be activated upon binding of Nrg3 (Hayes et al., 2008). ErbB2 is the preferred partner of ErbB4 and its signal induces responses that are different from those elicited by ErbB4 homodimers (Graus-Porta et al., 1997).

Analysis of the amino acid sequence of human Nrg3 reveals important homologies with Nrg1 (Zhang et al., 1997). Similar to Nrg1, the C-terminal hydrophobic segment may serve as the transmembrane domain and the N-terminal region may act as internal signal sequence. In contrast to many Nrg1 family members, however, the extracellular domain of Nrg3 lacks Ig-like domains. Nrg3 contains a unique Ala/Gly rich segment at the N-terminus, a mucin-like Ser/Thr rich region containing abundant sites for O-linked glycosylation, and an EGF motif (Figure 6). The last motif is distinct from those encoded by the Nrg1 and Nrg2 (Zhang et al., 1997). A soluble extracellular fragment of Nrg3 is released by post-translational proteolysis, and in vitro experiments have shown that it can activate Erbb4. Thus, the recombinant EGF domain of Nrg3 (rNrg3-EGF) is sufficient to induce Erbb4 receptor activation and phosphorylation (Zhang et al., 1997).

The expression of Nrg3 is highly restricted to the developing and adult nervous system, although it has been shown that Nrg3 is also expressed in the mammary gland during embryonic stages, where it controls its development (Kogata et al., 2013). The function of Nrg3 in brain development remains unclear. It has been recently shown that Nrg3 signaling may activate the protein tyrosine phosphatase non-receptor 21 (Ptpn21) to exert survival and neuritic elongation (Plani-Lam et al., 2015). Another study has proposed that Nrg3 may act as a chemorepellent for interneurons as they migrate from the MGE to cortical destinations (Li et al., 2012).

Like Nrg1, Nrg3 has been linked to the etiology of schizophrenia. In particular, multiple SNPs have been identified in the *Nrg3* locus. For instance, fine mapping of chromosome 10q22, a schizophrenia susceptibility locus, led to the identification of three

intronic SNPs in intron 1 of *Nrg3* that were associated with delusion symptom severity in patients with schizophrenia of Ashkenazi Jewish population (Chen et al., 2009). Association of these SNPs with schizophrenia was observed in a family-based study (Kao et al., 2010). Subsequently, more than 20 SNPs in *Nrg3* have been identified by case control studies and studies of rare copy-number variants (Meier et al., 2012; Wang et al., 2008; Xu et al., 2009). Some are significantly associated with psychotic symptoms and attention performance (Kao et al., 2010; Meier et al., 2012) or prefrontal cortical physiology in working memory (Tost et al., 2014), whereas others relate to better performance in the 'degraded-stimulus continuous performance' task, suggesting that Nrg3 may regulate attention processes for perceptual sensitivity and vigilance (Morar et al., 2011). A risk SNP that lies within a DNA ultra-conserved element strongly predicts elevated brain expression of *Nrg3* splice isoforms in schizophrenic patients compared to controls (Kao et al., 2010). In mice, elevated levels of Nrg3 expression in the prefrontal cortex have been linked with increased impulsivity (Loos et al., 2014).

4. Abnormal interneuron migration in neurological disease

A number of neurologic and psychiatric disorders are thought to result, at least in part, from the dysfunction of cortical interneurons. These conditions, recently termed "interneuronopathies" (Kato et al., 2005), include epilepsy, autism, schizophrenia, and even perhaps Alzheimer's disease (Rubenstein et al., 2003; Lewis et al., 2005; Chao et al., 2010; Marín, 2012; Verret et al., 2012; Rossignol, 2011). Although defects in the wiring, fine connectivity and circuit assembly of interneurons are likely behind the etiology of some of these dysfunctions, it is also possible that migration defects may influence some of these conditions. Both extrinsic factors and also SNPs and mutations in some key genes have been linked with abnormal interneuron migration. For example, prenatal stress in mice has been shown to impair the migration and final integration of interneurons in the cerebral cortex without affecting their production or survival (Stevens et al., 2012). It has been suggested that these defects are mediated by changes in the expression of key genes involved in the migration of interneurons, such as *Erbb4*. In fact, it seems reasonable to assume that variation in genes that control the development of subclasses of interneurons might confer susceptibility to neurologic disorders.

As previously described, ErbB4 is involved in sequential functions during the development of PV+ interneurons. First, it controls the tangential migration of interneurons towards the cerebral cortex in response to *Nrg1*, which acts as a chemoattractive molecule

for these cells (Flames et al., 2004). Immature interneurons fail to reach the cortex in normal numbers in the absence of ErbB4, and consequently the postnatal cortex of ErbB4 null mutant mice contains reduced numbers of GABAergic interneurons that express PV (Flames et al., 2004; Neddens et al., 2010). Thus, Nrg1/ErbB4 signaling at embryonic stages controls the normal allocation of PV+ interneurons in the cerebral cortex. Second, it controls the wiring of different populations of PV+ interneurons into specific cortical circuits. Conditional deletion of *Erbb4* in PV+ chandelier cells reduces the number of synapses that these cells make onto pyramidal cells (Fazzari et al., 2012), a phenotype that resembles post-mortem findings in schizophrenia (Woo et al., 1998). However this last aspect is more related with the integration of interneurons into circuits rather than with migration.

A feature related with interneuron migration is Nrg1 and Nrg2 signaling through ErbB4 receptors, a pathway necessary for the formation of the rostral migratory stream and the differentiation of GABAergic interneuron precursors in the adult mouse brain (Anton et al., 2004). Deficits in the migration and differentiation of interneurons in the olfactory system could influence olfactory perception. Interestingly, alterations in smell discrimination have been reported in patients with schizophrenia, depression and bipolar disorder (Moberg et al., 2003). If schizophrenic patients have a general deficit in odor identification and discrimination, these deficits could serve as an endophenotype for the disorder (Atanasova et al., 2008).

External factors such as fetal cocaine exposure result in impairment of interneuron migration. The effect of cocaine is thought to be mediated by BDNF, whose expression is decreased in cocaine-treated mice (McCarthy et al., 2011). Alternatively, cocaine has been shown to upregulate dopamine D2 receptors, whose activation reduces interneuron migration (Crandall et al., 2007). In contrast, exposure to relatively low levels of ethanol in utero enhances the sensitivity of interneurons to GABA, which, in turn, causes premature tangential migration (Cuzon et al., 2008). Thus, drug abuse and prenatal stress may increase susceptibility to mental disease by impacting the migration of cortical interneurons.

Genetic defects in humans may also disrupt the distribution of cortical interneurons. For example, interneuron defects have been described in humans carrying mutations in ARX, which causes X-linked lissencephaly with ambiguous genitalia (Bonneau et al., 2002; Marcorelles et al., 2010). In addition, fetuses with Miller– Dieker syndrome have a

significant reduction in the number of interneurons present in the cortex (Pancoast et al., 2005; Marcorelles et al., 2010). These defects are probably caused by migration abnormalities, as shown by mouse models carrying the corresponding mutations (Kitamura et al., 2002; Colasante et al., 2008; Gopal et al., 2010). Similarly, a mouse model of DiGeorge syndrome caused by the 22q11.2 deletion showed abnormalities in the distribution of PV-containing cortical interneurons (Meechan et al., 2009). Recent work suggests that these defects might be caused by a reduction in the level of Cxcr4, which would alter the timing of laminar allocation for PV-containing interneurons (Meechan et al., 2012). Toritsuka and colleagues (2013) have shown that the pivotal role of DiGeorge syndrome critical region gene 8 (Dgcr8) in miR-200a regulation is necessary for the maintenance of Cxcr4 levels.

Outstanding progress has been made to understand the mechanisms that regulate the migration of cortical interneurons, but there are important aspects of this process that are far from understood. For instance, we do not know whether interneurons are addressed to a particular region of the cortex already from their progenitor stage or if they are functionally able to integrate into any cortical area arbitrarily, the latest being a view supported by recent in vitro experiments (Lourenco et al., 2012). Through a series of culture and transplantation experiments it has been suggested that the incorporation of tangential migrating cells to the cortical circuitry follow cortical maturation gradients and might be related to regional expression patterns of positional cues. A second aspect of the migration of cortical interneurons that is largely unexplored is the process of CP invasion and layer distribution. Although it seems that pyramidal cells are instructing interneurons to find their final position in the cortex (Hevner et al., 2004; Pla et al., 2006; Lodato et al., 2011), none of the molecules involved in this process have yet been identified. The identification of the precise molecular mechanisms that control the allocation of interneurons within the cerebral cortex would help to advance our understanding of the integration of interneurons into specific cortical circuits.

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OBJECTIVES

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Objectives

The main goal of this work is to identify the molecular mechanisms that control the entry of GABAergic interneurons into the developing cortical plate and their subsequent arrangement into specific layers of the cerebral cortex. To this end, I have focused my research on the migration of MGE-derived interneurons and addressed the following specific aims:

1. To assess whether disruption of chemokine signaling is sufficient to promote the migration of MGE-derived interneurons into the developing cortical plate.

2. To develop a method to identify genes expressed by developing pyramidal cells that may influence the intracortical migration of interneurons, and to functionally assess one possible candidate.

3. To determine the precise temporal dynamics of cortical layering for late born MGE-derived interneurons using an inducible *Nkx2.1-CreER* mouse line.

4. To develop a method to identify genes that may regulate the final steps in the allocation of MGE-derived cortical interneurons migration in the cortex, and to functionally assess one possible candidate.

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METHODS

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Mice

We generated *Lhx6-Cre;ErbB4*$^{F/F}$ mice by breeding *Lhx6-Cre* mice with mice carrying loxP-flanked *ErbB4* alleles (Golub at al., 2004). We generated *Nex-Cre*;*Nrg3*$^{F/F}$ mice by producing knockout first mice (Mouse Biology Program, University of California, Davis; Skarnes et al, 2011; Figure 10) that we bred with *CAG-Flp* expressing mice (obtained from A. Nieto; Rodriguez C. et al., 2000) in order to obtain a conditional *Nrg3* allele. We next bred mice carrying loxP-flanked *Nrg3* alleles with *Nex-Cre* mice (Goebbels et al. 2006) to generate conditional mutants in which *Nrg3* is deleted from pyramidal cells (*Nex-Cre*;*Nrg3*$^{F/F}$). To genotype these mice we used the following primer sequences:

CSD-loxP: 5'-GAGATGGCGCAACGCAATTAATG-3'
CSD-Nrg3-SR1: 5'-AGTGCTGGAAATAAAAGCATGGTGGG-3'
CSD-Nrg3-wtF: 5'-CATATTACATACAGAATTCAAAGATAGGC-3'
CSD-Nrg3-wtR: 5'-CCAGTGCTGGAATTTGAATACAA-3'

CSD-loxP and CSD-Nrg3-SR1 primers were used to detect the knockout first allele. CSD-Nrg3-wtF and CSD-Nrg3-wtR were used to both detect the wild-type allele and the wild-type pre-conditional allele after exposure to CAG-*Flp* mice (Figure 10, Fw Rev primers, respectively).

Wild-type and GFP-expressing transgenic mice (Hadjantonakis et al.), maintained in a CD1 background, were used for confrontation assays experiments. *HER4*^{heart} transgenic mice, which express a human ErbB4 (*HER4*) cDNA under the control of the cardiac-specific HMC (myosin heavy chain) promoter, were maintained in a mixed C57b/6 and 129/SvJ background. The generation of *ErbB4* mutant mice (Gassmann et al., 1995) and *HER4heart* transgenic mice (Tidcombe et al., 2003) has been previously described.

Nkx2.1-Cre;tdTomato mice were generated by breeding *Nkx2.1-Cre* mice (Xu et al., 2008) with the *tdTomato* reporter line (ROSA26Sor^{tm9}[CAG-tdTomato]Hze/J) (Madisen et al., 2010). Pregnant females were used for in utero electroporation experiments. *Nkx2.1-CreER;RCE* mice were generated by breeding *Nkx2.1-CreER* mice (Taniguchi et al., 2011) with the *RCE* reporter line (Rosa26 Reporter CAG-boosted EGFP mice) (Sousa et al., 2009). These mice were used to isolate MGE-derived GFP+ cells with FACS.

Cx3cl1 mice (Cook et al., 2001) were maintained in two different backgrounds C57BL/6 and FVB.

The day of vaginal plug was considered to be embryonic day (E) 0.5 and the day of birth postnatal day (P) 0. Animal procedures were conducted in accordance with Spanish, United Kingdom and European regulations.

In situ hybridization and immunohistochemistry

For in situ hybridization, postnatal mice were perfused transcardially with 4% paraformaldehyde (PFA) in PBS and the dissected brains were postfixed overnight at 4°C in the same solution. Brains were then sectioned at 40 μm on a freezing microtome and free-floating coronal sections were subsequently hybridized with digoxigenin-labeled probes as described before (Flames et al., 2007).

The following cDNA probes were used in this study: *Nrg3* and *ErbB4* (kindly provided by Cary Lai, Indiana University, Bloomington, Indiana, USA), *GAD67* (kindly provided by John Rubenstein, UCSF, USA), *Lhx6* (kindly provided by V. Pachnis, The Crick Institute, London, UK), *Cxcr4* (Invitrogen, BG174412), *Cxcl12* (Invitrogen, clone number: 3483088), *Cdh6* and *Cdh9* (kindly provided by C Redies, University of Jena, Germany), *Ephb6* (Source BioScience, EST clone IMAGp998L1511952Q), *Epha6* (kindly provided by V. Borrell, Instituto de Neurociencias, Alicante, Spain), *Sema7a* (Source BioScience, EST clone IMAGp998I188236Q), *Cdh7* (Source BioScience), *Pcdh11x* (Source BioScience), *Rxfp1* (Source BioScience), *Robo2*, *Sema3a* and *Slit2* (kindly provided by M. Tessier-Lavigne, Rockefeller University, NY, USA), *Lgi2* (kindly provided by B. Rico, King's College London, UK), and *Cx3cl1* (Source BioScience, EST clone IMAGp998H139193Q).

For immunohistochemistry, postnatal mice were perfused transcardially with 4% PFA in PBS and the dissected brains were postfixed for 2 h at 4°C in the same solution. Brains were sectioned at 60 μm on a vibratome (VT1000S, Leica) or 40 μm on a freezing microtome and free-floating coronal sections were then subsequently processed for immunohistochemistry as previously described (Pla et al., 2006). The following primary antibodies were used: chicken anti-GFP (1:1000, GFP-1020, Aves Labs), rabbit anti-DsRed (1:500, 632496, Clontech), rat anti-BrdU (1:200, ab6326, Abcam) rabbit anti-PV (1:3000, Swant), rat anti-Somatostatin (1:200, MAB354, Millipore), rat anti-Ctip2 (1:500, ab18465, Abcam), rabbit anti-Cux1 (CDP-M222 1:100, Santa-Cruz), mouse anti-Satb2 (Abcam), and rabbit anti-Tbr1 (kindly provided by R. Hevner). The following secondary antibodies were used: goat anti-chicken 488, donkey anti-rabbit 555, donkey anti-mouse

488, and goat anti-rat IgG (H+L) Alexa Fluor® 555 conjugate (Molecular Probes). Cell nuclei were stained with 5 μM 4′′-6-diamidino-2-phenylindole (DAPI) in PBS and sections mounted with Mowiol (Sigma) with NPG (Calbiochem).

BrdU and Tamoxifen injections

In birthdating experiments, pregnant females received intraperitoneal injections at E12.5 (three injections in 18 h) or E15.5 (three injections in 12 h) with 50 mg/kg BrdU (5-bromo-2′-deoxyuridine, B5002 Sigma-Aldrich). *Nkx2.1CreERT2;RCE* pregnant females received a single intraperitoneal injection of tamoxifen (4 mg/kg) diluted in corn oil at E14.5.

In utero electroporation

E14.5 timed-pregnant ICR or *Nkx2.1-Cre;tdTomato* females were deeply anesthetized and the abdominal cavity cut open. Embryos were exposed in the uterus, and 1 μg/μl pCAG-*Gfp* or *Nrg3* (kindly provided by C. Lai, Indiana University, Bloomington, USA) plasmids were injected into the lateral ventricle of the telencephalon through the uterine wall. Square electric pulses of 45V and 50ms were passed through the uterus five times, spaced 950ms, using a square pulse electroporator (CUY21E, Nepa GENE). The uterine horns were placed back in the abdominal cavity, which was then suture closed and the female was allowed to recover.

Explant cultures

For COS cell confrontation assays, COS7 cell aggregates expressing *Rfp* alone, *Rfp* and *Cxcl12, Rfp and Nrg3, Rfp and CRD-Nrg1* and *Rfp and Ig-Nrg1* were prepared by diluting transfected cells with Matrigel in a 1:1 proportion. After jellification, COS cell aggregates were cut with a scalpel in small rectangular prisms of approximately 400x400x800 μm and confronted to explants of MGE (obtained from GFP-expressing transgenic mice) in Matrigel. The cDNA used for expression of *Cxcl12* was obtained from Invitrogen (clone number: 3483088; accession number: BC006640). *Nrg3* was kindly provided by Cary Lai (Indiana University, Bloomington, Indiana, USA). The sequences used for expression of type I NRG1 (*Ig-Nrg1*) and type III NRG1 (*CRD-Nrg1*) correspond to the accession numbers AY648976 and AY648975, respectively. For Cxcl12 chemokine-blocking experiments, SU6656 (Sigma, 330161-87-0) was added to the medium at the beginning of the culture period, at a final concentration of 15 μM.

In vitro focal electroporation

Coronal slice cultures were obtained as described previously (Anderson et al., 1997). A pCAGG based *dsRed* plasmid was pressure injected focally into the MGE of coronal slice cultures by a Pneumatic PicoPump (Narishige) through a glass micropipette. Slices were then electroporated within a setup of two horizontally oriented platinum electrodes (Protech International Inc.) powered by a T820 Electro Square Porator (BXT), as described before (Flames et al., 2004).

Time-lapse videomicroscopy

Slices were transferred to the stage of an upright Leica DMLFSA or inverted Leica DMIRE2 microscope coupled to a confocal spectral scanning head (Leica TCS SL) and viewed through 10–60X water immersion or 20X oil objectives. Slices were continuously superfused with warmed (32°C) artificial cerebrospinal fluid at a rate of 1 ml/minute or maintained in supplemented Neurobasal medium. To block Cxcl12 function, SU6656 (Sigma, 330161-87-0) was added to the medium at a final concentration of 15 μM.

Protein stripe assay

Purified CXCL12 protein was obtained from PeproTech (PeproTech, 250-20A) and used at 1 ng/ul. GST and EGF-Nrg3-GST were purified using standard protocols and used at 10 μg/ml. Alternating lanes, 50 μm wide, were laid down on a poly-lysine-coated plastic dish. Alexa555-labeled anti-rabbit IgGs were added to the GST, EGF-Nrg3-GST and CXCL12 protein solution for lane identification. The lanes were further coated with laminin. MGE explants were dissected out of GFP+ brain slices as described above, plated on top of the protein stripes, and incubated in methylcellulose-containing Neurobasal medium for 48 h.

Tissue Dissociation and FACS

After in utero electroporation in E14.5 pregnant ICR females, or tamoxifen injections in E14.5 *Nkx2.1-CreER;RCE* pregnant females, the sensorimotor cortex of E17.5 embryos and P4 pups was dissociated as described previously (Catapano et al., 2001). GFP+ cells were purified using fluorescent activated cell sorting (FACSARIA III, BD Biosciences) and the resulting pellet kept at -80 ºC.

Taqman gene expression assays

We isolated GFP+ pyramidal cells by FACS at E17.5 and P4 after in utero electroporation at E14.5. The mRNA was extracted using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. RNA quality was assessed using a bioanalyzer (Agilent Technologies) and then retro-transcribed into single-stranded cDNA. The RNA was sent to Unidad Genómica (Antonia Martín Gallardo, Fundación Parque Científico de Madrid) for quality control and retro-transcription. Relative gene expression of two independent samples was analyzed by custom designed TaqMan® low-density array (TLDA) plates (96 wells) (Micro Fluidic Cards, Applied Biosystems, Foster City, USA). Each plate contained duplicates for all the genes showed in the Table 2.1. Data were collected and analyzed using the threshold cycle (Ct) relative quantification method. The house keeping gene 18 RNA was included in the array for assessing RNA quality and sample normalization.

Microarrays

We isolated GFP+ interneurons by FACS at E17.5 and P4 following tamoxifen injections in E14.5 *Nkx2.1-CreER;RCE* pregnant females. We then carried out mRNA amplification and hybridized mouse whole genome Affymetrix® microarrays (GeneChip 430 2.0, Genomic and Proteomic Unit, Centro de Investigación del Cancer, Salamanca, Spain). Statistical significance of gene expression differences between interneurons populations was determined by pair-wise comparisons at each age using significance analysis of Microarrays (SAM) (Tusher et al., 2001, Anders and Huber, 2010), in which we considered genes differentially expressed with a False Discovery Rate (FDR) of <0.05 (the adjusted p-value, or q-value, of 0.05, implies that 5% of significant tests will result in false positives).

qPCR

qPCR was performed to confirm microarray data for Cx3cl1 and the absence of Nrg3 transcripts in *Nex-Cre; Nrg3$^{F/F}$* mice. To confirm microarray data, cortical tissue was collected from *Nkx2.1-CreER;RCE* mice at E17.5 and P4 after tamoxifen injections at E14.5, and the tissue was dissociated as previously described. To confirm the absence of *Nrg3* transcripts in *Nex-Cre;Nrg3$^{F/F}$* mice, cortical tissue was collected from control (*Nrg3$^{F/F}$*) and mutant (*Nex-Cre;Nrg3$^{F/F}$*) mice at P30 and dissociated. Total RNA from the somatosensory cortex was extracted using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. RNA was retro-transcribed into single-stranded cDNA using

SuperScript III Reverse Transcriptase and Oligo(dT)12-18 primers (Invitrogen) for 1 hour at 42°C. qPCR was carried out in an Applied Biosystems 7300 real-time PCR unit using TaqMan® probes (Life Technologies) according to the manufacturer's specifications. Each independent sample was assayed in triplicate. Gene expression levels were normalized using *GAPDH*. Probes were obtained from TaqMan® Life Technologies: *Cx3cl1*, 4331182, Mm00436454_m1 and *Nrg3*, 4331182, Mm01209104_m1.

Image analysis and quantification

Images were acquired using fluorescence microscopes (DM5000B, CTR5000 and DMIRB; Leica) coupled to digital cameras (DC500 or DFC350FX, Leica; OrcaR2, Hamamatsu), Apotome.2 (Zeiss) or an inverted Leica TCS SP8 confocal microscope. All images were analyzed with ImageJ (Fiji). For the quantification of migration in MGE explants, the distance migrated by the 30 furthest cells was measured. For the quantification of short-range chemoattraction, the colocalizing area between MGE and COS cells was measured. For the analysis of interneuron angle of migration, we draw a grid of virtual radial lines (lines perpendicular to the ventricular zone and the pial surface) and oriented each cell in relation to the most adjacent 'radial line'. Cells that deviated less than 25° from radial lines were considered as radially oriented; those that deviate more than 25° were designated as tangentially oriented. We systematically exclude from this analysis those cells located in the more lateral or medial regions of the cortex, so that the curvature of the slice in those regions would not interfere with our analysis (Martini et al., 2009). Stained sections in the somatosensory areas of control and mutant mice were imaged during the same imaging session using an inverted Leica TCS SP8 confocal microscope. Data acquisition was performed using the same laser power, photomultiplier gain, pinhole and detection filter settings (1024x1024 resolution, 12 bits). Quantifications were done using ImageJ (Fiji). Layers were drawn following nuclear staining. For in situ hybridization the area quantified was divided in ten equal bins and the percentage of cells in each bin was calculated. The bins were then converted to layers.

Statistical analyses

Statistical analysis was carried out in SPSS (SPSS Inc.). P values below 0.05 were considered statistically significant. Data are presented as mean and SEM throughout the Thesis. Normality and variance tests were first applied to all experimental data. When data follows a normal distribution, paired comparisons were analyzed with *t*-test, while multiple

comparisons were analyzed using either ANOVA with post-hoc Bonferroni correction (equal variances) or the Welch test with *post-hoc* Games-Howell (different variances). A χ^2-test was applied to analyze the distribution of cells in either bins or layers.

Tables 1-3: Results Part 1.

Sample	N° of cells	N° of FACS experiments	RNA value (RIN)	RNA concentration (ng/ul)
1) E17.5	783378	4 experiments	/	36.4
2) E17.5	951264	5 experiments	9.7	24
1) P4	703915	8 experiments	8.8	51.64
2) P4	843448	6 experiments	9.5	62

Table 1. Experiments of FACS and RNA extraction. Isolation of Gfp+ cells at two different stages of development (E17.5, P4) after in utero electroporation in the ventricular zone of E14.5 ICR embryos. Two independent replicates for condition. Reported number of total cells for each replicate, N° of FACS experiment performed, RNA quality values (RIN) and RNA concentration obtained.

Assay ID	Locus Link Gene Symbol	Public RefSeq
Mm00486918_m1	Cdh1,mCG20483	NM_009864.2
Mm00483213_m1	Cdh2,mCG141325	NM_007664.4
Mm01249209_m1	Cdh3,mCG20482	NM_007665.2
Mm00486926_m1	Cdh4,mCG116031	NM_009867.2
Mm03053719_s1	Cdh5	NM_009868.4
Mm00511182_m1	Cdhr5,mCG23289	NM_028069.3
Mm00483230_m1	Cdh6,mCG8950	NM_007666.3
Mm00556135_m1	Cdh7,mCG14554	NM_172853.2
Mm01242096_m1	Cdh8,mCG124257	NM_001039154.1
Mm00515462_m1	Cdh11,mCG125313	NM_009866.4
Mm01165359_m1	Cdh12,mCG19771	NM_001008420.2
Mm00490584_m1	Cdh13,mCG141363	NM_019707.4
Mm00483191_m1	Cdh15,mCG19581	NM_007662.2
Mm00483196_m1	Cdh16,mCG23406	NM_007663.2
Mm00490692_m1	Cdh17,mCG5094	NM_019753.4
Mm00457145_m1	Cdh20,mCG3576	NM_011800.4
Mm00558118_m1	Cdh22,mCG17522	NM_174988.3
Mm00465755_m1	Cdh23,mCG1819	NM_023370.2
Mm01313848_g1	Cdh24,mCG133655	NM_199470.2
Mm00547091_s1	Pcdh1,mCG142244	NM_029357.3
Mm00479579_m1	Pcdh7,mCG9825	NM_018764.2
Mm00480660_m1	Pcdh8,mCG19385	NM_001042726.3
Mm03038601_m1	Pcdh9	NM_001081377.1

Mm00477987_s1	Pcdh10,mCG7131	NM_001098171.
Mm01221603_m1	Pcdh11x,mCG51196	NM_001081385.1
Mm00450488_m1	Pcdh12,mCG18330	NM_017378.2
Mm00480870_m1	Pcdh15,mCG114141	NM_001142735.1
Mm00499890_m1	Pcdh18,mCG7322	NM_130448.3
Mm00724499_m1	Pcdh20,mCG17884	NM_178685.5
Mm00445804_m1	Epha1,mCG17082	NM_023580.4
Mm00438726_m1	Epha2,mCG10037	NM_010139.2
Mm00580743_m1	Epha3,mCG127999	NM_010140.3
Mm00433056_m1	Epha4,mCG119512	NM_007936.3
Mm00433074_m1	Epha5,mCG5337	NM_007937.3
Mm00433094_m1	Epha6,mCG127847	NM_007938.2
Mm00833876_m1	Epha7,mCG14600	NM_010141.3
Mm00433106_m1	Epha8,mCG9328	NM_007939.2
Mm00624498_m1	Epha10,mCG17241	NM_177671.5
Mm00557961_m1	Ephb1,mCG140739	NM_173447.3
Mm01181015_m1	Ephb2,mCG120083	NM_010142.2
Mm00802553_m1	Ephb3,mCG129784	NM_010143.1
Mm01201157_m1	Ephb4,mCG6855	NM_001159571.1
Mm00432456_m1	Ephb6,mCG4984	NM_001146351.1
Mm00438660_m1	Efna1,mCG17554	NM_010107.4
Mm00433011_m1	Efna2,mCG13393	NM_007909.3
Mm01212723_g1	Efna3,mCG17541	NM_010108.1
Mm00433013_m1	Efna4,mCG17548	NM_007910.2
Mm00438665_m1	Efna5,mCG50503	NM_010109.3
Mm00438666_m1	Efnb1,mCG51675	NM_010110.4
Mm00438670_m1	Efnb2,mCG17314	NM_010111.5
Mm00433016_m1	Efnb3,mCG20906	NM_007911.5
Mm01230580_g1	Sema3b,mCG18861	NM_001042779.1
Mm00443121_m1	Sema3c,mCG6382	NM_013657.5
Mm00712652_m1	Sema3d,mCG115650	NM_028882.4
Mm00809130_s1	Sema3e,mCG148351	NM_011348.2
Mm00441325_m1	Sema3f,mCG18872	NM_011349.3
Mm00803797_m1	Sema4b,mCG19462	NM_013659.4
Mm00443147_m1	Sema4d,mCG1273	NM_013660.3
Mm00442518_m1	Sema4g,mCG16919	NM_011976.1
Mm00436500_m1	Sema5a,mCG141513	NM_009154.2
Mm00443163_m1	Sema5b,mCG130168	NM_013661.2
Mm00444441_m1	Sema6a,mCG8025	NM_018744.2
Mm00441345_m1	Sema6c,mCG13711	NM_011351.1
Mm00441361_m1	Sema7a,mCG132078	NM_011352.2
Mm00436469_m1	Sema3a,mCG16225	NM_009152.3
Mm00443140_m1	Sema4a,mCG8826	NM_001163490.1
Mm00441343_m1	Sema4f,mCG126253	NM_011350.3
Mm00443176_m1	Sema6b,mCG22980	NM_001130456.1
Mm00553142_m1	Sema6d,mCG142100	N.R.
Mm00470649_m1	Plxdc2,mCG19758	NM_026162.5
Mm00501110_m1	Plxna1,mCG126649	NM_008881.2
Mm00801930_m1	Plxna2,mCG116593	NM_008882.2
Mm00501170_m1	Plxna3,mCG21221	NM_008883.2
Mm00558881_m1	Plxna4,mCG141681	NM_175750.3
Mm00555359_m1	Plxnb1,mCG16096	NM_172775.2

Mm00502216_m1	Plxnb3,mCG8090	NM_019587.2
Mm00450687_m1	Plxnc1,mCG4296	NM_018797.2
Mm01184367_m1	Plxnd1,mCG132454	NM_026376.3
Mm00507118_m1	Plxnb2,mCG140951	NM_138749.2
Mm00511436_m1	Plxdc1,mCG21901	NM_028199.3
Mm00810320_s1	Wnt1,mCG18420	NM_021279.4
Mm00470018_m1	Wnt2,mCG13463	NM_023653.5
Mm00437336_m1	Wnt3,mCG19162	NM_009521.2
Mm03053674_s1	Wnt5a	NM_009524.2
Mm00437350_m1	Wnt5b,mCG131712	NM_009525.3
Mm01209104_m1	Nrg3, mCG112807	NM_001190187.1
Mm01212130_m1	Nrg1, mCG130630	NM_178591.2
Mm00803929_m1	Slc12a5,mCG17512	NM_020333.2
Mm02619632_s1	Cxcr7	NM_007722.3
Mm01996749_s1	Cxcr4,mCG20049	NM_009911.3
Mm00436671_m1	Sst,mCG125080	NM_009215.1
Mm00748360_s1	Lhx6,mCG22275	NM_001083126.1
Mm00442874_m1	Htr3a,mCG3840	NM_001099644.1
Mm00501628_m1	Cux1,mCG18016	NM_009986.3
Mm00493433_m1	Tbr1,mCG15138	NM_009322.3

Table 2. TaqMan array low-density array (TLDA) genes. List of 95 genes run in the TLDA plates. Reported the AssayID (Applied Biosystems), Gene Symbol and Public RefSeq.

Gene	FC	pValue
Sema6b	1.354327	* **p <0.05**
Plxna1	2.027104	* **p<0.05**
Efna3	2.185114	* **p <0.05**
Sema4f	2.260192	*** **p <0,001**
Cdh6	2.548228	*** **p <0,001**
Pcdh15	2.592109	*** **p <0,001**
Ephb6	2.957104	*** **p <0,001**
Nrg3	3.026187	** **p <0.01**
Cdh20	3.301568	*** **p <0,001**
Pcdh11x	4.405326	*** **p <0,001**
Plxnd1	5.129877	*** **p <0,001**
Epha10	5.509796	*** **p <0,001**
Pcdh20	5.730245	*** **p <0,001**
Epha6	5.898654	*** **p <0,001**
Plxdc2	7.28674	*** **p <0,001**
Epha8	7.465011	*** **p <0,001**
Efna5	7.852177	** **p <0.01**

Table 3. Genes overexpressed in pyramidal cells during the integration of interneurons in the CP. List of 17 genes that are significantly more expressed by pyramidal cells at P4 compared to E17.5. Data were collected and analysed using the threshold cycle (Ct) relative quantification method. The house-keeping gene 18 sRNA was included in the array for assessing RNA

quality and sample normalization. t test, *p < 0.05; **p < 0.01; ***p < 0.001.

Tables 4-5: Results Part 2.

Sample	N of cells/ N of FACS exp	RNA value (RIN)	RNA concentration (ng/ul)
E17.5) 1	204743 cells / 3 FACS	8.6	2.2
E17.5) 2	141659 cells / 4 FACS	9.5	0.5
E17.5) 3	128127 cells / 4 FACS	9.5	0.9
P4) 1	152987 cells / 6 FACS	9.7	1.1
P4) 2	160609 cells / 5 FACS	9.8	1.6
P4) 3	154488cells / 5 FACS	9.5	0.4

Table 4. Experiments of FACS and RNA extraction. Isolation of Gfp+ cells at two stages of development (E17.5 and P4) after tamoxifen injection in *Nkx2.1-CreER*; *RCE* mice at E14.5. Three indipendent replicates for condition. Reported number of total cells for each replica, N° of FACS experiment performed, RNA quality values (RIN) and RNA concentration obtained.

Gene Name	Probeset ID	D. Value	P. Value	Q. value	R fold
AI593442	ENSMUSG00000078307	-38.352688	7.12E-05	0.051299	24.58748
Cdh7	ENSMUSG00000026312	-34.119748	0.000109	0.051299	24.22043
Spock3	ENSMUSG00000054162	-18.455287	0.000838	0.053417	23.89853
Cdh9	ENSMUSG00000025370	-41.183677	4.61E-05	0.051299	22.49083
Grin3a	ENSMUSG00000039579	-24.544684	0.000348	0.052376	16.47004
AC116825.1	ENSMUSG00000074341	-46.863311	3.77E-05	0.051299	16.44491
Ppargc1a	ENSMUSG00000029167	-25.178135	0.000318	0.052376	16.15217
Me3	ENSMUSG00000030621	-30.291438	0.000142	0.052376	15.63507
4930431L04Rik	ENSMUSG00000061864	-17.257471	0.000959	0.053473	14.92861
AF529169	ENSMUSG00000039313	-33.211412	0.000117	0.052376	14.73086
Klhl14	ENSMUSG00000042514	-18.973663	0.000737	0.053417	14.1291
Adcy8	ENSMUSG00000022376	-34.905991	0.000105	0.051299	13.16556
Sstr1	ENSMUSG00000035431	-39.55023	6.28E-05	0.051299	12.95226
Lgi2	ENSMUSG00000039252	-19.062632	0.000729	0.053417	11.23648
Plcb1	ENSMUSG00000051177	-18.720106	0.000783	0.053417	9.879695
Olfm3	ENSMUSG00000027965	-19.977789	0.000637	0.052649	9.494824
Mkx	ENSMUSG00000061013	-25.528265	0.000302	0.052376	9.17637
Ak5	ENSMUSG00000039058	-17.207422	0.000972	0.053473	8.932042

Synpr	ENSMUSG00000056296	-38.903235	6.7E-05	0.051297	8.719978
9330182L06Rik	ENSMUSG00000056004	-21.731839	0.000486	0.052376	8.654999
Rxfp1	ENSMUSG00000034009	-21.549576	0.000494	0.052376	8.583995
Plekhh2	ENSMUSG00000040852	-23.448936	0.000402	0.052376	8.556274
Sstr4	ENSMUSG00000037014	-17.66682	0.000917	0.053417	8.077973
Garnl3	ENSMUSG00000038860	-28.766708	0.000176	0.052376	8.038019
Pcdh11x	ENSMUSG00000034755	-65.586981	1.68E-05	0.051299	8.017279
Luzp2	ENSMUSG00000063297	-26.226094	0.000247	0.052376	7.635815
Kcnip2	ENSMUSG00000025221	-21.323765	0.000519	0.052376	7.599791
Kcnd2	ENSMUSG00000060882	-26.621593	0.00023	0.052376	7.569809
Cntnap5a	ENSMUSG00000070695	-24.928171	0.000331	0.052376	7.448733
Unc13c	ENSMUSG00000062151	-18.625005	0.000804	0.053417	7.215458
Ahr	ENSMUSG00000019256	-28.602101	0.00018	0.052376	7.206574
Cx3cl1	ENSMUSG00000031778	-25.470068	0.00031	0.052376	6.889467
Prmt8	ENSMUSG00000030350	-18.342069	0.000858	0.053417	6.87771
Pvt1	ENSMUSG00000072566	-20.538847	0.00059	0.052376	6.779918
Mgat4c	ENSMUSG00000019888	-36.248086	8.79E-05	0.051299	6.678472
Mgll	ENSMUSG00000033174	-21.821794	0.000477	0.052376	6.648967
Spnb1	ENSMUSG00000021061	-25.826464	0.000276	0.052376	6.556382
Acsl1	ENSMUSG00000018796	-25.655709	0.000289	0.052376	6.498551
Alcam	ENSMUSG00000022636	-21.469017	0.000503	0.052376	6.480723
Rnf152	ENSMUSG00000047496	-27.577391	0.000218	0.052376	6.383545
Fam134b	ENSMUSG00000022270	-18.57662	0.000817	0.053417	6.245415
Sema3a	ENSMUSG00000028883	-37.176695	8.38E-05	0.051299	6.217023
Igsf11	ENSMUSG00000022790	-27.676863	0.000214	0.052376	6.030843
Mme	ENSMUSG00000027820	-76.058208	4.19E-06	0.051299	6.00319
Cbln4	ENSMUSG00000067578	-22.176068	0.000444	0.052376	5.778953
Cyp46a1	ENSMUSG00000021259	-17.650043	0.000921	0.053417	5.659849
Ell2	ENSMUSG00000001542	-18.969453	0.000741	0.053417	5.643712
Limch1	ENSMUSG00000037736	-19.313099	0.000699	0.053416	5.636185
Thrb	ENSMUSG00000021779	-67.586475	8.38E-06	0.051299	5.523606
Rcan2	ENSMUSG00000039601	-17.902781	0.000892	0.053417	5.51513

Ppp2r2c	ENSMUSG00000029120	-16.557880	0.001064	0.053673	5.454809
Prickle1	ENSMUSG00000036158	-23.772513	0.000394	0.052376	5.274728
Oxtr	ENSMUSG00000049112	-23.308012	0.000406	0.052376	5.182945
Etl4	ENSMUSG00000036617	-28.237588	0.000193	0.052376	5.158861
Fam3c	ENSMUSG00000029672	-30.545503	0.000134	0.052376	5.02356
Tmeff2	ENSMUSG00000026109	-25.672669	0.000285	0.052376	4.995402
Camk2d	ENSMUSG00000053819	-25.627849	0.000293	0.052376	4.826848
Gabrg3	ENSMUSG00000055026	-18.377347	0.000854	0.053417	4.818257
Tbc1d4	ENSMUSG00000033083	-46.162269	4.19E-05	0.051299	4.766013
Cntn4	ENSMUSG00000064293	-24.834343	0.000335	0.052376	4.723962
Glt8d4	ENSMUSG00000030074	-25.968072	0.000268	0.052376	4.440002
Adcy2	ENSMUSG00000021536	-17.387984	0.000946	0.053417	4.436557
Id4	ENSMUSG00000021379	-20.576987	0.000582	0.052376	4.425381
Rasgef1a	ENSMUSG00000030134	-29.735838	0.000151	0.052376	4.396209
Mid2	ENSMUSG00000000266	-18.762329	0.000766	0.053417	4.379123
Glrb	ENSMUSG00000028020	-17.854759	0.0009	0.053417	4.363083
Klf5	ENSMUSG00000005148	-19.717628	0.000666	0.052912	4.332054
Ncald	ENSMUSG00000051359	-18.382118	0.00085	0.053417	4.300609
Gabra3	ENSMUSG00000031343	-35.046042	0.000101	0.051299	4.144777
AC100382.1	ENSMUSG00000029483	-16.920568	0.001022	0.053473	4.138205
Grin2d	ENSMUSG00000002771	-17.250857	0.000963	0.053473	4.036203
Gabbr2	ENSMUSG00000039809	-51.283527	2.93E-05	0.051299	4.023868
Ppp2r2b	ENSMUSG00000024500	-24.014732	0.000369	0.052376	4.015713
Btbd11	ENSMUSG00000020042	-29.542015	0.000163	0.052376	3.908717
St8sia5	ENSMUSG00000025425	-29.573568	0.000155	0.052376	3.796367
Atp1b1	ENSMUSG00000026576	-21.903439	0.000465	0.052376	3.757363
Frmd3	ENSMUSG00000049122	-17.329853	0.000951	0.053417	3.699325
Kndc1	ENSMUSG00000066129	-16.895192	0.001026	0.053473	3.685219
Kcnip1	ENSMUSG00000053519	-21.028475	0.000536	0.052376	3.630925
Slc2a3	ENSMUSG00000003153	-18.198075	0.000871	0.053417	3.627648
9930013L23Rik	ENSMUSG00000052353	-17.586863	0.00093	0.053417	3.591155
Pla2g4e	ENSMUSG00000050211	-23.27819	0.00041	0.052376	3.544342

Dnm1	ENSMUSG00000026825	-26.028075	0.00026	0.052376	3.526246
Tmem117	ENSMUSG00000063296	-23.747451	0.000398	0.052376	3.518755
Trps1	ENSMUSG00000038679	-19.080120	0.000724	0.053417	3.498355
Ank1	ENSMUSG00000031543	-20.054933	0.000632	0.052649	3.498248
Nell1	ENSMUSG00000055409	-21.942227	0.000461	0.052376	3.483445
Rimbp2	ENSMUSG00000029420	-18.432867	0.000846	0.053417	3.472516
Rgs17	ENSMUSG00000019775	-17.051468	0.000988	0.053473	3.470908
Slit2	ENSMUSG00000031558	-20.566784	0.000586	0.052376	3.439645
AC139023.2	ENSMUSG00000033204	-37.552242	7.54E-05	0.051299	3.41719
Slc16a14	ENSMUSG00000026220	-17.02874	0.000997	0.053473	3.373908
Kcnq3	ENSMUSG00000056258	-18.43710	0.000842	0.053417	3.350545
Fam171b	ENSMUSG00000048388	-25.570589	0.000297	0.052376	3.307217
Ptchd1	ENSMUSG00000041552	-26.551439	0.000235	0.052376	3.281418
Galnt13	ENSMUSG00000060988	-29.109986	0.000168	0.052376	3.246302
Fgf14	ENSMUSG00000025551	-16.970692	0.001013	0.053473	3.19224
Fam163b	ENSMUSG00000009216	-18.933412	0.00075	0.053417	3.152961
Fut8	ENSMUSG00000021065	-32.716905	0.000121	0.052376	3.133098
Prss23	ENSMUSG00000039405	-18.745729	0.000775	0.053417	3.089334
B4galt6	ENSMUSG00000056124	-18.505791	0.000825	0.053417	3.069643
Tmem132c	ENSMUSG00000034324	-20.587835	0.000574	0.052376	3.020942
AC122281.2	ENSMUSG00000074942	-18.632828	0.0008	0.053416	2.99366
Dcbld2	ENSMUSG00000035107	-28.515849	0.000184	0.052376	2.908097
Tmem130	ENSMUSG00000043388	-39.862512	5.86E-05	0.051299	2.859852
Sh3bgrl2	ENSMUSG00000032261	-23.943375	0.000373	0.052376	2.818409
Slc35f3	ENSMUSG00000057060	-18.507092	0.000821	0.053417	2.806469
Wasf3	ENSMUSG00000029636	-17.230124	0.000967	0.053473	2.804347
Grem1	ENSMUSG00000074934	-25.830448	0.000272	0.052376	2.797435
Enpp1	ENSMUSG00000037370	-18.709089	0.000787	0.053417	2.761269
Atp1a1	ENSMUSG00000033161	-27.806659	0.000205	0.052376	2.74732
Ptprr	ENSMUSG00000020151	-24.360287	0.00036	0.052376	2.740528
Tram1	ENSMUSG00000025935	-19.519631	0.000678	0.052912	2.707271
Myrip	ENSMUSG00000041794	-17.536109	0.000934	0.053417	2.700432

Bai3	ENSMUSG00000033569	-19.932634	0.000649	0.052649	2.694988
Ndst3	ENSMUSG00000027977	-20.467968	0.000599	0.052376	2.687811
Mafb	ENSMUSG00000074622	-23.778621	0.000385	0.052376	2.679302
Kcns3	ENSMUSG00000043673	-20.721792	0.000565	0.052376	2.665249
Grid1	ENSMUSG00000041078	-20.425369	0.000603	0.052376	2.651197
Gpr176	ENSMUSG00000040133	-21.857832	0.000473	0.052376	2.594976
Sfmbt2	ENSMUSG00000061186	-67.130484	1.26E-05	0.051299	2.583612
Kcnh7	ENSMUSG00000059742	-20.093879	0.000628	0.052649	2.531468
Lhfpl3	ENSMUSG00000058361	-23.899379	0.000377	0.052376	2.518662
Klhl5	ENSMUSG00000054920	-26.035370	0.000255	0.052376	2.360548
Rora	ENSMUSG00000032238	-20.856059	0.000553	0.052376	2.340936
Daam1	ENSMUSG00000034574	-30.329742	0.000138	0.052376	2.332617
Galnt9	ENSMUSG00000033316	-20.862168	0.000549	0.052376	2.331981
Cyp2u1	ENSMUSG00000027983	-19.878680	0.000653	0.052649	2.316851
Chd5	ENSMUSG00000005045	-16.633328	0.001051	0.053673	2.295999
Hook1	ENSMUSG00000028572	-16.655893	0.001043	0.053670	2.29044
Tmem106b	ENSMUSG00000029571	-18.456477	0.000833	0.053417	2.245763
Pitpnm3	ENSMUSG00000040543	-40.418436	5.44E-05	0.051299	2.220421
Syt10	ENSMUSG00000063260	-28.48692	0.000188	0.052376	2.202084
Syt1	ENSMUSG00000035864	-27.869444	0.000201	0.052376	2.19344
Slc30a4	ENSMUSG00000005802	-36.126139	9.63E-05	0.051299	2.187058
Cplx2	ENSMUSG00000025867	-21.163289	0.000532	0.052376	2.150409
Mef2a	ENSMUSG00000030557	-26.2316	0.000243	0.052376	2.149714
Tmem65	ENSMUSG00000062373	-24.993245	0.000322	0.052376	2.114353
Esrrg	ENSMUSG00000026610	-25.990488	0.000264	0.052376	2.099457
Sh2d5	ENSMUSG00000045349	-19.413564	0.000687	0.052912	2.057716
Rims1	ENSMUSG00000041670	-25.498609	0.000306	0.052376	2.049284
Lonrf2	ENSMUSG00000048814	-18.704344	0.000791	0.053417	2.049041
Atp1a3	ENSMUSG00000040907	-18.490777	0.000829	0.053417	2.014124
Robo2	ENSMUSG00000052516	-18.1979	0.000875	0.053417	2.001616
Slc9a6	ENSMUSG00000060681	-23.773576	0.000389	0.052376	1.988564
Nptn	ENSMUSG00000032336	-18.594143	0.000808	0.053417	1.970175

Pcdhb8	ENSMUSG00000045876	-22.24033	0.00044	0.052376	1.939669
Timp2	ENSMUSG00000017466	-23.251537	0.000415	0.052376	1.896965
Agbl4	ENSMUSG00000061298	-24.734918	0.000339	0.052376	1.851024
Myo1c	ENSMUSG00000017774	-20.47106	0.000595	0.052376	1.816455
Atp2b3	ENSMUSG00000031376	-23.812169	0.000381	0.052376	1.800645
Slc24a4	ENSMUSG00000041771	-16.981462	0.001005	0.053473	1.731099
Stxbp1	ENSMUSG00000026797	-19.652113	0.00067	0.052912	1.710777
Clip4	ENSMUSG00000024059	-26.090476	0.000251	0.052376	1.653156
BC022960	ENSMUSG00000081137	-17.122889	0.00098	0.053473	1.627862
Manba	ENSMUSG00000028164	-19.086181	0.00072	0.053417	1.624745
Slc26a6	ENSMUSG00000023259	-17.187653	0.000976	0.053473	1.598479
Pgr	ENSMUSG00000031870	-16.703128	0.001039	0.053670	1.524042
Wdr17	ENSMUSG00000039375	-17.920734	0.000888	0.053417	1.517059
Ntn4	ENSMUSG00000020019	-19.971000	0.000641	0.052649	1.508373
Kcnh2	ENSMUSG00000038319	-27.705597	0.000209	0.052376	1.505656
Ocrl	ENSMUSG00000001173	-20.294437	0.00062	0.052649	1.502595
Sc5d	ENSMUSG00000032018	-24.710915	0.000343	0.052376	1.48146
D1Ertd622e	ENSMUSG00000044768	-17.405197	0.000942	0.053417	1.476509
AL671335.3	ENSMUSG00000081308	-37.229738	7.96E-05	0.051299	1.325801
Eml2	ENSMUSG00000040811	-24.483902	0.000352	0.052376	1.307813

Table 5. Genes obtained with genome Affymetrix® microarrays.
166 genes significantly higher at P4 compared to E17.5 stage of development, with R fold values between 24.58 and 1.3. In the table are reported the Gene Name, Probeset ID, D value (Delta value), P value, Q value (adjusted P-value) and R fold. Statistical significance of gene expression differences between interneurons populations was determined by pairwise comparisons at each age using significance analysis of Microarrays (SAM) considering the genes differentially expressed with a False Discovery Rate (FDR) of <0.05.

RESULTS

UNIVERSITAS
Miguel
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Part I.

Molecular mechanisms controlling cortical plate entry for MGE-derived interneurons

Blocking chemokine signaling promotes interneuron invasion of the cortical plate

Chemokines are responsible for maintaining migrating interneurons within the MZ and the SVZ as they disperse tangentially throughout the cortex. Cells in the meninges and in the SVZ express Cxcl12, a potent chemoattractant for MGE-derived cells, (Tham et al., 2001; Stumm et al., 2003; Daniel et al., 2005; Tiveron et al., 2006) while interneurons express Cxcr4 and Cxcr7, two receptors for this chemokine (Tiveron et al., 2006; Lopez-Bendito et al., 2008; Wang et al., 2011). Loss of Cxcr4 and Cxcr7 function does not prevent interneurons from reaching the cortex in normal numbers, but it disrupts their distribution in the neocortex (Abe et al., 2014; Vogt et al**.,** 2014; Sánchez-Alcañiz et al., 2011; Tiveron et al., 2006). Specifically, loss of chemokine signaling cause premature interneuron entry into the CP, which disrupts their normal laminar and regional distribution (Abe et al., 2014; Sánchez-Alcañiz et al., 2010, Wang et al. 2011; Li et al. 2008; Lopez-Bendito et al. 2008). To directly assess whether disruption of chemokine signaling is sufficient to direct tangentially migrating interneurons into the cortical plate, we performed time-lapse experiments in slices while blocking their response to Cxcl12. To this end, we acutely disrupted Cxcl12 signaling downstream of Cxcr4 and Cxcr7 receptors by inhibiting Src kinase activation (Cabioglu et al. 2005). We first tested the effect of the Src inhibitor SU6655 in confrontation assays in three-dimensional matrices in which MGE explants obtained from E13.5 GFP-expressing embryos (Hadjantonakis et al., 1998) were cultured together with aggregates of COS cells transfected with control or *Cxcl12* encoding plasmids (Figure 1A). These experiments demonstrate that blocking Src inhibits Cxcl12-induced migration in MGE cells (Figure 1B –1F; Control: MGE versus mock, n = 32 explants; MGE versus Cxcl12, n = 40, one-way ANOVA, ***$p < 0.001$; SU6655: MGE versus mock, n = 36 explants; MGE versus Cxcl12, n = 39, one-way ANOVA, $p > 0.05$; 0.243).

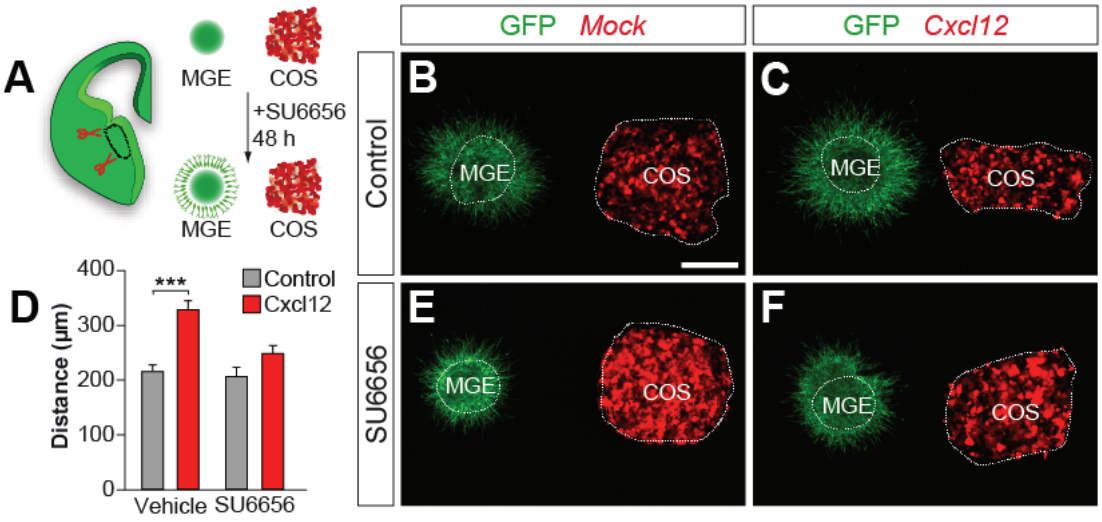

Figure 1. Blocking Src inhibits Cxcl12-induced migration in MGE cells
(A) Schematic of the experimental design. (B–F) Migration of MGE-derived cells in control situation (B and C) and after SU6656 addition (E and F) in response to mock-transfected (B and E) or Cxcl12-transfected (C and F) COS cells. COS cell aggregates cultured in collagen matrices for 48 hr. Dotted lines indicate the limits of the explants and COS cell aggregates. (D) Quantification of confrontation assays. Control: MGE versus mock, *n* = 32 explants; MGE versus Cxcl12, *n* = 40, one-way ANOVA, ***p < 0.001; SU6655: MGE versus mock, *n* = 36 explants; MGE versus Cxcl12, *n* = 39, one-way ANOVA, p > 0.05. Histograms show average ± SEM. Scale bar equals 200 µm.

We then performed similar experiments in acute slices in which MGE-derived cortical interneurons were previously labeled by focal electroporation of a plasmid encoding td-Tomato (Figure 2A). We observed that addition of SU6655 leads to a rapid disorganization of the tangential migratory routes, with a concomitant four-fold increase in the number of interneurons migrating radially towards the cortical plate (Figure 2B, 2D and 2E; 2 independent experiments, *n* = 240 cells analyzed for each condition, *t*-test ***p < 0.001). Time-lapse analysis of tangentially migrating neurons confirmed these observations. Compared to control experiments, SU6655 induced the formation of new branches at very wide angles in tangentially migrating interneurons (Figure 2D and 2F), which led to a rapid transition in the direction of migration from tangential to radial, and invasion of the cortical plate. These results led us to hypothesize that pyramidal cells express chemoattractive signals that promote the invasion of the CP by interneurons, which would only normally act once interneurons stop responding to the chemokines present in their routes of tangential dispersion.

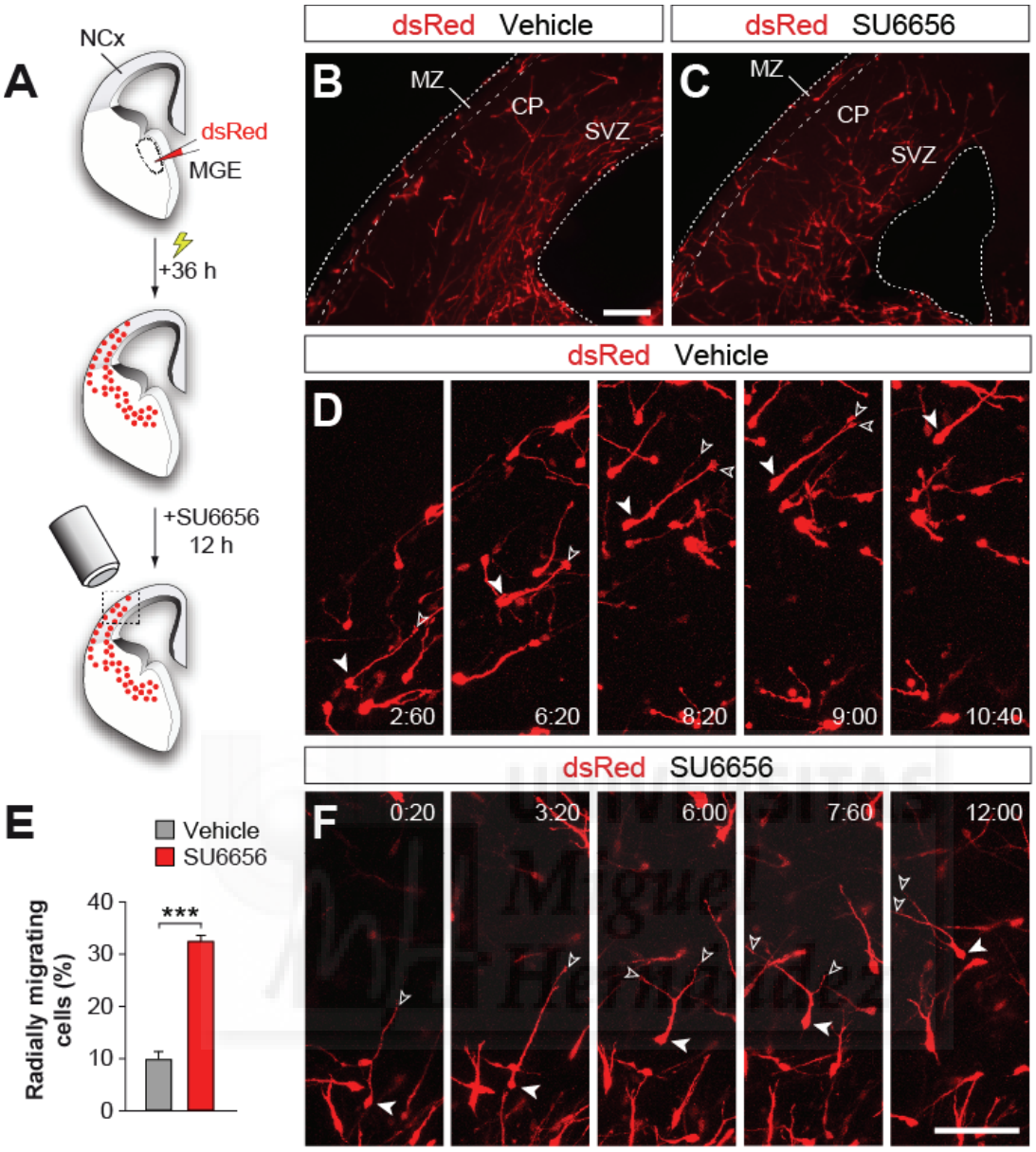

Figure 2. Blocking chemokine signaling promotes interneuron invasion of the cortical plate

(A) Schematic of the experimental design. (B-C) Focal electroporation of td-Tomato in acute slices at E13.5 after 36 hours in culture. Vehicle (B) and with addition of SU6655 in the media (C). (E) Quantifications of interneurons radially migrating towards the cortical plate. 2 independent experiments, *n* = 240 cells analyzed for each condition, *t*-test ***p < 0.001. (D-F) Time-lapse analysis of tangentially migrating neurons. (D) Vehicle. (F) SU6655 addition. (D-F) Time is depicted in hours: minutes. The white arrowheads mark the soma of interneurons, the smaller and empty arrowheads mark the formation of new branches. Scale bar equals 100 µm.

A candidate gene approach to identify factors regulating cortical plate invasion by interneurons

We took a candidate gene approach to investigate the molecular mechanisms regulating the migration of interneurons into the CP. We hypothesized that factors relevant for this process might be upregulated in pyramidal cells during early postnatal stages, when interneurons invade the CP. To analyze gene expression in equivalent cohorts of pyramidal cells, we performed in utero electroporation experiments with a plasmid encoding GFP targeting the dorsal pallium of embryonic day (E) 14.5 mice (Figure 3A). We then used fluorescence activated cell sorting (FACS) to isolate GFP+ pyramidal cells from these experiments at two different stages: E17.5, when pyramidal cells are still migrating towards the CP, and postnatal day (P) 4, when pyramidal cells are already reached their final position (Figure 3B–3D, Table1, see *Methods*). To examine the differential gene expression at these two stages in the development of pyramidal cells, we customized a TaqMan array with ~100 genes known to be involved in neuronal migration, adhesion and axon guidance during corticogenesis (Table 2), including members of the eph, ephrin, semaphorin, plexin, cadherin, protocadherin and neuregulin families. We also include several genes known to encode proteins expressed in pyramidal cells, such as *Cux1* and *Tbr1*, as positive controls, and genes that encode proteins exclusively expressed in interneurons, such as *Cxcr7*, *Cxcr4*, *Sst*, *Lhx6* and *Htr3a*, as negative controls.

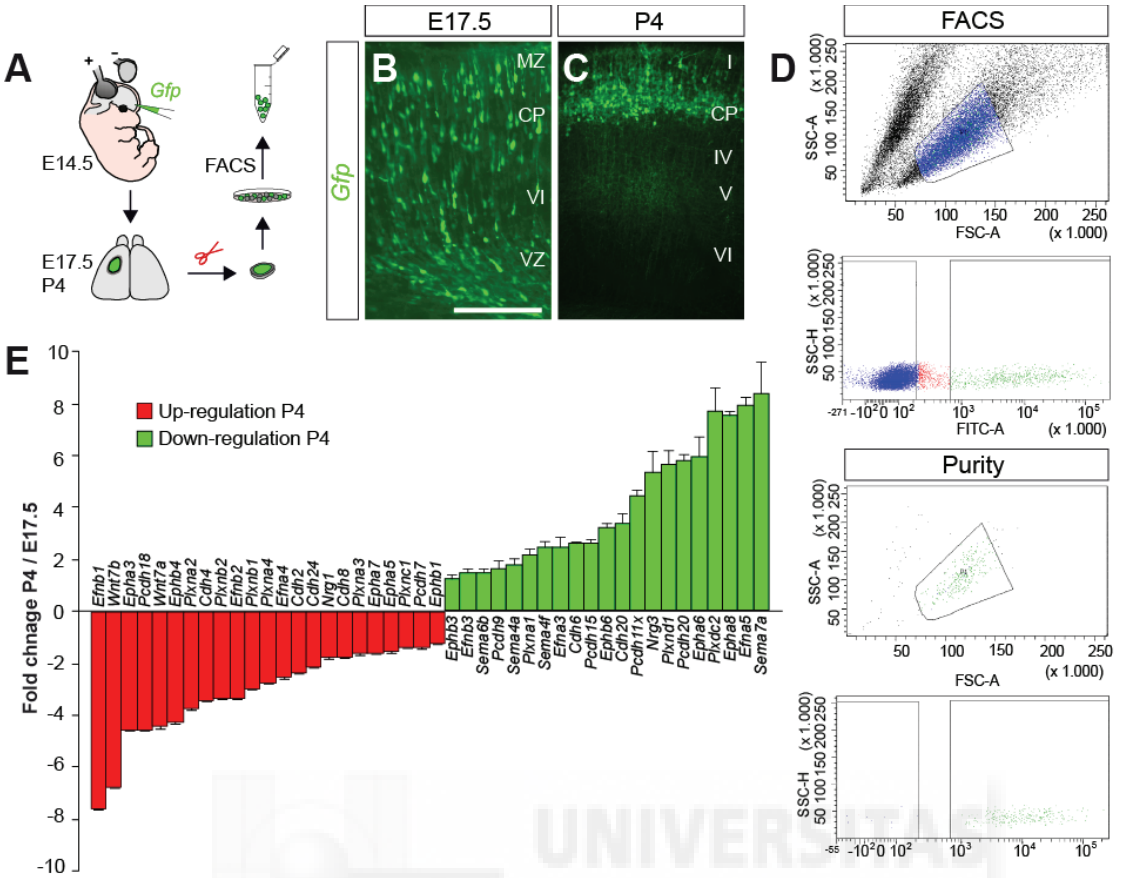

Figure 3 Genes overexpressed in pyramidal cells during the integration of interneurons in the cortical plate. (A) Schematic of the experimental design. (B-C) In utero electroporation to specifically label pyramidal cells born at E14.5 at E17.5 (B) and P4 (C). (D) FACS sorting after in utero electroporation to isolate fluorescent GFP+ cells at E17.5 and P4. (E) Quantitative real-time PCR (Taqman array, Applied Biosystems) for 96 genes involved in axon guidance and neuronal migration. Graph comparing relative concentration of RNA at E17.5 and P4. We identified 44 genes that are differentially expressed between the two stages. p values for genes more expressed at P4 in Table 3., *Methods* (*t*-test). Scale bar equals 250 µm.

We identified 44 genes that are differentially expressed between the two stages (Figure 3E). We focused our attention on 17 genes that are significantly more expressed by pyramidal cells at P4 compared to E17.5 (Table 3), because these are more likely to be involved in the chemoattraction of interneurons into the CP. To examine the pattern of expression of these genes, we performed in situ hybridization at E17.5 and P4 for this later list of candidate genes (*Cdh6*, *Cdh20*, *Epha6*, *Epha10*, *Ephb2*, *Ephb3*, *Ephb6*, *Efna3*, *Efna5*, *Efnb3*, *Plxndc2*, *Plxna1*, *Plxnd1*, *Pcdh9*, *Pcdh15*, *Pcdh20*, *Nrg3*, and *Sema7a*). Analysis of the expression of candidate genes revealed different patterns. For example, some genes were preferentially expressed in superficial layers of the cortex, and their expression increased during early postnatal stages (Figure 4A–4F). In other cases, candidate genes were expressed throughout all layers of the neocortex (Figure 4G and 4H).

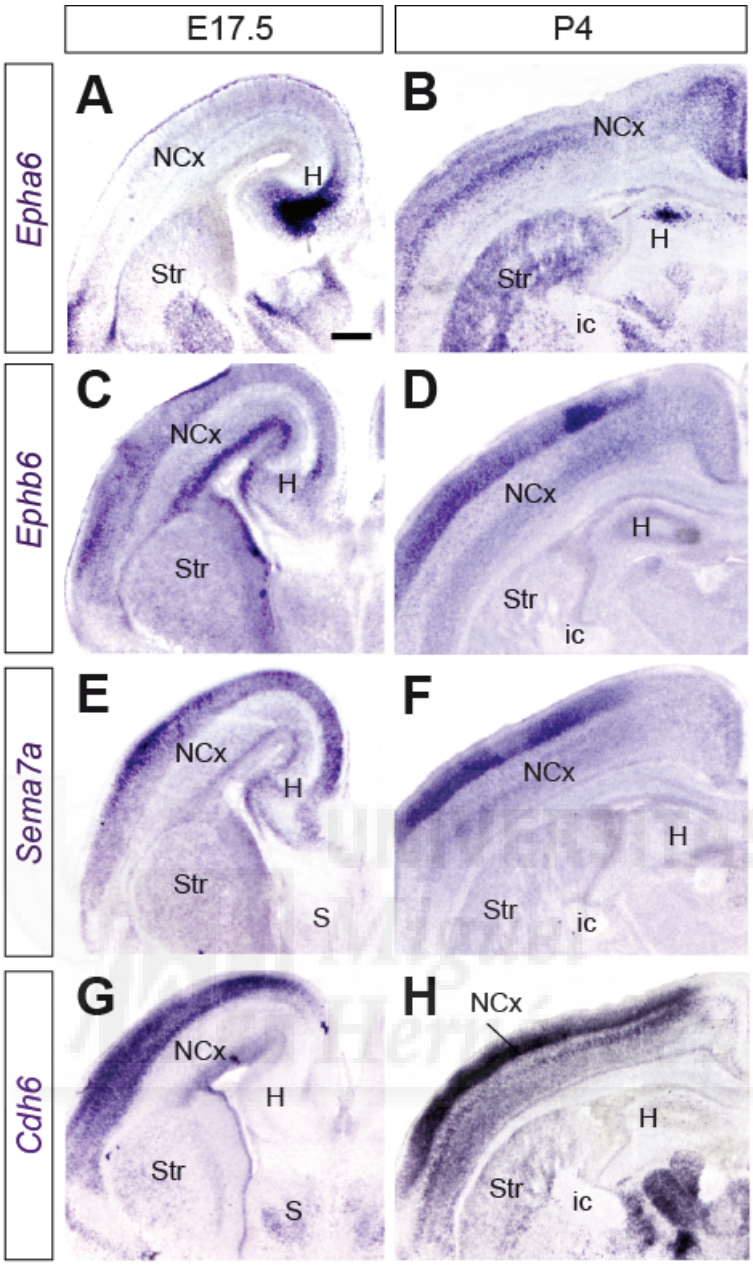

Figure 4 Expression of genes significantly more expressed at P4 compared to E17.5. (A-H) Coronal sections through the telencephalon of E17.5 and P4 cortex showing mRNA expression for: *EphA6* (A-B), *EphB6* (C-D), *Sema7a* (E-F), *Cdh6* (G-H). H, Hippocampus; ic, internal capsule; NCx, neocortex; S, Septum; Str, striatum. Scale bar equals 250 µm.

Nrg3 is expressed in the developing cortical plate

We noticed that one of the genes that is expressed throughout the CP and that is significantly more expressed by pyramidal cells at later stages is *Nrg3*, a member of the neuregulin family. Neuregulins are a family of four structurally related proteins (Nrg1, Nrg2, Nrg3 and Nrg4) that are part of the EGF family of proteins and are ligands for receptor tyrosine kinases of the ErbB family. They are involved in several processes of neural development, including cell migration and axon guidance (Rio et al., 1997, Anton et. al, 1997; Flames et al., 2004; Lopez-Bendito et al., 2006). In particular, different isoforms of Nrg1 have been previously implicated in the tangential migration of GABAergic interneurons from the subpallium to the developing cortex (Flames et al. 2004). Moreover, Nrg3 has been reported to bind preferentially to ErbB4 receptors (Zhang et al, 1997, PNAS), which are highly enriched in migrating cortical interneurons and excluded from pyramidal cells (Yau et al., 2003, Flames et al. 2004; Vullhorst et al., 2009 JN; Fazzari et al., 2010). Based on this evidence, we hypothesized that Nrg3 might regulate the intracortical migration of GABAergic interneurons, and focused our subsequent work on this molecule.

We investigated the pattern of expression of *Nrg3* in the developing cortex from mid-embryonic until early postnatal stages using in situ hybridization (Figure 5). We observed that *Nrg3* is highly expressed in the developing CP in pyramidal cells since its inception, and that Nrg3 expression is maintained in pyramidal cells as they mature and start forming differentiated layers. Pyramidal cells therefore express *Nrg3* as soon as they reach the CP, and its expression is subsequently maintained throughout all layers of the neocortex, including the subplate. *Nrg3* is however largely absent from the MZ and the SVZ at all stages examined (Figure 5). These results support the hypothesis that Nrg3 might be involved in the regulation of interneuron migration into the CP.

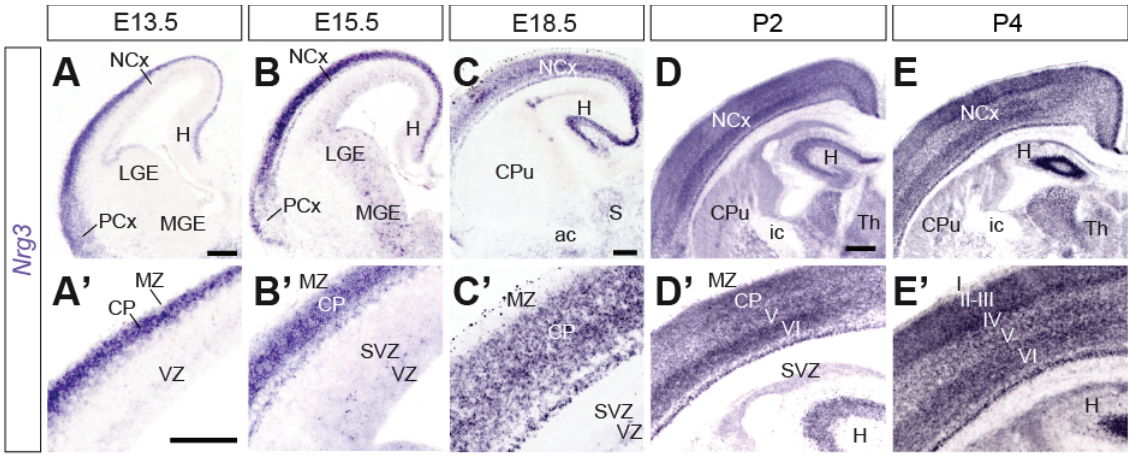

Figure 5. Nrg3 Expression in the Developing Mouse Cortex. (A–E') Coronal sections through the telencephalon of E13.5 (A-A'), E15.5 (B-B'), E18.5 (C-C'), P2 (D-D'), P4 (E-E') embryos showing mRNA expression for *Nrg3.* Ac, anterior commissure; CP, cortical plate; CpU, Caudate Putamen (Striatum); H, Hippocampus; ic, internal capsule; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, marginal zone; NCx, neocortex; Pcx, Piriform cortex; Str, striatum; SVZ, subventricular zone; th, thalamus; VZ, ventricular zone, S, Septum. Layers I; II-III; IV; V; VI. Scale bars equal Scale bar equals 250 µm.

Nrg3 functions as a short-range chemoattractant for MGE-derived interneurons

We have previously shown that different isoforms of Nrg1 act both as short- and long-range chemoattractive molecules for tangentially migrating interneurons (Flames et al, 2004). To examine whether Nrg3 may exert a similar effect on cortical interneurons, we performed confrontation assays in three-dimensional matrices in which we cultured MGE explants obtained from E13.5 GFP-expressing embryos (Hadjantonakis et al., 1998) together with aggregates of COS cells transfected with control or *Nrg3* encoding plasmids (Figure $6A_1$). In parallel experiments, we carried out co-cultures in which COS cells were transfected with *Nrg1-Ig*, which encodes for a diffusible form of Neuregulin-1. As described before (Flames et al., 2004), we observed that Ig-Nrg1 exerts a prominent chemoattractive response in MGE-derived cells (Figures 6B, 6C, and 6H; n = 19 and 24 mock and *Ig-Nrg1* explants, respectively; one-way ANOVA, $^{**}p < 0.01$). In contrast, we found no difference in the response of MGE-derived cells to *Nrg3* compared to controls (Figures 6B, 6D, and 6H; n = 19 and 20 mock and *Nrg3* explants, respectively; one-way ANOVA). Thus, *Nrg3* does not seem to function as a long-range chemoattractant for MGE-derived interneurons.

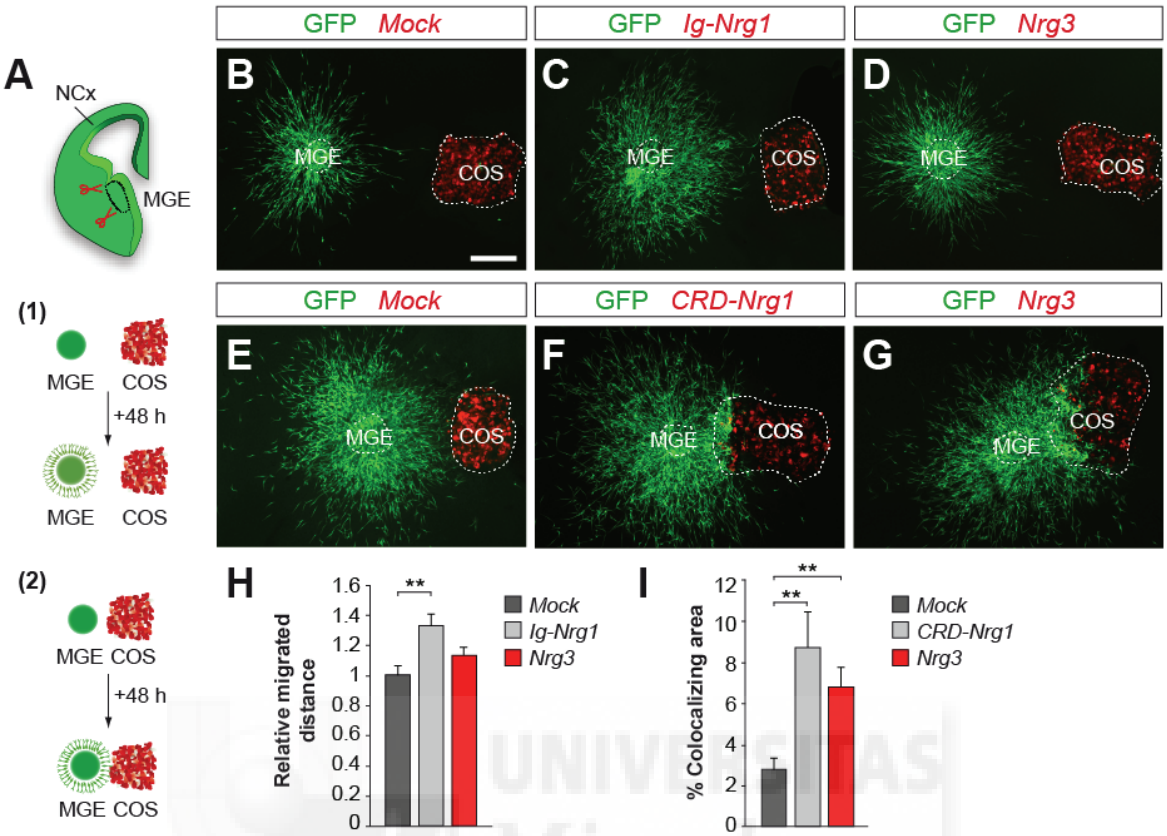

Figure 6. Nrg3 functions as a short-range chemoattractant for MGE-derived interneurons. (A) Schematic of the experimental design. **(A_1)** Migration of MGE-derived cells in long-range (B–D) and **(A_2)** short-range distance (E-G) in response to mock-transfected (B and E), *Ig-Nrg1* (C), *CRD-Nrg1* (F) or Nrg3 (D-G). COS cell aggregates cultured in collagen matrices for 48 hr. Dotted lines indicate the limits of the explants and COS cell aggregates. (H) Quantification of long-distance confrontation assays. Control: MGE versus mock n = 19; MGE versus *Ig-Nrg1* n=24; one-way ANOVA, $^{**}p < 0.01$. MGE versus *Nrg3*, n = 20, one-way ANOVA, $p > 0.05$. (I) Quantification of short-distance confrontation assays. Control: MGE versus mock n = 29; MGE versus *CRD-Nrg1* n=24 MGE versus *Nrg3*, n = 27; one-way ANOVA, $^{**}p < 0.01$. Histograms show average ± SEM. Scale bar equals 200 µm

To investigate whether Nrg3 may function as a short-range chemoattractant for migrating interneurons, we carried a new set of co-culture experiments in which COS cell aggregates, transfected with control or *Nrg3* encoding plasmids, were placed at a relatively short distance from MGE explants (Figure 6A_2). In this new set of experiments we used COS cells transfected with *CRD-Nrg1* as a positive control, because this membrane bound form of Neuregulin-1 has been shown to induce short-range chemoattraction during the migration of cortical interneurons through the subpallium (Flames et al., 2004). We found that both CRD-Nrg1 and Nrg3 evoke a potent chemoattractive effect on migrating

interneurons, which can be visualized by the great abundance of cells accumulating around the proximal side of COS cell aggregates (Figure 6E–6G and 6I; n = 29, 24 and 27 mock, *CRD-Nrg1* and *Nrg3* explants, respectively; one-way ANOVA, $^{**}p < 0.01$ for both comparisons). Thus, Nrg3 induces a potent short-range chemoattractive effect on MGE-derived interneurons.

Nrg3 chemoattraction requires ErbB4 function

Because Nrg3 is thought to bind preferentially to ErbB4 receptors (Zhang et al, 1997, PNAS), we next examined whether ErbB4 function mediates the chemoattractive responses elicited by Nrg3 in MGE-derived interneurons. To this end, we carried out a new set of co-culture experiments with MGE explants obtained from control and *Erbb4* mutant embryos (Figure 7A). Because loss of ErbB4 causes early lethality due to cardiac defects, *Erbb4* mutants carried a human transgene under a cardiac-specific myosin promoter ($HER4^{heart}$) to circumvent this problem (Tidcombe et al., 2003). In contrast to controls, we observed that Nrg3 does not exert any effect on MGE cells derived from *Erbb4* mutant embryos (Figure 7B–7F; n = 21 $Erbb4^{+/+};HER4^{heart}$ versus *mock*; n = 25 $Erbb4^{+/+};HER4^{heart}$ versus *Nrg3*, n = 15 $Erbb4^{-/-};HER4^{heart}$ versus *mock*; n = 14 $Erbb4^{-/-};HER4^{heart}$ versus *Nrg3*; one-way ANOVA, $^{***}p < 0.001$). Thus, ErbB4 is necessary for the short-range chemotaxis of cortical interneurons in response to Nrg3. Altogether, these experiments indicate Nrg3 exert a chemoattractive effect on migrating interneurons that is mediated by ErbB4 function.

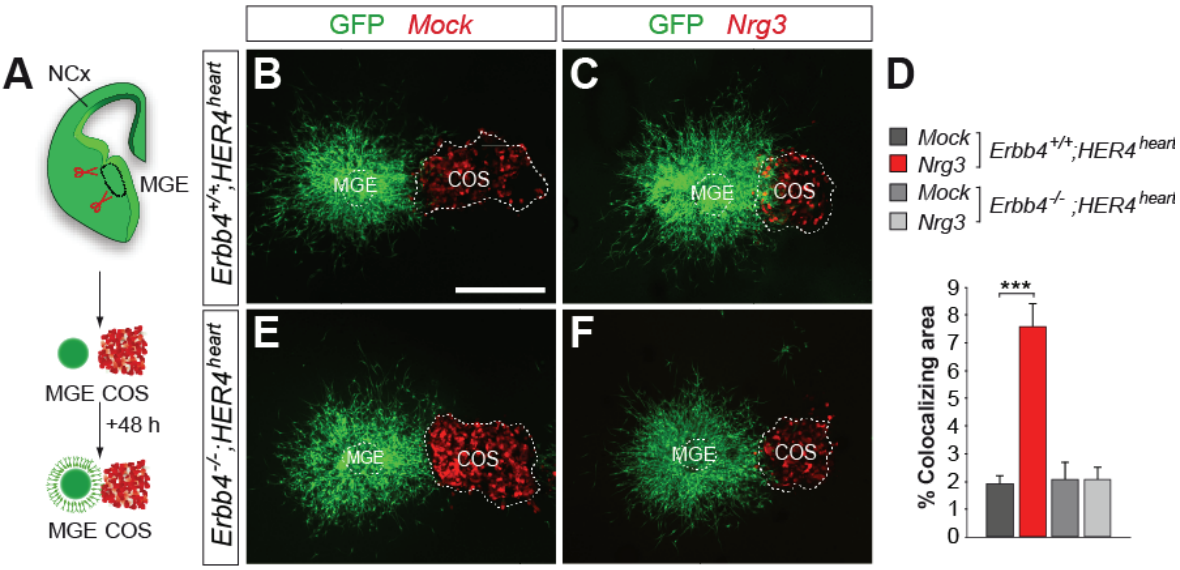

Figure 7. **Nrg3 short-range chemoattraction requires ErbB4 function.** A) Schematic of the experimental design. (B-C) Migration of MGE-derived cells derived from *Erbb4*$^{+/+}$*;HER4*^{heart} mice in response to mock-transfected (B) and in response to *Nrg3* (C). (E-F) Migration of MGE-derived cells derived from *Erbb4*$^{-/-}$*;HER4*^{heart} in response to mock-transfected (E) and in response to *Nrg3* (F). COS cell aggregates cultured in collagen matrices for 48 hr. Dotted lines indicate the limits of the explants and COS cell aggregates. (D) Quantification of confrontation assays. *Erbb4*$^{+/+}$*;HER4*^{heart} versus *mock*, *n* = 25 *Erbb4*$^{+/+}$*;HER4*^{heart} versus *Nrg3*, *n* = 15 *Erbb4*$^{-/-}$*;HER4*^{heart} versus *mock*; *n* = 14 *Erbb4*$^{-/-}$*;HER4*^{heart} versus *Nrg3*; one-way ANOVA, ***$p < 0.001$. Histograms show average ± SEM. Scale bar equals 300 µm.

Tangentially migrating MGE-derived interneurons prefer Cxcl12 to Nrg3

It has been previously shown that the chemokine Cxcl12 strongly promotes the tangential migration of MGE-derived cells throughout the embryonic cortex. Cxcl12 is expressed by the meninges and in the SVZ of the pallium during embryonic development (Stumm et al., 2003; Tiveron et al., 2006; Borrell and Marín, 2006), and it maintains migrating interneurons within their main migratory streams during tangential dispersion (Stumm et al., 2003; Tiveron et al., 2006; Li et al., 2008; López-Bendito et al., 2008, Sanchez-Alcañiz et al., 2011; Wang et al., 2011). Since Nrg3 is expressed in the CP from early stages of development (Figure 5), tangentially migrating interneurons encounter both cues as they reach the embryonic cortex. Both Cxcl12 and Nrg3 seem to function as chemoattractive factors for migrating MGE-derived interneurons, so we explored whether interneurons display a preference for any of these molecules. To this end, we cultured MGE explants obtained from E13.5 embryos from GFP-expressing mice together with aggregates of COS

cells placed at a short-distance and transfected with either a mock plasmid, *Nrg3* or both *Nrg3* and *Cxcl12* together (Figure 8A$_1$). As expected, we observed that Cxcl12 enhances the migration of MGE-derived interneurons (Figure 8B–8E; n = 18, 15 and 27 mock, *CRD-Nrg1* and *Nrg3* explants, respectively; one-way ANOVA, ***$p < 0.001$). In addition, we found that Cxcl12 does not block the chemoattractive effect elicited by Nrg3 (Figure 8B–8D and 6F; n = 18, 15 and 27 mock, *CRD-Nrg1* and *Nrg3* explants, respectively; one-way ANOVA, ***$p < 0.001$, *$p < 0.05$). These results suggested that tangentially migrating MGE-derived interneurons are equipped to respond to both Cxcl12 and Nrg3 simultaneously.

We next wondered whether MGE-derived interneurons display any preference for Cxcl12 or Nrg3. To answer this question, we performed stripe choice assays using recombinant proteins, as described previously (Walter et al., 1987). In brief, E13.5 MGE explants obtained from GFP-expressing embryos were placed on top of stripes coated with a control peptide (GST), Nrg3-GST or recombinant Cxcl12 in alternate combinations (Figure 8A$_2$), and their lane preference scored after 48 h. As expected, MGE-derived cells showed no migratory preference when alternative stripes were coated with the same recombinant protein (GST/GST, Nrg3/Nrg3 or Cxcl12/Cxcl12) in control experiments (Figure 8I and data not shown). In contrast, MGE-derived interneurons displayed a strong preference for Nrg3-coated stripes compare to control lanes (Figure 8G and 8I; n = 24; one-way ANOVA, ***$p < 0.001$). Remarkably, MGE-derived cells exhibited a strong preference towards Cxcl12 when they were given the possibility to migrate on alternating stripes containing Nrg3 and Cxcl12 (Figure 8H and 8I; n = 20; one-way ANOVA, ***$p <$ 0.001). Altogether, these experiments suggest that tangentially migrating interneurons can respond simultaneously to Cxcl12 and Nrg3, but they display stronger affinity for the chemokine. These observations are consistent with the in vivo behavior of MGE-derived interneurons, which initially disperse through the cortex via Cxcl12-rich territories (MZ and SVZ) without accumulating in the CP (López-Bendito et al., 2008).

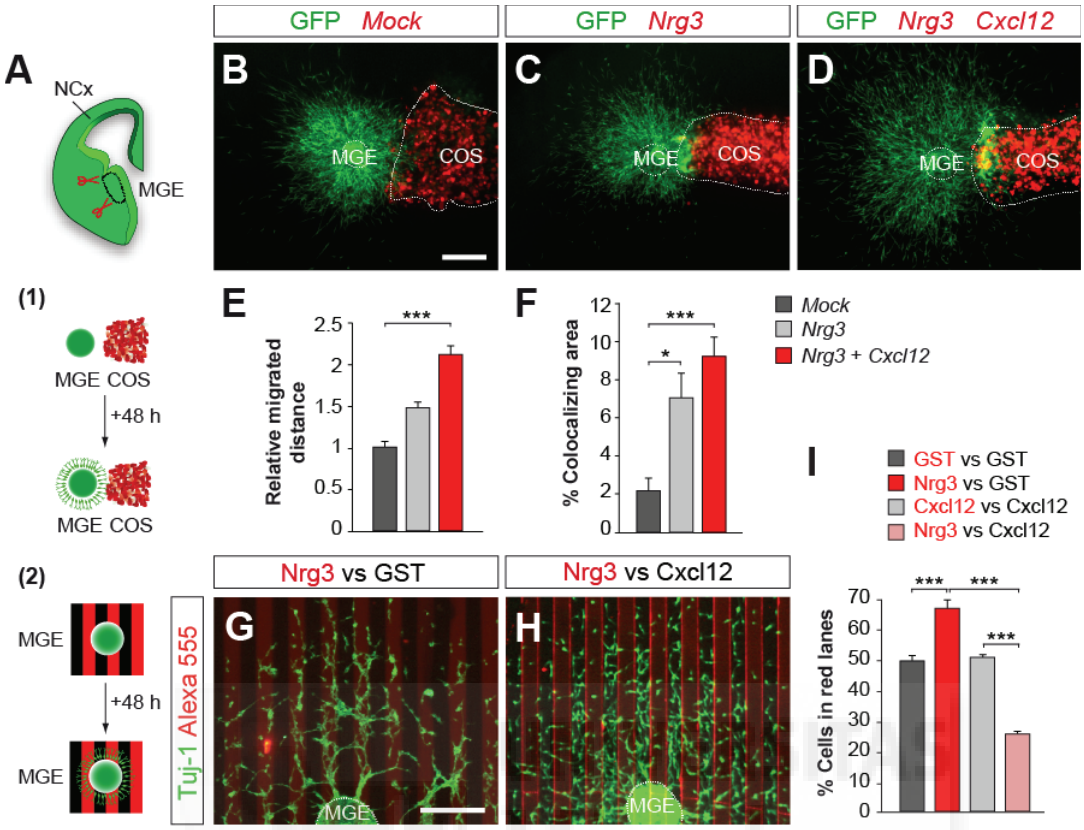

Figure 8. Tangentially migrating MGE-derived interneurons respond to both Cxcl12 and Nrg3 but prefer Cxcl12 in the Stripe Choice Assay. (A) Schematic of the experimental design. **A_1)** Migration of MGE-derived cells in short-range (B–F) in response to mock-transfected (B), *Nrg3*-transfected (C) or *Nrg3* together with *Cxcl12* (D). (E) Quantification of MGE-cells migrating away from explants. *n* = 18, 15 and 27 mock, *CRD-Nrg1* and *Nrg3* explants, respectively; one-way ANOVA, ***$p < 0.001$ (F) Quantification of short-distance confrontation assays. *n* = 18, 15 and 27 mock, *CRD-Nrg1* and *Nrg3* explants, respectively; one-way ANOVA, ***$p < 0.001$, *$p < 0.05$. COS cell aggregates cultured in collagen matrices for 48 hr. Dotted lines indicate the limits of the explants and COS cell aggregates. **A_2)** Migration of MGE-derived cells in the stripe choice-assay (G-I). MGE explants placed on top of stripes coated with *Nrg3-GST* (G) or recombinant *Cxcl12* and *Nrg3* in alternate combinations (H). (I) Quantification of stripe choice assay. *Nrg3-GST* coated stripes, n = 24; one-way ANOVA, ***p < 0.001. *Cxcl12-Nrg3* coated stripes, n = 20; one-way ANOVA, ***p < 0.001. Scale bar equals 200 µm.

Nrg3 overexpression enhances interneuron invasion of the CP in vivo

We next wondered whether unbalancing the normal levels of Nrg3 in the developing CP interferes with the migration of cortical interneurons in vivo. To this end, we electroporated the ventricular zone of the pallium in E14.5 *Nkx2.1;R26R*^{tdTomato} embryos with either *Gfp* expressing plasmids or a combination of *Gfp* and *Nrg3* (Figure 9A), and examined the distribution of MGE-derived interneurons (labeled with tdTomato) at E18.5.

We observed that overexpression of Nrg3 does not seem to disrupt the migration of pyramidal cells (Figure 9B and 9E). In contrast, overexpression of Nrg3 promotes the invasion of the CP by MGE-derived interneurons (Figure 9C, 9D and 9F; *Gfp*: 3607.64 ± 166.63 cells/mm^2, *Nrg3*: 4740.32 ± 143.3 cells/mm^2; $n = 6$, *t*-test ***$p < 0.001$). Thus, these results suggested that Nrg3 promotes the intracortical migration of MGE-derived interneurons in vivo.

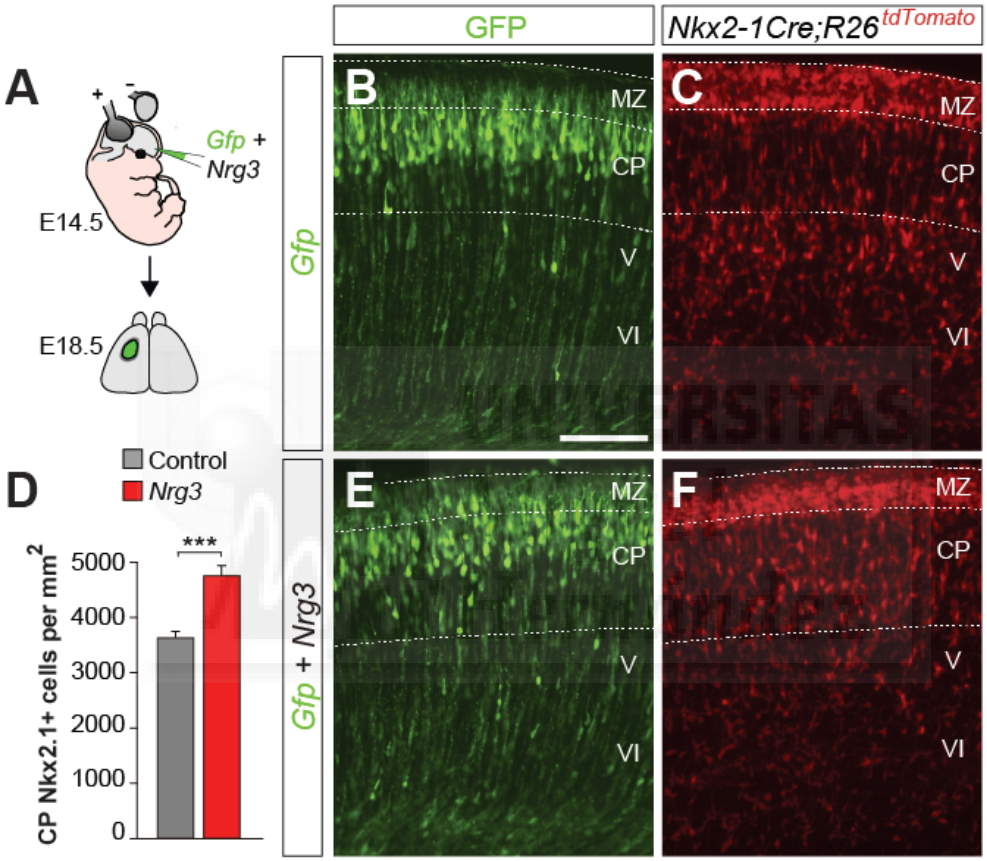

Figure 9. **Nrg3 overexpression in pyramidal cells enhances interneuron invasion of the CP in vivo** A) Schematic of the experimental design. (B-F) In utero electroporation in the ventricular zone of E14.5 *Nkx2.1;R26R*^{tdTomato} embryos with *Gfp* expressing plasmids (A) or combination of *Gfp* and *Nrg3* (B). Analysis of the distribution of MGE-derived interneurons (tomato labeling) in the CP, in control situation (C) or after *Nrg3* overexpression (F). (D) Quantification of migrating MGE-derived cells in the CP. *Gfp*: 3607.64 ± 166.63 cells/mm^2, *Nrg3*: 4740.32 ± 143.3 cells/mm^2; $n = 6$, *t*-test ***$p < 0.001$. Scale bar equals 200 µm.

Conditional deletion of Nrg3 disrupt the normal lamination of cortical interneurons

To examine the long-term consequences of disrupting Nrg3 signaling in vivo, we generated conditional *Nrg3* mutants from a *Nrg3* knockout first allele generated by the

Knockout Mouse Program of the University of California, Davis (Figure 10; Skarnes et al., 2011). To delete *Nrg3* specifically from developing pyramidal cells, we used *Nex-Cre* mice, in which exon 2 of the *NeuroD6* locus has been replaced by Cre recombinase (Goebbels et al., 2006).

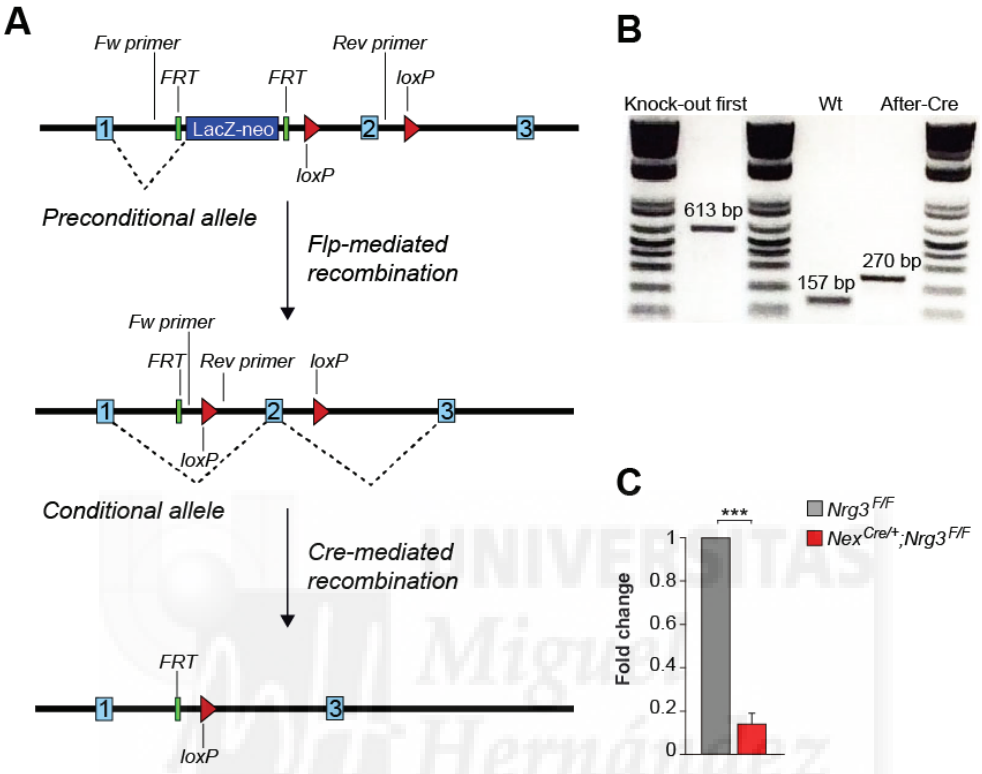

Figure 10. Generation of conditional *Nrg3* mutant mice (A) Schema showing the generation of conditional *Nrg3* mutant mice. A trapping cassette flanked by "FRT" sites has been inserted within an intron upstream of Nrg3 exon2, flanked by "loxP" sites. The allele has been converted to a wild-type pre-conditional allele by exposure to Flp recombinase. Subsequent deletion of *Nrg3* from developing pyramidal cells has obtained through the usage of *Nex-Cre* mice. (B) PCR bands showing the generation of the conditional *Nrg3* mutant mice. Band of 613 bp corresponding to knock-out first allele. After Flp recombination, in the wt allele the band size is 157bp. If recombination has occurred a band of 270bp appears. Primers binding site are shown in the schema (A), the sequences are shown in *Methods* (see "Mice".) (C) Real-time PCR showing decrease of *Nrg3* transcript in Nrg3 conditional mice after crossing with *Nex-Cre* mice. n=4 t-test ***$p < 0.001$.

Analysis of the distribution of *Erbb4*-expressing neurons in the somatosensory cortex of control and conditional *Nrg3* mutants at P30 revealed no differences in the density of *Erbb4*+ cells (Controls: 783.28 ± 48.18 cells/mm^2, *Nrg3* mutants: 923.69 ± 56.15 cells/mm^2; n = 4, t-test $p > 0.05$). However, we observed that the laminar location of *Erbb4*-expressing neurons was significantly different between control and conditional

Nrg3 mutants, with a deep to superficial layer swift in the distribution of *Erbb4*-expressing cells in mutants compared to controls (Figure 11A–11C; Controls: 10.13 ± 0.73 % [I], 13.65 ± 2.04% [II-III], 13.53 ± 1.05% [IV], 24.86 ± 1.14% [V], 37.83 ± 0.78% [VI]; *Nrg3* mutants: 9.38 ± 0.70 % [I], 19.37 ± 0.37% [II-III], 18.65 ± 0.95% [IV], 21.96 ± 0.33% [V], 30.73 ± 1.61% [VI]; $n = 4$, $*p < 0.05$, χ^2 test). Similar results were obtained when we analyzed the distribution of PV+ interneurons at P30, although in this case only the abnormal number of superficial layer interneurons reached statistical significance (Figure 11D–11F). To determine where these differences in the laminar distribution of cortical interneurons were already present in the early postnatal cortex, we examined the distribution of *Lhx6*-expressing neurons in the somatosensory cortex of control and conditional *Nrg3* mutants at P4. We found that the number of *Lhx6*-expressing cells located in the prospective superficial layers of the cortex was significantly higher in conditional *Nrg3* mutants compared to controls (Figure 11G–11I; layers II-IV: 30.17 ± 1.71% in controls, 35.14 ± 1.39% in *Nrg3* mutants; n = 5, $*p < 0.05$, χ^2 test). Thus, conditional deletion of Nrg3 from pyramidal cells during development impairs the intracortical migration of cortical interneurons and disrupts their normal laminar distribution.

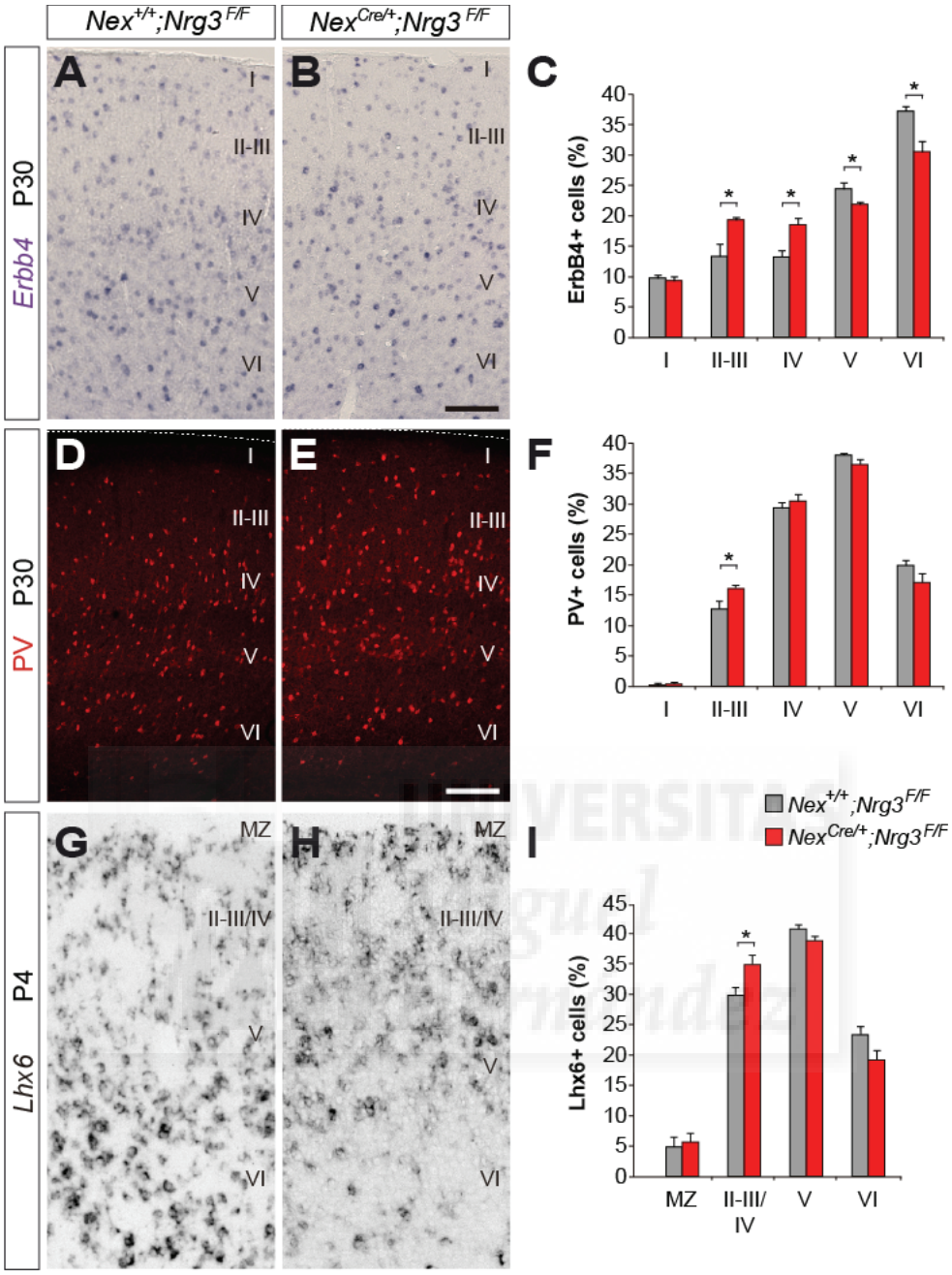

Figure 11. Conditional deletion of Nrg3 from pyramidal cells disrupts the normal lamination of cortical interneurons (A-B) *Erbb4* expressing neurons in somatosensory cortex of $Nex^{+/+}$; $Nrg3^{F/F}$ (A) and mutants $Nex^{Cre/+}$; $Nrg3^{F/F}$ (B) P30 mice. (D-E) *PV* expressing neurons in somatosensory cortex of $Nex^{+/+}$; $Nrg3^{F/F}$ (A) and mutants $Nex^{Cre/+}$; $Nrg3^{F/F}$ (B) P30 mice (C) Quantification of the distribution of *Erbb4*-expressing cells in layers in $Nex^{+/+}$; $Nrg3^{F/F}$ mice: n= 4; 10.13 ± 0.73 % [I], 13.65 ± 2.04% [II-III], 13.53 ± 1.05% [IV], 24.86 ± 1.14% [V], 37.83 ± 0.78% [VI]; and $Nex^{Cre/+}$; $Nrg3^{F/F}$ mutants: 9.38 ± 0.70 % [I], 19.37 ± 0.37% [II-III], 18.65 ± 0.95% [IV], 21.96 ± 0.33% [V], 30.73 ± 1.61% [VI]; n = 4, $^*p < 0.05$, χ^2 test. (G-I) *Lhx6* expression in somatosensory cortexes of $Nex^{+/+}$; $Nrg3^{F/F}$ (G) and $Nex^{Cre/+}$; $Nrg3^{F/F}$ P4 mice (H). (I) Quantification of the distribution of Lhx6 positive cells in layers: layers II-IV: 30.17 ± 1.71% in controls, 35.14 ± 1.39% in *Nrg3* mutants; n = 5, $^*p < 0.05$, χ^2 test). Histograms show average ± SEM. Scale bar equals 200 µm.

Conditional deletion of ErbB4 in MGE-derived interneurons disrupts lamination

We have previously shown embryonic loss of ErbB4 disrupts the tangential migration of interneurons and reduces their number in the postnatal cortex (Flames et al., 2004). Conditional deletion of *Erbb4*, however, does not affect the number of MGE-derived interneurons reaching the cortex, most likely because complete removal of ErbB4 does not occur before cells have reached the cortex (Fazzari et al., 2010). In *Lhx6-Cre;Erbb4*$^{F/F}$ mutant mice, for example, the number of MGE-derived interneurons was reported to be normal (del Pino et al., 2013), but the laminar distribution of interneurons have not been explored in detail. To confirm that the defects observed in conditional *Nrg3* mutants are mediated by ErbB4, we examined the laminar distribution of PV+ interneurons in the somatosensory cortex of control and *Lhx6-Cre;Erbb4*$^{F/F}$ mutant mice at P30 (Figure 12A). Analysis of the distribution of PV+ neurons in the somatosensory cortex of control and conditional *Erbb4* mutants at P30 confirmed no differences in the density of these cells. In contrast, we observed a clear swift in the laminar distribution of PV+ interneurons in conditional *Erbb4* mutants, with fewer cells in deep layers and more cells in superficial layers than in controls (Figure 12B–12F; layers II-IV: 144.3 ± 19.2 cells per mm^2 in controls, 193.3 ± 7.9 cells per mm^2 in *Erbb4* mutants; layer VI: 147.4 ± 12.5 cells per mm^2 in controls, 79.9 ± 10.05 cells per mm^2 in *Erbb4* mutants; $n = 5$, *$p < 0.05$, **p < 0.01, *t* test). We next wondered whether this defect reflect an overall swift in the normal allocation of cortical interneurons, most likely cause by their delay entry in the CP. To test this idea, we examined the laminar distribution of specific cohorts of PV+ cells by injecting BrdU in control and conditional *Erbb4* mutants at E12.5 or E15.5 (Figure 12A). We found that interneurons born at E12.5 and E15.5 tend to occupy deep and superficial layers of the cortex, respectively, in both controls and *Erbb4* mutants (Figure 12B–12E). However, the distribution of PV+ interneurons was shifted towards progressively more superficial layers for both cohorts of cells in conditional *Erbb4* mutants compared to controls (Figure 12G and 12H; E12.5 BrdU, layer V: 43.47 ± 5.18% in controls, 58.34 ± 3.34% in *Erbb4* mutants; layer VI: 38.19± 2.44% in controls, layer VI: 21.55± 1.85% in *Erbb4* mutants; E15.5 BrdU, layer II/III: 53.29 ± 5.86% in controls, 74.35 ± 2.65% in *Erbb4* mutants; layer IV: 42.6± 3.42% in controls, 17.41± 2.3% in *Erbb4* mutants;
$n = 5$, *$p < 0.05$, χ^2 test). These results indicate that ErbB4 mediates the function of Nrg3 in the intracortical migration of interneurons, and that this signaling system is required for the appropriate timing of laminar allocation for these cells.

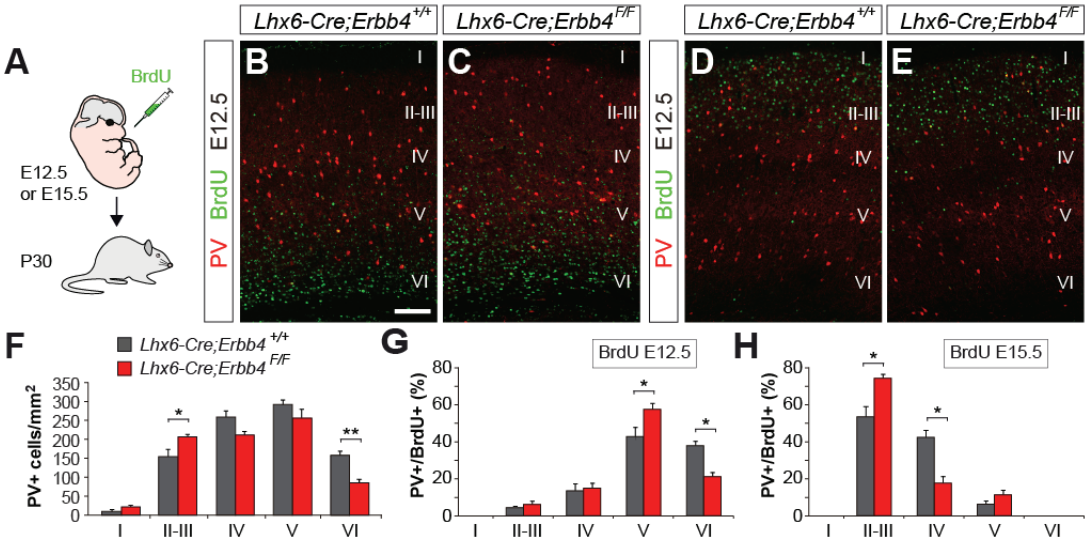

Figure 12. Conditional deletion of *ErbB4* in MGE-derived interneurons disrupts their lamination A) Schematic of the experimental design. (B-E) Laminar distribution of PV+ and specific cohort of PV cells (BrdU-injections at E12.5 and E15.5) in the somatosensory cortex of control and *Lhx6-Cre;Erbb4*$^{F/F}$ mutant mice at P30. (F) Quantification of the distribution of PV+ interneurons in the somatosensory cortex of control and *Lhx6-Cre;Erbb4*$^{F/F}$ mutant mice in layers. (Layers II-IV: 144.3 ± 19.2 cells per mm^2 in controls, 193.3 ± 7.9 cells per mm^2 in *Erbb4* mutants; layer VI: 147.4 ± 12.5 cells per mm^2 in controls, 79.9 ± 10.05 cells per mm^2 in *Erbb4* mutants; n = 5, *p < 0.05, **p < 0.01, t test). (G-H) Quantification of the distribution of specific cohorts of PV+ cells in conditional *Erbb4* mutants compared to controls (E12.5 BrdU, layer V: 43.47 ± 5.18% in controls, 58.34 ± 3.34% in *Erbb4* mutants; layer VI: 38.19± 2.44% in controls, layer VI: 21.55± 1.85% in *Erbb4* mutants; E15.5 BrdU, layer II/III: 53.29 ± 5.86% in controls, 74.35 ± 2.65% in *Erbb4* mutants; layer IV: 42.6± 3.42% in controls, 17.41± 2.3% in *Erbb4* mutants; n = 5, *p < 0.05, χ^2 test). Histograms show average ± SEM. Scale bar equals 200 µm.

In sum, the experiments reported in this part of the Thesis suggest that Nrg3 is one of the molecules that contribute to attract interneurons into the CP. We defined a new role for Nrg3 in the allocation of interneurons in the cerebral cortex. Analysis of the distribution of MGE-derived interneurons in the cortex of P4 and P30 conditional *Nrg3* mutants revealed defects in the laminar organization of these cells. We observed similar laminar defects in conditional *Erbb4* mutants, which reinforces the view that Nrg3 regulates the intracortical migration of interneurons through the ErbB4 receptor.

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Part 2.

Molecular mechanisms regulating the laminar positioning of MGE-derived interneurons

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The results of the experiments described in the first part of the thesis suggest that Nrg3 plays a role in attracting MGE-derived interneurons into the developing CP of the cerebral cortex. Once in this region, interneurons sort out into different layers and adopt their final location in the cortex, but the molecular mechanisms regulating this process remain largely unknown. The aim of the second part of this Thesis was to identify proteins involved in these final events in the allocation of cortical interneurons.

It is has been previously shown that the allocation of interneurons is disrupted in mice in which the laminar distribution of pyramidal cells is compromised. For example, the laminar distribution of interneurons is abnormal in *reeler* mice (Hevner et al., 2004), and this is not due to the cell automomous loss of Reelin signaling in migrating interneurons (Pla et al., 2006). In conceptually similar experiments, it has been shown that different projection neurons ectopically placed in the cortex can affect interneuron positioning (Lodato et al., 2011). Interestingly, interneurons recruited into these areas containing ectopic pyramidal cells match the subtype-specific identity of the projection neurons, rather than their day of birth (Lodato et al., 2011). Together, these experiments suggested that MGE-derived interneurons occupy deep or superficial layers of the neocortex in response to specific signals provided by pyramidal cells found in these layers. We hypothesized that specific cohorts of MGE-derived interneurons exist for deep and superficial layers of the cortex, and that each of these populations of interneurons begin to express protein(s) that allow them to recognized specific cue(s) provided by pyramidal cells in the corresponding target layers once they have finished their tangential migration throughout the cortex. To begin testing this hypothesis, we performed experiments aimed at identify genes fulfilling this premise.

Characterization of Nkx2.1-CreER mice to label late born interneurons

Several lines of evidence indicate that there is a very good correlation between the birthdate of MGE-derived interneurons and their final laminar allocation in the neocortex (Hevner et al., 2004, Pla et al., 2006; Lopez-Bendito et al., 2008). We took advantage of this observation to identify the molecules that are differentially expressed by the same cohort of MGE-derived interneurons before and after they adopt their final laminar position.

We focused on late born MGE-derived interneurons that colonize the upper layers of the cortex because we envisioned a strategy to isolate this population of cells from other

interneurons in a consistent manner. In brief, we crossed *Nkx2.1-CreER* mice (Taniguchi et al., 2011) with the *RCE* reporter line ((Rosa26 Reporter CAG-boosted EGFP mice) (Sousa et al., 2009) and injected pregnant females with tamoxifen at E14.5. Since Nkx2.1 is rapidly turned down by interneurons as soon as they begin migrating away from the MGE (Nóbrega-Pereira et al., 2008), in these experiments recombination of the reporter gene is restricted to progenitor cells present in the MGE at E14.5, which should give rise to interneurons almost exclusively populating the upper layers of the cortex. To confirm this hypothesis and to characterize the timing of CP invasion by late born MGE-derived interneurons, we analyzed the distribution of GFP+ interneurons at different stages of development following intraperitoneal tamoxifen injections at E14.5 (Figure 1, A-D). Analysis of mouse embryos at E18.5 revealed MGE interneurons throughout the entire thickness of the cortex, with many interneurons still confined to the MZ and SVZ, the routes of tangential migration. By contrast, between P0 and P4 interneurons become progressively restricted to the superficial layers of the cortex. By this later stage, most MGE interneurons labeled at E14.5 are mainly located in layers II/III and IV. Thus, late born MGE interneurons transition from tangential to radial migrating roughly around birth, and end up occupying their final laminar position by P4.

To evaluate the peak of Cre recombination after tamoxifen administration we carried out BrdU injections at the same time, 6, 12 or 24 hours after tamoxifen injection, and we then counted the number of BrdU/GFP double labeled cells at E18.5 (Figure 1 E-G). The results of these experiments revealed a peak of colocalization about 6 hours after tamoxifen injection (Figure 1G-H). Therefore, we concluded that tamoxifen injections at E14.5 primarily label cells that are born within 6 hours of the injection.

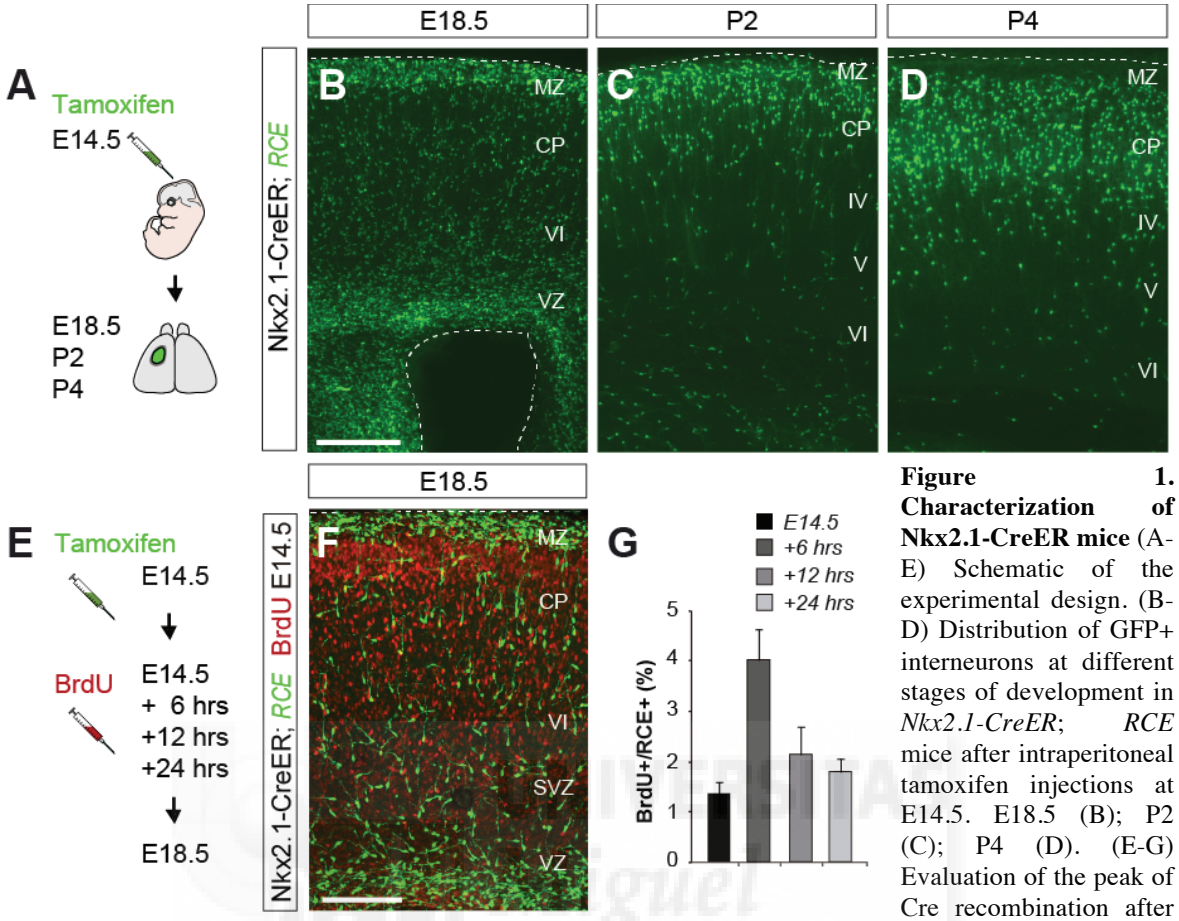

Figure 1. Characterization of Nkx2.1-CreER mice (A-E) Schematic of the experimental design. (B-D) Distribution of GFP+ interneurons at different stages of development in *Nkx2.1-CreER*; *RCE* mice after intraperitoneal tamoxifen injections at E14.5. E18.5 (B); P2 (C); P4 (D). (E-G) Evaluation of the peak of Cre recombination after tamoxifen administration. BrdU injection at the same time than tamoxifen injection (E). (H) Quantification the number of BrdU/GFP double labeled cells at E18.5 (Figure 1 E-G). Histograms show average ± SEM. Scale bar equals 250 μm.

Isolation of late born interneurons and analysis of differentially expressed genes

We decided to follow an unbiased approach to identify genes that are differentially expressed in late born interneurons before (E17.5) and after (P4) they adopt their final position in the neocortex. We choose P4 as the stage of cortical lamination because although the final allocation of interneurons is only completed around P6 (data not shown), the genes that control this process must be expressed already by P4.

To isolate MGE interneurons at these two different stages we injected tamoxifen in *Nkx2.1-CreER*;*RCE* pregnant females at E14.5 and dissect out the cortex of their progeny at E17.5 and P4. We then used FACS to isolate GFP+ cells (Figure 2A–2B), performed mRNA amplifications and hybridized mouse whole genome Affymetrix® microarrays (GeneChip 430 2.0) at the Genomic and Proteomic Unit of the Centro de Investigación del Cancer in Salamanca, Spain. After completing three independent experiments (i.e., three

biological replicas) for each dataset, we obtained a list of 224 genes that are differentially expressed between the two stages using SAM analysis (Anders and Huber, 2010) with a False Discovery Rate (FDR) below 0.05. This basically means that only less than 5% of the significant tests will result in false positives. Among these genes, we found 166 genes that are significantly higher at P4 compared to E17.5, with R fold between 24.58 and 1.3 (Table 5, see *Methods*).

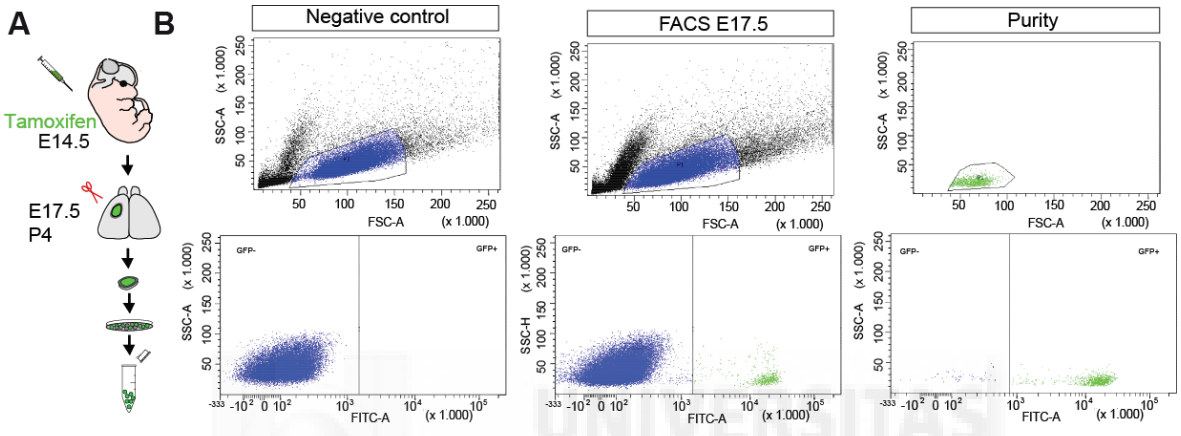

Figure 2. Isolation of late born interneurons through FACS. A) Schematic of the experimental design. (B) Isolation of fluorescent GFP+ cells in *Nkx2.1-CreER*; *RCE* mice after tamoxifen injection at E14.5 through FACS sorting. Negative control, E17.5 and purity panels are shown.

Validation of target genes

We focus our analysis in those genes that are significantly more expressed at P4 than at E17.5 (Table 5, see *Methods*). Among these, we identified several genes that have been previously shown to regulate axon guidance, including *Slit2*, *Robo2*, *Sema3a*, *Netrin4* (Brose and Tessier-Lavigne, 2000; Nakamura et al., 2000; Quin et al., 2007). Other interesting candidates include the gene encoding the chemokine Cx3cl1 and genes coding homophilic cell adhesion protein such as Cdh7, Pcdh11x and Pcdhb8. In addition, we also identified genes linked to metalloprotease function like Mme and Timp2 and some phosphatases, such as Ptprr and Ppp2r2c. Another large family of genes that are upregulated in late born interneurons as they adopt their final position are several channels, including *Kcnh2*, *Kcnd2*, *Kcnq3*, *Kcns3*, *Kcnh7*, a sodium/potassium/calcium exchanger, Slc24a4, and two Kv channels, Kcnip1, Kcnip2.

In addition, we identified a member of proteoglycan family, Spock3 (also known as Testican), Cbln4, and one member of the leucin-rich family of genes previously linked to epilepsy, Lgi2 (Sepp¨al¨a et al., 2011).

We also found two members of the contactin family of genes, Contactin4 and 5a. Contactin family of genes has been shown to be involved in several processes including axon guidance and axon targeting (Osterhout et al., 2015; Kleijer et al., 2015). Many members of this family have also been linked to autism spectrum disorder (Cottrell et al., 2011, Gdalyahu et al. 2015, Chiocchetti et al., 2015). We also identified a growth factor, Fgf14, and several other transmembrane proteins including Tmeff2, Tmem117, Tmem132c, Tmem130, Tmem106b and Tmem65.

Finally, another group of genes upregulated in late born MGE interneurons at P4 is related to synaptic function. This includes Syt10 and Syt1, one gene regulating synapses exocytosis, Rims1, and the synaptoporin gene, Synpr. In addition, we identified several genes encoding GABA (Gabrg3 and Gabbr2), Somatostatin (Sstr1 and Sstr4) and Glutamate (Grid1 and Grin3a) receptors.

We checked the expression pattern of selected genes at P4 using the Allen Brain Atlas (http://www.brain-map.org/). For some of the genes we observed interesting pattern in the cortex with specific expression in layers at P30. For instance, expression of *Alcam*, *Nrp2*, *Sema3a*, *Synpr* is largely restricted to the superficial layers of the cortex, whereas *Cdh7*, *Grin3a*, *Nrp1*, *Slit2*, *Spock3*, *Sstr1* and *Ppargc1a* seem to be expressed more abundantly in deep layers. In contrast, cells expressing *Cx3cl1*, *Kcnq3*, *Ncald*, *Pcd11x*, *Ppp2r2c*, *Robo2* and *Timp2* were found throughout the neocortex at P30. We also performed in situ hybridization experiments to analyze the expression pattern of a small selection of candidate genes at both E17.5 and P4 (Figure 3).

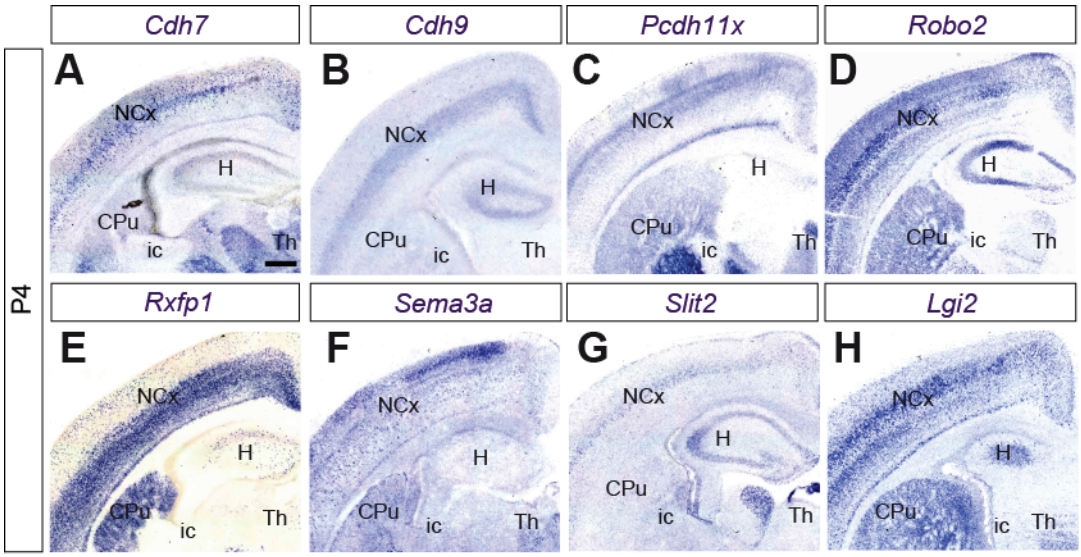

Figure 3. Expression of genes obtained from the Affymetrix® microarrays in the developing mouse cortex. (A–H) Coronal sections through the telencephalon of P4 mice showing mRNA expression for *Cdh7* (A), *Cdh9* (B), *Pcdh11x* (C), *Robo2* (D), *Rxfp1* (E), *Sema3a* (F), *Slit2* (G), *Lgi2* (H). NCx, neocortex; H, Hippocampus; Th, Thalamus; CpU, Caudate Putamen (Striatum); ic, internal capsule. Scale bars equal 250 μm.

Cx3cl1 expression in the developing cerebral cortex

Among those genes differentially expressed by late born interneurons between E17.5 and P4 we focus our attention on the chemokine Cx3cl1, also named Fractalkine (Figure 4). Cx3cl1 exists in two forms, soluble and membrane-bound, and was previously shown to be expressed in forebrain neurons (Tarozzo et al., 2003). The fractalkine receptor, Cx3cr1 is expressed by microglia and astrocytes, which suggested a possible role in signaling between neurons and glia (Nishiyori et al., 1998). Moreover, recent studies have shown that defects in the distribution of microglia in the cortex affect the laminar positioning of cortical interneurons (Squarzoni et al., 2014). We therefore hypothized that Cx3cl1 may play a role in the laminar positioning of cortical interneurons.

The expression of *Cx3cl1* has already been described in the developing mouse brain (Tarozzo 2003). Nevertheless, we performed ISH at different stages of development, from E14.5 to P4 (Figure 4). At E14.5, *Cx3cl1* is expressed in the MGE, in the piriform cortex and in the subplate of the developing neocortex (Figure 4A and 4A'). At E17.5, *Cx3cl1* is strongly expressed by cells in the basal ganglia, piriform cortex, and throughout the neocortex. Expression in the subplate remains strong at this stage (Figure 4B and 4B'). At P2, Cx3cl1 expression is found in the hippocampus and in the neocortex, predominantly in

the somatosensory cortex, where is particularly strong in layer V and in the subplate. By P4, *Cx3cl1* expression in the neocortex is largely restricted to layer V.

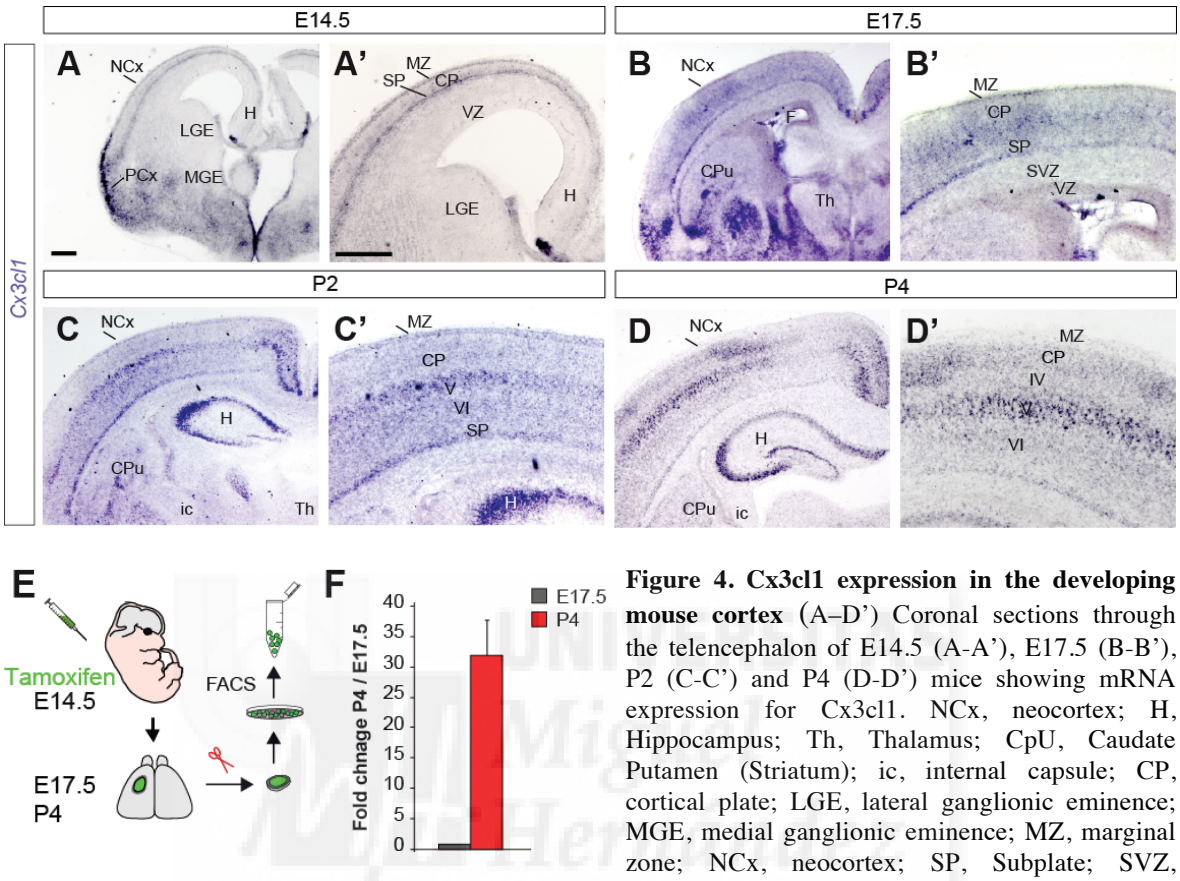

Figure 4. Cx3cl1 expression in the developing mouse cortex (A–D') Coronal sections through the telencephalon of E14.5 (A-A'), E17.5 (B-B'), P2 (C-C') and P4 (D-D') mice showing mRNA expression for Cx3cl1. NCx, neocortex; H, Hippocampus; Th, Thalamus; CpU, Caudate Putamen (Striatum); ic, internal capsule; CP, cortical plate; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, marginal zone; NCx, neocortex; SP, Subplate; SVZ, subventricular zone; VZ, ventricular zone; Pcx, Piriform cortex. Layers IV, V and VI. Scale bars equal 250 μm. (E-F) Real-time PCR to confirm *Cx3cl1* expression in MGE interneurons. (E) Schematic of the experimental design. (F) Fold change (P4/E17.5) confirming the Affymetrix® microarrays data.

To confirm that *Cx3cl1* is differentially upregulated at P4 compared to E17.5 in late born MGE interneurons, we isolated GFP+ cells after FACS, using the Nkx2.1-CreER inducible mice crossed with the RCE reporter, as described previously. We then performed real-time PCR starting with RNA extracted from E14.5 late born interneurons. The results of this analysis confirmed the Affymetrix® microarrays data, indicating that *Cx3cl1* is more abundantly expressed in MGE interneurons at P4 than E17.5 (Figure 3).

Analysis of Cx3cl1 mutant mice

To investigate the role of the chemokine Cx3cl1 in the laminar position of cortical interneurons, we analyzed the distribution of MGE-derived interneurons in the cortex of *Cx3cl1* null mice. *Cx3cl1* mutant mice have been bred in two different backgrounds, C57BL/6 and FVB (see *Methods*). Analysis of Cx3cl1 mutants in the C57BL/6 genetic background revealed no gross anatomical or behavioural abnormalities (Cook et al., 2001). However, *Cx3cl1* mutant mice in the background FVB develop serious behavioural abnormalities around two months of age (S. Lira, unpublished observations), including seizures and abnormal aggressive behavior, and about 70% of them died precociously. For this reason, we focused our analysis in *Cx3cl1* mutant mice in the background FVB.

First, we performed immunohistochemistry for markers of specific cortical layers to determine whether cortical lamination of pyramidal cells was abnormal in *Cx3cl1* mutants. In particular, we carried out immunostaining for Ctip2 (which is expressed at high levels in subcerebral neurons in layer V and at lower levels in corticothalamic neurons in layer VI; Arlotta, et al. 2005), Cux1 (a marker of pyramidal cells in layers II-III and IV), Tbr1 (mainly expressed by corticothalamic pyramidal cells in layers V and VI) and Satb2 (expressed in callosal pyramidal cells through layers II-VI) (Figure 6). We did not observe any differences in the distribution of pyramidal cells in Cx3cl1 mutants compared to controls (Figure 6).

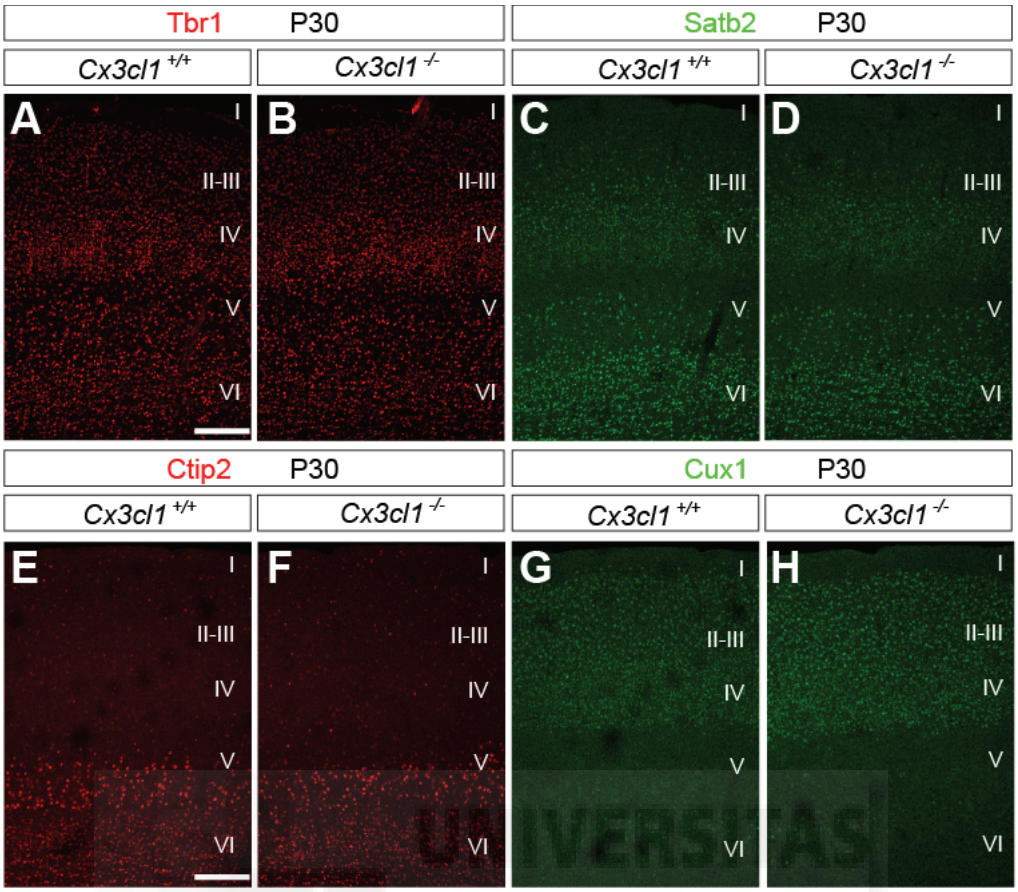

Figure 5. Expression of pyramidal markers of cortical layers is not altered in *Cx3cl1* mutants.(A-H) Immunohistochemistry for markers specific for cortical layers in the mouse somatosensory cortex at P30. Tbr1 expression in controls (A) and Cx3cl1$^{-/-}$ mutants (B); Satb2 in controls (C) and Cx3cl1$^{-/-}$ mutants (D); Ctip2 expression in controls (E) and Cx3cl1$^{-/-}$ mutants (F); Cux1 expression in controls (G) and Cx3cl1$^{-/-}$ mutants (H). Layers I, II-III, IV, V and VI. Scale bar equals 200 µm.

Next, we examined the distribution of cortical interneurons in the somatosensory cortex of control and mutant mice at P4 and at P30 in both genetic backgrounds. No differences were observed in the distribution of *Gad67* and *Lhx6* mRNA at P4 (Figure 7I, 7L, 7M and 7N). Similarly, analysis of the distribution of PV+ and SST+ interneurons in the somatosensory cortex at P30 revealed no significant differences between genotypes (Figure 7A–7H and 7O).

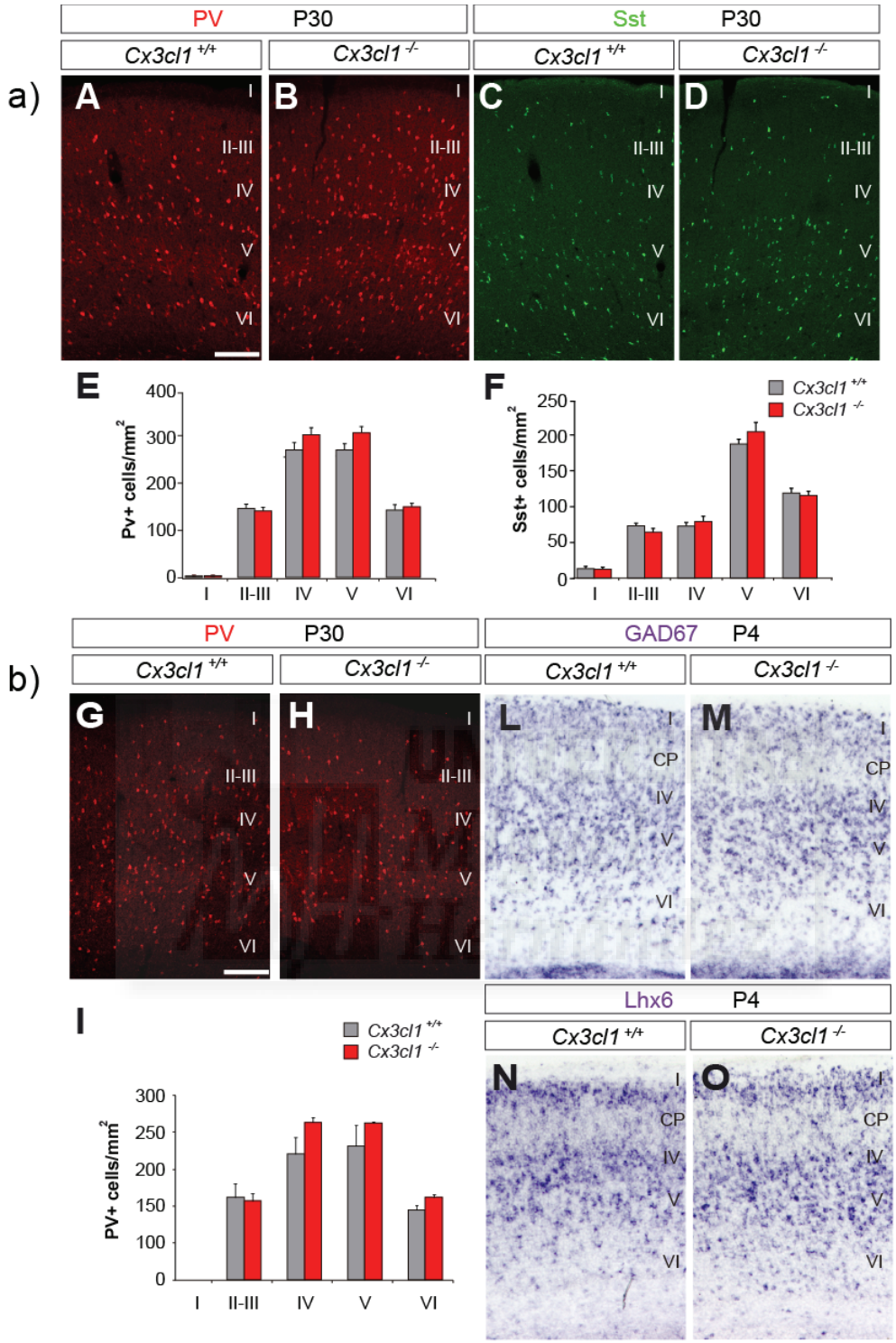

Figure 6. Laminar distribution of interneurons is not altered in *Cx3cl1* mutants.

a) FVB background b) C57BL/6 background. a) (A-D) PV and Sst expression in mice somatosensory cortex at P30 in control (A-C) and Cx3cl1$^{-/-}$ mutants (B-D). (E-F) Quantification of PV (E) and Sst (F) density expressed as cells/mm^2. b) (G-H) PV expression in mice somatosensory cortex at P30 in control (G) and Cx3cl1$^{-/-}$ mutants (H). (I) Quantification of PV density expressed as cells/mm^2. (L-O) mRNA expression for GAD67 in controls (L) and mutants (M); mRNA expression for Lhx6 in controls (N) and mutants (O); somatosensory cortex of P4 mice. CP, cortical plate; Layers I, II-III, IV, V and VI. Scale bar equals 200 μm.

Part 3.

Integration of GABAergic interneurons into cortical cell assemby: Lessons from embryos and adult

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Review

Integration of GABAergic Interneurons into Cortical Cell Assemblies: Lessons from Embryos and Adults

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In the forebrain, cortical structures consist of networks of excitatory and inhibitory neurons born in distant locations. Understanding how these two major classes of neurons integrate into unique functional cell assemblies may shed light on the organization of cortical circuits. In this review, we provide an overview of the mechanisms used by GABAergic interneurons to reach their final position, with an emphasis on the final steps of this process. To this end, we analyze similarities and differences between the integration of GABAergic interneurons in the developing cerebral cortex and in the postnatal brain, using the neocortex and the olfactory bulb as model systems.

Introduction

From a reductionistic perspective, many brain circuits have evolved as hierarchical networks of excitatory glutamatergic neurons and γ-aminobutyric acid-containing (GABAergic) interneurons. In the telencephalon, for example, cortical structures consist of excitatory and inhibitory neuronal assemblies independent of their complexity and function. Accordingly, functional circuits in regions as disparate as the olfactory bulb, hippocampus, and neocortex rely on relatively similar cell assemblies of glutamatergic neurons and GABAergic interneurons. Glutamatergic neurons are the main excitatory units in these networks, typically linked through multiple recurrent connections that are critical for computational performance (Binzegger et al., 2004; Somogyi et al., 1998). GABAergic interneurons, on the other hand, comprise a highly heterogeneous group of neurons that maintain the stability of cortical networks through synaptic inhibition. In addition, interneurons modulate network activity by shaping the spatiotemporal dynamics of different forms of synchronized oscillations (Klausberger and Somogyi, 2008).

The organization of neuronal assemblies in the cortex seems to obey certain rules that guarantee a critical balance between excitation and inhibition while maximizing their computational ability. In the cerebral cortex, for example, the ratio between excitatory and inhibitory neurons is relatively constant across regions and species (Fishell and Rudy, 2011; Hendry et al., 1987; Sahara et al., 2012). In the adult olfactory bulb, where interneurons are continuously added throughout life, the proportion of newborn neurons that integrates into the mature network is tightly regulated (Kohwi et al., 2007; Winner et al., 2002). In addition, GABAergic interneurons in the cerebral cortex and olfactory bulb come in a rich variety of classes, each having highly stereotypical laminar arrangements, unique patterns of connectivity, and functions (Fishell and Rudy, 2011; Klausberger and Somogyi, 2008; Lledo et al., 2008). This enormous variety of interneuron classes provides cortical circuits with the required flexibility to carry out complex computational operations during information processing.

Considering the highly stereotypical organization of cortical networks, the most striking aspect of their assembly is that their cellular ingredients are born in separate locations. While glutamatergic neurons of the olfactory bulb and the cerebral cortex are generated locally by progenitor cells in the developing pallium (Molyneaux et al., 2007; Rakic, 2007), GABAergic interneurons populating these structures derive from the subpallium, the base of the telencephalon (Batista-Brito and Fishell, 2009; Gelman and Marín, 2010; Wonders and Anderson, 2006). Consequently, glutamatergic neurons and GABAergic interneurons follow very different strategies to reach their final destination. Glutamatergic neurons migrate radially to form the different layers of cortical structures (Rakic, 2006). In contrast, interneurons first migrate tangentially from their birthplace to the cerebral cortex and olfactory bulb and subsequently switch their mode of migration to radial to adopt their final position in these structures (Marín and Rubenstein, 2001). How these apparently disconnected processes synchronize during development is arguably one of the most fascinating questions on the assembly of neuronal circuits in the mammalian brain.

The purpose of this review is to summarize our current understanding of the mechanisms controlling the coordinated integration of glutamatergic neurons and GABAergic interneurons into cortical networks. The emphasis is on those aspects related to the final settlement of GABAergic interneurons in the cerebral cortex and olfactory bulb, and not so much on the mechanisms controlling their tangential migration to their target structures (reviewed in Belvindrah et al., 2009; Marín, 2013). The developing neocortex is used here as a model for the coordinated integration of glutamatergic neurons and GABAergic interneurons into nascent cortical circuits, while the adult olfactory bulb illustrates the ability of newborn GABAergic interneurons to integrate into fully mature networks.

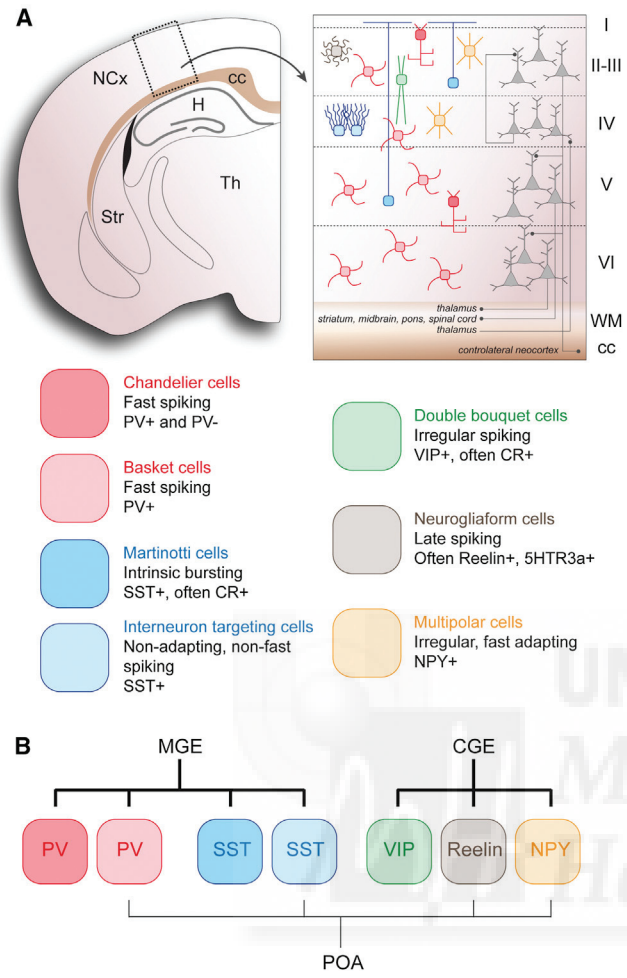

Figure 1. Major Classes of Neocortical Interneurons and Their Developmental Origins

(A) Schematic of a coronal section through the mouse cerebral cortex showing the main classes of GABAergic interneurons and their respective laminar allocation. Fast-spiking PV^+ basket cells are distributed throughout all cortical layers except for layer I. Chandelier cells localize primarily to the border between layers I and II/III, and in layer V. SST^+ Martinotti cells are mainly found in layers II/III and V and extend their axon toward layer I. Non-fast-spiking, nonadapting SST^+ interneurons are restricted to layer IV. Rapidly adapting VIP^+ interneurons and late-spiking neurogliaform cells are particularly abundant in layer II/III. Finally, multipolar cells that often contain NPY are found through layers II/III and IV. The laminar organization of pyramidal cells is also schematically represented.

(B) Grouping of the main classes of cortical interneurons according to their developmental origins. cc, corpus callosum; HC, hippocampus; NCx, neocortex; Str, striatum; Th, thalamus; WM, white matter; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; POA, preoptic area; I–VI, cortical layers I to VI; 5HTR3a, ionotropic serotonin receptor 3a.

Integration of GABAergic Interneurons in the Developing Cortex

Glutamatergic pyramidal cells and inhibitory GABAergic interneurons constitute the main cellular elements of each of the individual modules or microcircuits of the cerebral cortex. Pyramidal cells represent about 80% of the neurons in the cortex and specialize in transmitting information between different cortical areas and to other regions of the brain. GABAergic interneurons, on the other hand, control and orchestrate the activity of pyramidal cells.

Pyramidal cells are a highly heterogeneous group of neurons with different morphological, neurochemical, and electrophysiological features. A basic classification of pyramidal cells is based on their connectivity, which is roughly linked to their laminar location in the cortex (Jones, 1984) (Figure 1). Subcortical projection pyramidal cells are the main neurons in layers V and VI. They target the thalamus (layer VI) and other telencephalic and subcerebral regions, such as the striatum, midbrain, pons, and spinal cord (layer V pyramidal cells). Pyramidal cells in layer IV, the granular layer, are associative neurons that project to pyramidal cells in layers II/III. Finally, callosal projection pyramidal cells project to the contralateral cortex and are particularly abundant in layers II/III. Some of these pyramidal cells are also present in layers V and VI. Layer II/III pyramidal cells also project abundantly to infragranular pyramidal cells.

More than 20 different classes of interneurons have been identified in the hippocampus and neocortex, each of them with distinctive spatial and temporal capabilities to influence cortical circuits (Fishell and Rudy, 2011; Klausberger and Somogyi, 2008). The classification of interneurons is a remarkably complicated task because their unequivocal identification requires a combination of morphological, neurochemical, and electrophysiological properties (Ascoli et al., 2008; DeFelipe et al., 2013). For the purpose of this review, neocortical interneurons can be broadly classified into five categories (Figure 1). The most abundant group consists of interneurons with the electrophysiological signature of fast-spiking neurons. It includes two main classes of interneurons: basket cells and chandelier cells (Markram et al., 2004). Most fast-spiking interneurons express the calcium binding protein parvalbumin (PV), although many chandelier cells do not (Taniguchi et al., 2013). A second group of interneurons is characterized by the expression of the neuropeptide somatostatin (SST). It includes interneurons with intrinsic-burst-spiking or adapting nonfast-spiking electrophysiological profiles and includes at least two different classes of interneurons. Martinotti cells, with a characteristic axon extending into layer I, are the most abundant SST^+ interneurons (Ma et al., 2006; Xu et al., 2013). In addition, a second class of SST^+ interneurons with axons that branch abundantly near the cell soma has been identified (Ma et al., 2006; Xu et al., 2013). The third major group of neocortical interneurons includes rapidly adapting interneurons with bipolar or double-bouquet morphologies, which typically express the vasointestinal peptide (VIP) and may also contain the calcium binding protein calretinin (CR) (Rudy et al., 2011). Neurogliaform cells constitute a fourth large group of neocortical interneurons (Armstrong et al., 2012). They have a very characteristic morphology, with highly branched short dendrites and a defining dense local axonal plexus. Neurogliaform cells have a late-spiking firing pattern, and many express Reelin and the ionotropic serotonin receptor 3a. Finally, a fifth group of interneurons consists of multipolar cells with irregular or rapidly adapting electrophysiological properties that often contain neuropeptide Y (NPY) (Lee et al., 2010). As explained below, the different classes of interneurons distribute through the cerebral cortex following highly specific regional and laminar patterns. This remarkable degree of organization suggests that the functional

integration of interneurons into specific neuronal circuits is largely dependent on their precise positioning within the cortex.

Pyramidal cells and interneurons are organized along two main dimensions in the cerebral cortex. The first axis divides the cortex into a variable number of layers depending on the cortical area. Neurons within the same cortical layer share important features, including general patterns of connectivity (Dantzker and Callaway, 2000; Molyneaux et al., 2007). The second axis reflects the vertical organization of neuronal circuits within a column of cortical tissue. Neurons within a given column are stereotypically interconnected in the radial dimension, share extrinsic connectivity, and function as the basic units underlying cortical operations (Mountcastle, 1997). Thus, any given cortical area consists of a sequence of columns in which their main cellular constituents, pyramidal cells and interneurons, share a common laminar organization. From this perspective, the integration of GABAergic interneurons within the organized matrix of layers and columns that compose the cortex might be better understood as a sequence of events that first determine the specific rostrocaudal and mediolateral coordinates of interneurons in the tangential plane (i.e., regional distribution) and subsequently determine their precise layering within the radial axis (i.e., laminar distribution).

Regional Distribution of Cortical Interneurons

As local circuit neurons, interneurons could be potentially incorporated in any cortical region. The question is whether interneurons are specified to migrate to precise locations or they just colonize the cerebral cortex without being targeted to specific coordinates. In other words, is there a correlation between their site of origin within the subpallium and their distribution along the rostrocaudal and mediolateral dimensions of the cortex?

Multiple lines of evidence suggest that the different classes of cortical interneurons are born in specific regions of the subpallium (Gelman and Marín, 2010; Wonders and Anderson, 2006) (Figure 1). In brief, the embryonic subpallium has five major proliferative regions: the lateral, medial, and caudal ganglionic eminences (LGE, MGE, and CGE, respectively), the preoptic area (POA), and the septum. The large majority of PV^+ and SST^+ interneurons derive from the MGE (Butt et al., 2005; Flames et al., 2007; Fogarty et al., 2007; Inan et al., 2012; Taniguchi et al., 2013; Wichterle et al., 2001; Xu et al., 2004, 2008). In turn, the CGE gives rise to most of the remaining interneurons, including bipolar VIP^+ interneurons, most neurogliaform neurons, and NPY^+ multipolar interneurons (Butt et al., 2005; Miyoshi et al., 2010; Nery et al., 2002; Xu et al., 2004). Finally, the POA generates a small, but diverse, contingent of PV^+, SST^+, and NPY^+ interneurons (Gelman et al., 2009, 2011).

Although the vast majority of cortical interneurons originate in the embryonic subpallium and migrate as postmitotic cells toward the cortex, postnatal sources of cortical interneurons seem to exist. One of these has been identified in the dorsal white matter and comprises what seems to be an expanding pool of progenitor cells possibly derived from the LGE and/or CGE (Riccio et al., 2012; Wu et al., 2011). Interestingly, these interneurons appear to follow a unique specification program and differentiate later than interneurons born in the embryo. Interneurons from this source populate primarily the lower layers of the anterior cingulate cortex. In addition, the adult subventricular zone (SVZ), the main postnatal source of olfactory bulb interneurons, also seems to give rise to some interneurons that populate forebrain structures other than the olfactory bulb, including the neocortex, caudoputamen nucleus, and nucleus accumbens (Inta et al., 2008). Intriguingly, some of the SVZ-derived interneurons that populate the deep layers of the frontal cortex share some morphological and functional features with olfactory bulb interneurons. They are small, axonless neurons that establish dendrodendritic synapses and integrate into the network in an experience-dependent manner (Le Magueresse et al., 2011).

These studies suggest that specific classes of interneurons derive from distinct regions of the subpallium to later colonize multiple cortical structures. Fast-spiking interneurons are a clear example of this circumstance. Transplantation and genetic fate-mapping studies have shown that the MGE is the origin of fast-spiking interneurons found in the amygdala, striatum, piriform cortex, hippocampus, and neocortex (Marín et al., 2000; Pleasure et al., 2000; Tricoire et al., 2011; Wichterle et al., 2001; Xu et al., 2008). Several lines of evidence suggest that distinct pools of progenitor cells within the MGE are specified to produce interneurons for each of these telencephalic structures. For instance, striatal and cortical interneurons seem to derive from different progenitor pools within the MGE (Flandin et al., 2010). Consistent with this notion, striatal and cortical interneurons are specified to reach their targets by expressing different complements of guidance receptors (Marín et al., 2001; Nóbrega-Pereira et al., 2008; van den Berghe et al., 2013). In addition, the hippocampus contains certain classes of interneurons that do not seem to have a clear homolog in the neocortex, such as PV^+/SST^+ bistratified cells (Buhl et al., 1994). Similarly, VIP^+ interneurons populate the cortex and the hippocampus but are absent from the striatum. Thus, it is conceivable that different pools of progenitor cells within the subpallium are specified to generate interneurons that migrate to specific subdivisions of the telencephalon (i.e., striatum, amygdala, neocortex, hippocampus).

Does the same rule apply for different neocortical regions? If this were the case, then one would expect to observe a topographical relationship between the origin of a specific class of interneurons within the subpallium and their final distribution in the neocortex. Transplantation experiments in slices have shown that the mediolateral distribution of GABAergic interneurons in the neocortex is not topographically related to their birthplace. So, irrespective of the site of origin in the MGE, interneurons tend to colonize the neocortex following a lateral to medial progression (Lourenço et al., 2012), in parallel to the normal maturation gradient of pyramidal cells (Bayer and Altman, 1987). Consistent with this notion, PV^+ interneurons within the same layer are, on average, younger in the lateral third of the somatosensory cortex than in the medial third (Rymar and Sadikot, 2007).

The mechanisms that control the regional distribution of neocortical interneurons are presently unclear, but several lines of evidence suggest that this process is related to the transition of interneuron migration from tangential to radial or, more precisely, to its timing (Figure 2). On their entry into the pallium, interneurons do not immediately target the cortical plate, where developing pyramidal cells are beginning to differentiate. Instead, interneurons continue their tangential spread using the

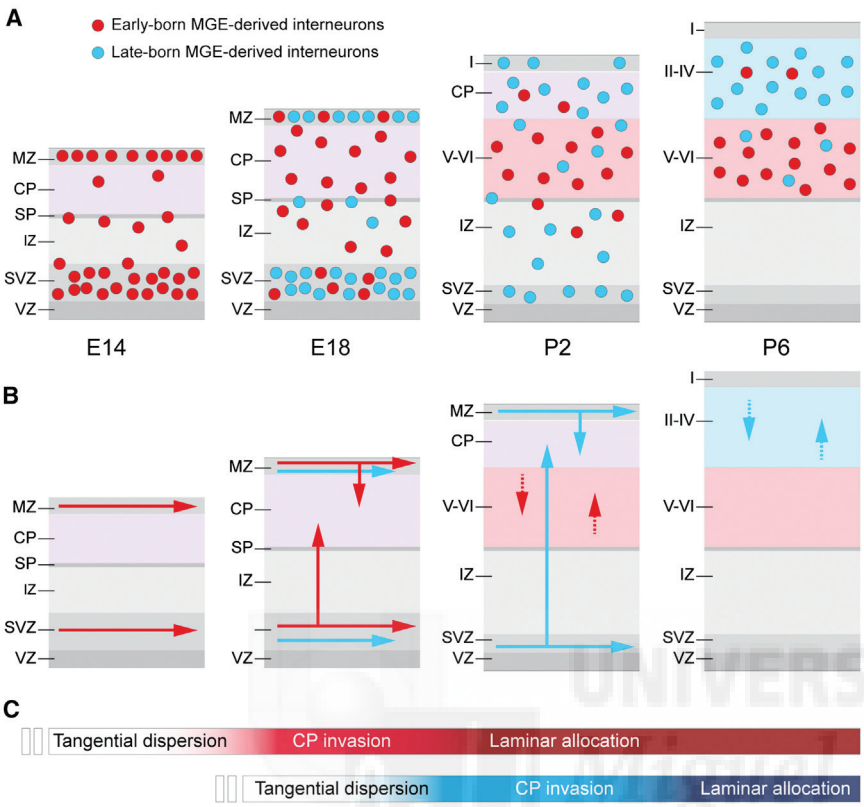

Figure 2. Integration of MGE-Derived Interneurons into Cortical Layers

(A–C) Schematic representation of the different phases underlying the integration of GABAergic interneurons in the neocortex. Circles in (A) schematically represent the distribution of MGE-derived interneurons, while arrow lines in (B) represent the migratory trajectories followed by interneurons. Early- and late-born MGE-derived interneurons are depicted in red and blue, respectively. The figure shows schematic representations of the mouse neocortex at different developmental stages (E14, E18, P2, and P6). Three distinct phases can be observed for each cohort of interneurons: tangential dispersion, cortical plate (CP) invasion, and laminar allocation. These consecutive phases seem common to all MGE-derived interneurons, but their timing varies depending on the age of interneurons (C). Sorting of interneurons into different layers of the cortex seems to follow a two-step process. First, interneurons seem generally attracted to the CP (purple); subsequently, they restrict their distribution to particular layers (light blue and light red), so that early-born MGE-derived interneurons primarily settle in infragranular layers, while late-born MGE-derived interneurons populate the superficial layers. This later phase appears to depend on signals released by pyramidal cells. MZ, marginal zone; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; I–VI, cortical layers I to VI.

marginal and subventricular zones of the cortex (Lavdas et al., 1999; Marín and Rubenstein, 2001; Wichterle et al., 2001). Eventually, interneurons switch their mode of migration from tangential to radial and invade the cortical plate, where they take residence. This suggests that the mediolateral and rostrocaudal position of an interneuron during this transition determines its final coordinates in the neocortex.

The chemokine Cxcl12 regulates the tangential dispersion of interneurons throughout the neocortex. This molecule is expressed by the meninges and intermediate progenitor cells in the subventricular zone of the cortex and contributes to maintain interneurons within the tangential migratory streams (Daniel et al., 2005; Stumm et al., 2003; Tham et al., 2001; Tiveron et al., 2006). Interneurons respond to Cxcl12 using two G protein couple receptors, Cxcr4 and Cxcr7. In mouse mutants for these receptors, interneurons leave the migratory streams and enter the cortical plate prematurely, which disrupts their regional distribution within the neocortex (Li et al., 2008; López-Bendito et al., 2008; Meechan et al., 2012; Sánchez-Alcañiz et al., 2011; Tanaka et al., 2010). These studies strongly suggest that the timing of exit from the migratory streams—and so the final distribution of neocortical interneurons—is directly linked at a molecular level with the loss of responsiveness to Cxcl12.

Laminar Allocation of Cortical Interneurons

The laminar organization of pyramidal cells has been studied for several decades, and important progress has been made in understanding the mechanisms controlling their ordered allocation into specific layers. The characteristic six-layered structure of the neocortex emerges during development in an inside-out pattern that is universal among mammalian species (Rakic, 2007). Newborn pyramidal cells always migrate through previous cohorts of pyramidal neurons, so that early-born cells end up located in deep (i.e., infragranular) layers, and late-born cells populate superficial (i.e., supragranular) layers of the cortex. A signaling pathway elicited by Reelin, a glycoprotein expressed by Cajal-Retzius cells at the surface of the cortex, controls the ordered migration of pyramidal cells (Franco and Müller, 2011; Soriano and Del Río, 2005). This pattern of migration allows the organization of particular classes of pyramidal cells into coherent groups with similar functional properties. In other words, pyramidal cells exhibit comparable—although not necessarily identical—patterns of axonal connections within each of the cortical layers, which contribute to the establishment of reproducible circuits within each column of the cerebral cortex.

A superficial analysis of the distribution of GABAergic interneurons may lead to the premature conclusion that these cells distribute uniformly throughout all layers of the cerebral cortex. There is, however, a remarkable degree of sophistication in the laminar distribution of neocortical GABAergic interneurons (Figure 1). For instance, PV^+ interneurons are absent from layer I (Rymar and Sadikot, 2007), while Martinotti cells are particularly abundant in layers V and VI, and to a minor extent in layers II/III, but nearly absent from layer IV (Ma et al., 2006). In addition, most bipolar or double-bouquet interneurons reside in the supragranular layers of the cortex (Rymar and Sadikot, 2007), while chandelier cells are almost exclusively found in layers II and V

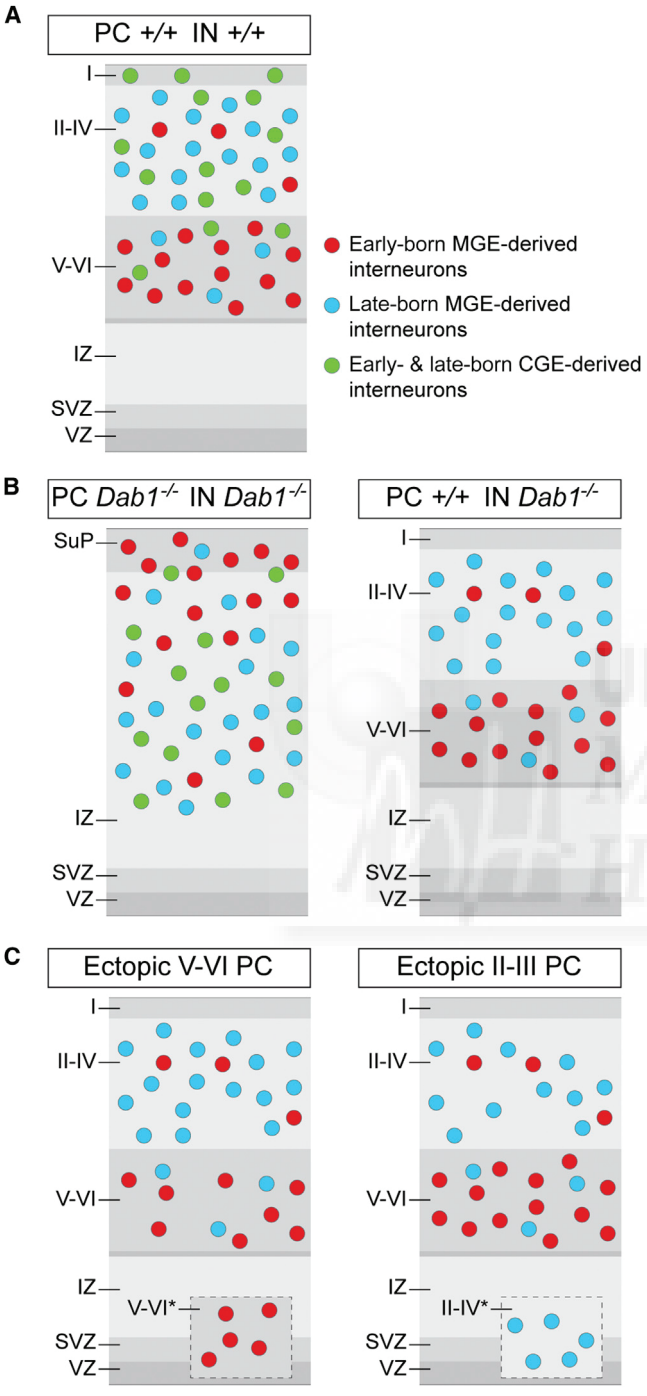

Figure 3. Pyramidal Cells Control the Distribution of GABAergic Interneurons in the Neocortex

(A) Schematic diagram illustrating the laminar distribution of MGE- and CGE-derived interneurons in the neocortex. Similar to pyramidal cells, MGE-derived interneurons distribute in a roughly inside-out pattern: early-born MGE-derived interneurons (red circles) are mainly located in infragranular layers, while late-born MGE-derived interneurons (blue circles) occupy the superficial layers. CGE-derived interneurons (green circles) distribute primarily throughout supragranular layers independently of their birthdate.

(B) Abnormal distribution of pyramidal cells in *Dab1*$^{-/-}$ mice disturbs the laminar organization of MGE-derived interneurons (left panel). This phenotype is due to the abnormal location of PN in *Dab1*$^{-/-}$ mice, because when *Dab1*$^{-/-}$ interneurons are transplanted into wild-type mice, they adopt a normal distribution (right panel).

(C) Pyramidal cells selectively recruit local interneurons based on their subtype-specific identity. The generation of ventricular zone (VZ) ectopias containing infragranular (left panel) or supragranular (right panel) pyramidal cells is sufficient to recruit early- and late-born interneurons, respectively, to this abnormal location. IN, interneurons; IZ, intermediate zone; PC, pyramidal cells; SuP, superplate; SVZ, subventricular zone; VZ, ventricular zone; I–VI, cortical layers I to VI; V–VI* and II–IV*, ectopic infragranular and supragranular pyramidal cells, respectively.

in the rodent neocortex (Taniguchi et al., 2013). Even those interneurons that seem to distribute more or less uniformly through most cortical layers, such as PV^{+} basket cells, display distinct patterns of connectivity according to their laminar position (Tremblay et al., 2010). This remarkable degree of organization suggests that precise developmental mechanisms control the laminar distribution of cortical interneurons.

The laminar distribution of MGE-derived interneurons follows a sequence that is similar to that followed by pyramidal cells. Thus, early-born MGE-derived interneurons primarily populate the infragranular layers of the neocortex, while late-born interneurons colonize the supragranular layers (Fairén et al., 1986; Miller, 1985; Pla et al., 2006; Rymar and Sadikot, 2007; Valcanis and Tan, 2003) (Figure 3). This seems to imply that the time of neurogenesis largely determines the laminar allocation of interneurons. However, several lines of evidence suggest that this is actually not the case. First, CGE-derived interneurons largely concentrate in supragranular layers of the cortex, independently of their birthdate (Miyoshi et al., 2010; Rymar and Sadikot, 2007; Xu et al., 2004). This indicates that the birthdate is not a universal predictor of laminar allocation for interneurons. Second, the distribution of MGE-derived interneurons is directly influenced by the position of pyramidal cells (Hevner et al., 2004; Lodato et al., 2011; Pla et al., 2006). For example, the laminar distribution of interneurons is abnormal in *reeler* mice (Hevner et al., 2004), and this is not due to the loss of Reelin signaling in interneurons (Pla et al., 2006) (Figure 3). These studies led to an alternative hypothesis to explain the laminar distribution of interneurons, according to which interneurons would adopt their laminar position in response to cues provided by specific classes of pyramidal cells. Direct support for this idea derives from experiments in which the laminar position of MGE-derived interneurons was specifically altered by disrupting the laminar distribution of specific classes of pyramidal cells, independently of their birthdate (Lodato et al., 2011) (Figure 3). Thus, MGE-derived interneurons appear to occupy deep or superficial layers of the cortex in response to specific signals provided by pyramidal cells located in these layers. Consequently, this process is perhaps only correlatively, but not causally, linked to the time of neurogenesis.

Recent studies on the generation of cortical lineages have shed further light on the chemical matching hypothesis for the laminar distribution of neocortical interneurons. The classical view of cortical development is based on the premise that pyramidal cells in all layers of the neocortex originate from the same lineage (Woodworth et al., 2012). In other words, cortical progenitors are multipotent and give rise to any class of pyramidal cell, but are gradually restricted to producing neurons for

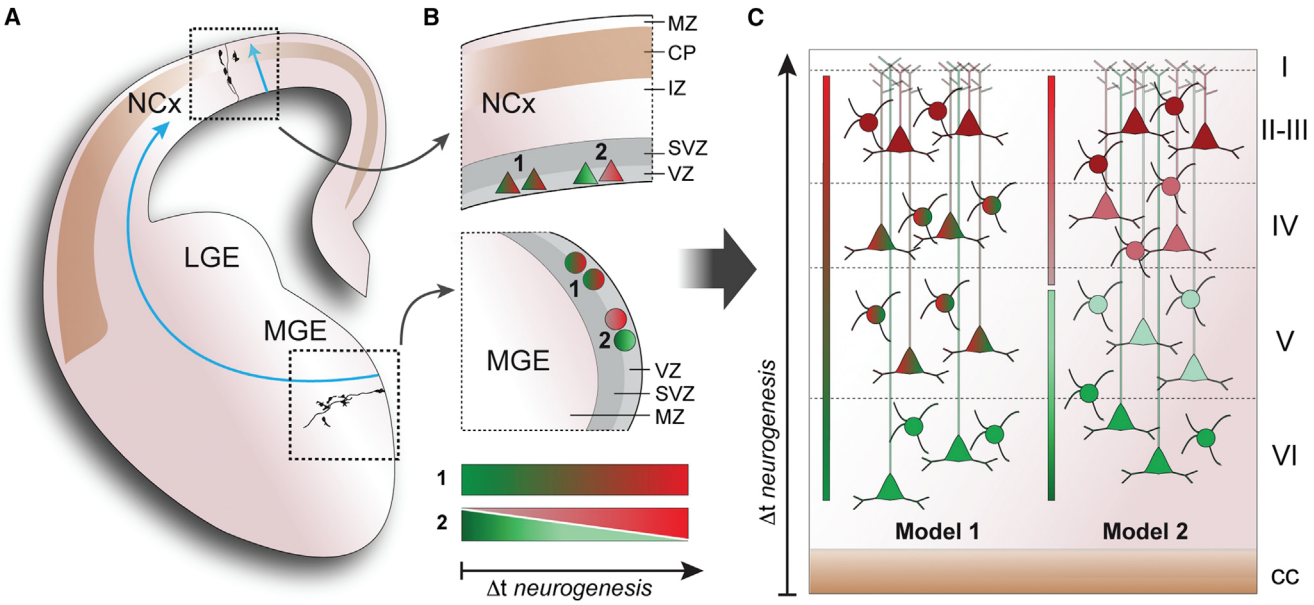

Figure 4. Lineages and Ontogenic Organization of the Neocortex

(A and B) Schematic diagram of a coronal section through the mouse telencephalon during embryonic development. The boxed areas in (A) correspond to the schemas shown in (B), which illustrate two models (1 and 2) of neurogenesis for pyramidal cells and MGE-derived interneurons. According to the classical model (model 1), progenitor cells in the embryonic cortex (triangles) and in MGE (circles) are multipotent. Each progenitor cell in these regions has the potential to generate pyramidal cells and interneurons, respectively, for all cortical layers. The fate potential of progenitor cells is progressively restricted along neurogenesis so that they give rise first to deep cortical neurons and later on to progressively more superficial neurons (transition from green to red in model 1). Model 2 is based on the observation that at least two classes of progenitor cells seem to exist for pyramidal cells (triangles) and interneurons (circles), each one committed to generate neurons with specific laminar allocations. In this model, the two lineages coexist in the proliferative regions, but their relative proportion and/or neurogenic potential changes during development.

(C) Schematic diagram of a coronal section through the adult neocortex, showing lineage relationships and neuron distributions for model 1 (left) and model 2 (right). In model 1, lineages of pyramidal cells and interneurons are organized along the columnar dimension of the neocortex. In model 2, lineages of pyramidal cells and interneurons are primarily organized along the laminar dimension of the neocortex. Color codes in the figure do not represent any developmental program but simply reflect the fate of cells according to their laminar position. In addition, note that both models are not incompatible. cc, corpus callosum; NCx, neocortex; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; I–VI, cortical layers I to VI.

progressively more superficial layers (Noctor et al., 2001; Rakic, 1988). Recent work on the organization of interneuron lineages also led to the conclusion that MGE-derived interneurons that extend throughout multiple layers of the cortex derive from the same progenitor cells (Brown et al., 2011) (Figure 4, model 1).

This view of cortical neurogenesis has recently been challenged by the identification of different classes of progenitor cells for both pyramidal cells and interneurons (Ciceri et al., 2013; Franco et al., 2012; Stancik et al., 2010) (Figure 4, model 2). In the pallium, two classes of progenitor cells in the neocortex might exist: one largely responsible for the generation of pyramidal cells in deep (V and VI) layers and another one for pyramidal cells in superficial (II and IV) layers (Franco et al., 2012). Similarly, recent work on the organization of progenitor cells in the subpallium suggests that MGE-derived interneurons originate from at least two separate lineages: one that primarily produces interneurons for deep (V and VI) layers of the cortex and another one that generates interneurons for superficial (II and IV) layers (Ciceri et al., 2013) (Figure 4). According to this model, the relative proportion of the different types of progenitor cells varies with time, and this determines the classes of pyramidal cells and interneurons that are being produced at a particular developmental stage. Furthermore, these experiments suggest that MGE-derived interneurons might be generated to mirror the laminar organization of pyramidal cells.

Early Functional Interactions

The distribution of GABAergic interneurons into the cerebral cortex also relies on functional interactions between these cells and the networks into which they integrate. Initially, these interactions rely on the ability of migrating interneurons to sense the combined extracellular levels of GABA and glutamate, and so they precede the onset of synaptogenesis in the cortex. Both neurotransmitters enhance neuronal migration in the embryo because they depolarize the membrane of interneurons and stimulate the generation of calcium transients (Cuzon et al., 2006; Manent et al., 2005). However, the reversal potential for chloride ions changes in interneurons as they mature, and so GABA becomes hyperpolarizing when this occurs. This change turns ambient GABA into a stop signal for migrating interneurons, because hyperpolarizing GABA decreases the frequency of intracellular calcium transients (Bortone and Polleux, 2009). The potassium/chloride exchanger KCC2 mediates the reversal potential of chloride ions in maturing neurons (Ben-Ari, 2002), and so the mechanisms controlling the upregulation of this transporter are likely linked to the termination of migration (Bortone and Polleux, 2009). Consistently, interneurons upregulate

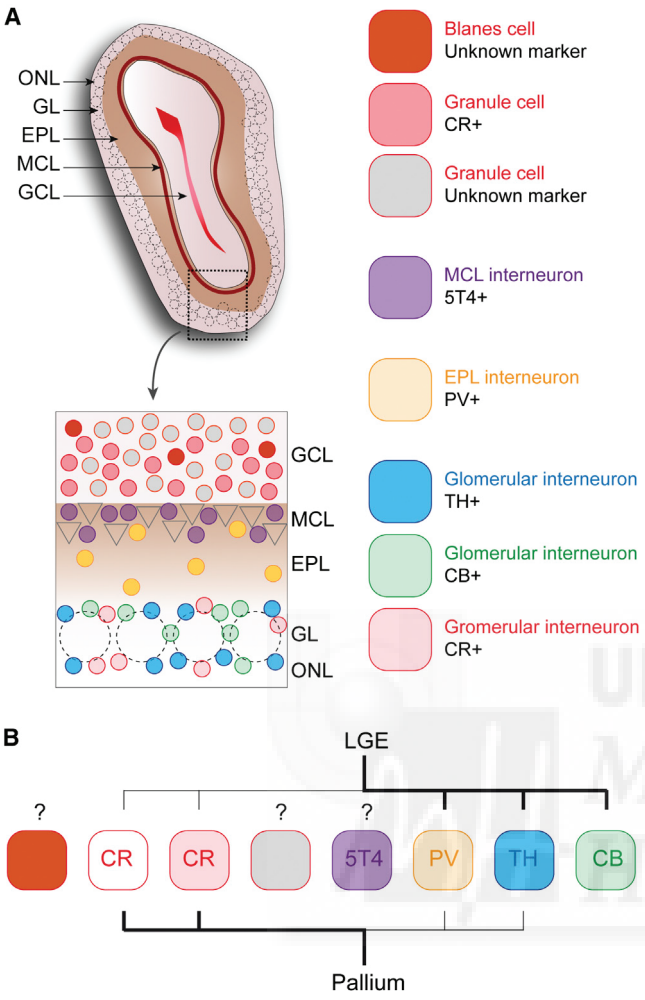

Figure 5. Major Classes of Olfactory Bulb Interneurons and Their Developmental Origins

(A) Schematic of a coronal section through the mouse olfactory bulb showing the main classes of GABAergic interneurons and their respective laminar allocation. Granule cells include at least three different classes: Blanes cells, CR^+ granule cells preferentially located in the most superficial aspect of the granule cell layer, and granule cells without a known specific marker. The mitral cell layer contains interneurons that express the glycoprotein 5T4. The external plexiform layer contains PV^+ interneurons. Periglomerular interneurons comprise at least three classes based on their neurochemical content: TH^+, CB^+, and CR^+ cells.

(B) Grouping of the main classes of cortical interneurons according to their developmental origins. ONL, olfactory nerve layer; GL, glomerular layer: EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer; LGE, lateral ganglionic eminence.

KCC2 expression during their radial sorting in the cortex (Miyoshi and Fishell, 2011); however, it is presently unclear how this process is integrated with the laminar allocation of interneurons. One possibility is that interneurons get preferentially immobilized in layers with increased network activity, in which modification of calcium dynamics might be more prominent (de Lima et al., 2009). Alternatively, the layer-specific cues that are thought to control the final distribution of interneurons might also regulate the expression of KCC2 in these cells. In agreement with this hypothesis, factors released by cortical cells decrease the mobility of embryonic interneurons in culture (Inamura et al., 2012). In any case, early patterns of activity seem to play a clear role in the final settlement of interneurons, independently of their origin (Bortone and Polleux, 2009; De Marco García et al., 2011).

Integration of GABAergic Interneurons in the Adult Olfactory Bulb

The adult olfactory bulb represents a good model to study the ability of newly generated GABAergic interneurons to integrate into mature networks. Similar to the cerebral cortex, the olfactory bulb is organized as an assembly of excitatory and inhibitory neurons distributed through layers (Zou et al., 2009). However, olfactory interneurons outnumber excitatory neurons in an ~100:1 proportion, perhaps because the primary function of the olfactory bulb is to discriminate sensory information. In addition, neural circuits in the olfactory bulb are continuously remodeled by the addition of new GABAergic interneurons, generated through the process of adult neurogenesis. This circumstance makes the adult olfactory bulb an ideal model for studying how GABAergic interneurons integrate into mature neuronal circuits. Transplantation experiments have shown that embryonic cortical interneurons also have the ability to migrate and functionally integrate in the adult cortex (Alvarez-Dolado et al., 2006; Wichterle et al., 1999), which suggests that this might be a rather general characteristic of GABAergic interneurons.

Two classes of excitatory neurons are present in the olfactory bulb, mitral cells and tufted cells, which are confined to a single layer that lies between the external plexiform and granule cell layers (Figure 5). Both classes of neurons are glutamatergic, but they comprise several different populations that diverge in the spatial organization of their connections and molecular markers (Mizuguchi et al., 2012; Mori and Sakano, 2011). Mitral cells and tufted cells send their primary dendrites into single glomeruli, where they receive inputs from olfactory sensory neurons. In turn, they convey this information to other brain centers in the telencephalon through the lateral olfactory tract (Igarashi et al., 2012). Hence, as in the cortex, excitatory neurons are the main projection neurons in the olfactory bulb.

The olfactory bulb contains several classes of GABAergic interneurons, grouped in three main populations: granule cells, external plexiform layer interneurons, and periglomerular cells (Figure 5) (Batista-Brito et al., 2008). It is worth noting that olfactory bulb interneurons have not been as extensively characterized as cortical interneurons, and so their classification largely relies on marker analyses at this point. Granule cells are the most abundant GABAergic neurons in the olfactory bulb. They have a small soma and make dendrodendritic connections with excitatory neurons (Price and Powell, 1970). Several classes of neurons have been identified within the granule cell layer, including external granule cells, whose soma is located within the mitral cell layer and expresses the glycoprotein 5T4, CR^+ granule cells located in the external aspect of the granule cell layer, and Blanes cells (Imamura et al., 2006; Pressler and Strowbridge, 2006). This later population of interneurons is specialized in inhibiting granule cells, thereby controlling the strength of inhibition on the excitatory neurons (Pressler and Strowbridge, 2006). Many granule cells do not express any known markers,

which suggests an even larger diversity within this population. The most common population of interneurons in the external plexiform layer contains PV (Kosaka and Kosaka, 2008), but several other classes of interneurons seem to exist in this layer (Huang et al., 2013; Krosnowski et al., 2012; Liberia et al., 2012). Interneurons in this layer are thought to provide inhibition to mitral and tufted cells (Huang et al., 2013), probably by targeting their apical dendrites. Finally, three distinct subtypes of interneurons have been identified in the glomerular layer of the mouse, based on the expression of tyrosine hydroxylase (TH), calbindin (CB), and CR, respectively (Kohwi et al., 2007; Kosaka and Kosaka, 2005). These interneurons receive direct input from olfactory receptor neuron axons and synapse with the dendrites of mitral and tufted cells (Kosaka and Kosaka, 2005).

The organization of olfactory bulb interneurons into distinct layers is directly related to their function in the neural circuit, processing olfactory information (Zou et al., 2009). Interneurons in the glomerular layer receive synapses from olfactory receptor neuron axons and, in turn, synapse with the dendrites of mitral cells and tufted cells. In turn, granule cells established dendrodendritic synapses with excitatory neurons in the external plexiform layer. Consequently, the laminar allocation of interneurons largely determines their function within the neural circuits that underlie the processing of sensory information in the olfactory bulb.

Sources of Adult-Born Olfactory Bulb Interneurons

Olfactory interneurons are born remotely in the subpallium and reach their final destination through tangential migration (Altman, 1969; Belvindrah et al., 2009; Luskin, 1993). During embryonic stages, the olfactory bulb emerges as a protrusion of the rostral tip of the telencephalon that is continuous with the region of the subpallium that gives rise to its interneurons (Gong and Shipley, 1995). As development proceeds, however, interneurons must migrate increasing distances to reach their destination. Importantly, many interneurons continue to be generated through adulthood (Lois and Alvarez-Buylla, 1994), which poses a notable challenge for the transit of new inhibitory neurons to the olfactory bulb.

The origin of olfactory interneurons has been classically associated with the LGE, a region that was shown to contribute to the SVZ of the lateral ventricles in the postnatal telencephalon (Stenman et al., 2003; Wichterle et al., 2001). However, recent evidence indicates that the diversity of OB interneurons derives from a more extensive and heterogeneous germinal region than previously thought (Lledo et al., 2008). Genetic fate-mapping analyses have confirmed that the LGE is the main contributor to the adult SVZ. Thus, the majority of dividing cells in the SVZ derive from lineages expressing the subpallial marker Gsh2, and nearly 70% of the olfactory bulb interneurons emerge from these progenitors (Young et al., 2007). The remaining interneurons derive from a lineage of progenitor cells that express the transcription factor Emx1 and are therefore classically considered pallial derivatives (Young et al., 2007). However, this should be interpreted with caution because LGE progenitors may also contain low levels of Emx1 (Waclaw et al., 2009). Independently of their origin, $Emx1^+$ progenitors in the adult are located in the regions of the lateral ventricular wall facing the corpus callosum, from where neurosphere-forming stem cells have been obtained (Ventura and Goldman, 2007; Willaime-Morawek et al., 2006). Finally, a very small fraction of olfactory bulb interneurons (~1%) seem to derive from a lineage of SVZ progenitor cells that express the transcription factor Nkx2-1 (Young et al., 2007), a marker of the MGE.

LGE and pallial progenitors contribute differently to the diversity of olfactory bulb interneurons (Figure 5). For instance, periglomerular cells are produced by both sets of progenitors, although in different proportions. LGE-derived progenitors contribute many TH^+ interneurons and the large majority of CB^+ cells, whereas pallium-derived progenitors produce most CR^+ neurons (Kohwi et al., 2007; Stenman et al., 2003; Young et al., 2007). PV^+ interneurons in the external plexiform layer are also generated from both classes of progenitors, although most seem to derive from the LGE (Li et al., 2011). In the granular cell layer, most CR^+ interneurons develop from pallial progenitors, while the remaining cells are likely derived from the LGE (Kohwi et al., 2007; Merkle et al., 2007; Young et al., 2007).

Each population of olfactory bulb interneurons is produced in a unique temporal pattern and turnover rate (Lledo et al., 2008). This suggests that the neurogenic processes occurring during development and in the adult are not directly equivalent (De Marchis et al., 2007; Lemasson et al., 2005). Interestingly, bromodeoxyuridine (BrdU) labeling experiments revealed that the relative ratio of the different subtypes of olfactory bulb interneurons remains relatively constant from birth to adulthood, although they seem to be produced at different rates. For instance, CR^+ cells make up the largest proportion of newborn neurons in adult mice (Batista-Brito et al., 2008), while TH^+ and CB^+ periglomerular interneurons are produced to a lesser extent, and PV^+ interneurons are not significantly turned over in the adult (Kohwi et al., 2007; Li et al., 2011). It is presently unclear what physiological circumstances determine the precise turnover of the different classes of olfactory bulb interneurons in the adult.

Regional and Laminar Distribution of Adult-Born Olfactory Bulb Interneurons

The mechanisms controlling the migration of embryonic interneurons to the olfactory bulb resemble in many aspects that of cortical interneurons (Long et al., 2007) and will not be considered here in detail. However, the migration of interneurons to the olfactory bulb changes dramatically as the brain matures, because the brain parenchyma becomes progressively less permissive for migration. Adult-born interneurons migrate to the olfactory bulb through the rostral migratory stream (RMS), a highly specialized structure in which chains of migrating neuroblasts are ensheathed by astrocytes (Doetsch and Alvarez-Buylla, 1996; Jankovski and Sotelo, 1996; Lois et al., 1996; Thomas et al., 1996) (Figure 6). Interneurons migrate, crawling into each other in a process that is known as chain migration (Wichterle et al., 1997). Many factors have been shown to influence the tangential migration of olfactory neuroblasts through the RMS (reviewed in Belvindrah et al., 2009), but very little is known on the mechanisms that control the final distribution of newborn interneurons in the olfactory bulb.

Newborn interneurons seem to distribute uniformly throughout the rostrocaudal extent of the olfactory bulb (Lemasson et al., 2005). In contrast, interneurons target a specific layer within the olfactory bulb, according to their fate, in a process that is likely determined at the time of their specification. In agreement

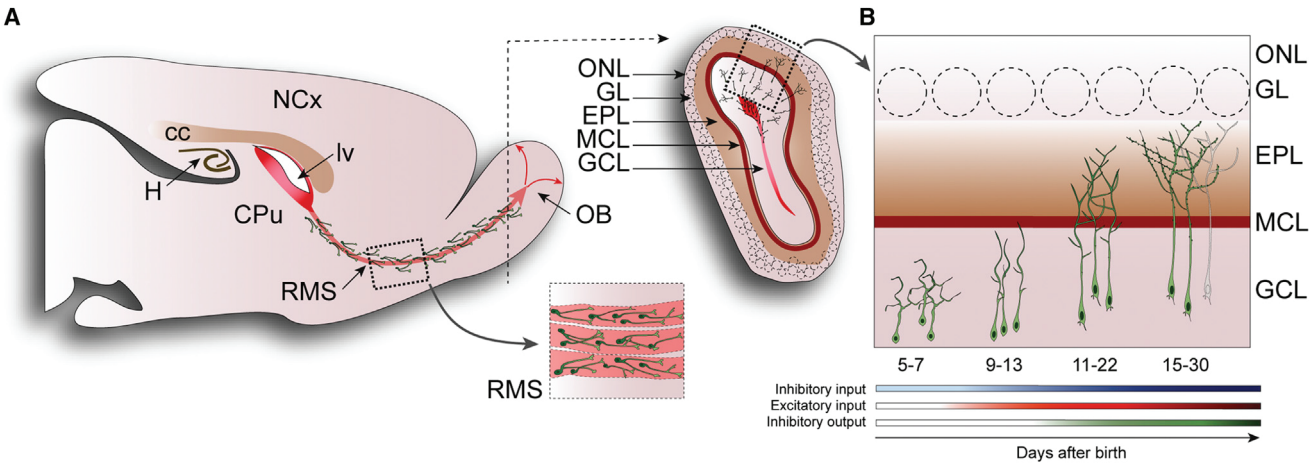

Figure 6. Integration of Adult-Born Interneurons into the Olfactory Bulb
(A) Schematic of sagittal section through the mouse brain illustrating the migration and integration of adult-born GABAergic interneurons into the olfactory bulb. Olfactory bulb interneurons are produced in the SVZ and reach the olfactory bulb though the rostral migratory stream (RMS).
(B) Schematic of a coronal section depicting the laminar organization of the adult olfactory bulb. The inset illustrates different stages in the maturation of granule cells, from their arrival to the olfactory bulb to their integration into functional circuits. The numbers refer to their approximate age in days. ONL, olfactory nerve fiber layer; GL, glomerular layer: EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer; cc, corpus callosum; CPu, caudoputamen nucleus; H, hippocampus; lv, lateral ventricle; NCx, neocortex.

with this notion, overexpression of the transcription factor Pax6 in migrating neuroblasts promotes their differentiation to periglomerular TH$^+$ cells at the expense of other interneuron classes (Hack et al., 2005). These results reinforce the view that the laminar allocation is largely linked to the fate of cells originating from different progenitor cells. Since granular and periglomerular interneurons play very distinct roles in the processing of olfactory information (Chen and Shepherd, 2005; Shepherd et al., 2007), the precise targeting of these cells to their appropriate layer seems critical for the function of the olfactory bulb.

Important differences seem to exist in the mechanisms underlying the laminar distribution of cortical and olfactory bulb interneurons. First, olfactory bulb interneurons reside in layers that lack projection neurons, which is in sharp contrast to most of their neocortical counterparts (with the exception of cortical layer I). This suggests that the hypothetical mechanism proposed to regulate the allocation of most neocortical interneurons is unlikely to apply in the olfactory bulb. Second, adult-born interneurons reach their final position by traversing a territory that is largely populated by fully mature, differentiated neurons. This indicates that the mechanisms regulating the integration of interneurons into their appropriate target layer in the olfactory bulb are maintained through adulthood, at least for periglomerular and granule cells.

Reelin is the only factor identified to date that seems to influence the laminar positioning of olfactory bulb interneurons. In contrast to the cerebral cortex, where Reelin regulates the distribution of pyramidal cells and only affects the location of GABAergic interneurons in a non-cell-autonomous manner (Pla et al., 2006), this glycoprotein seems to directly control the migration of olfactory bulb interneurons. Indeed, mitral and tufted cells adopt their final position independently of this signaling system (Devor et al., 1975). Conversely, Reelin produced by these cells is required for interneurons to detach from the RMS and adopt their normal laminar position (Hack et al., 2002; Hellwig et al., 2012). In *reeler* mutants, for example, some TH$^+$ and CB$^+$ interneurons fail to reach the glomerular layer and instead reside in the external plexiform layer; some defects have also been reported in the distribution of CR$^+$ interneurons in the granular layer (Hellwig et al., 2012). Nevertheless, the position of PV$^+$ interneurons in the external plexiform layer, and most periglomerular interneurons, is unaffected by the loss of Reelin signaling, which suggests that the correct laminar distribution of olfactory bulb interneurons depends on additional factors. Consistent with this idea, a population of glial cells located in the olfactory nerve layer, the olfactory ensheathing cells, releases a chemoattractive activity that attracts migrating neuroblasts in vitro (Zhu et al., 2010). This suggests that olfactory ensheathing cells may contribute to regulate the radial distribution of interneurons in the surface of the olfactory bulb.

Functional Integration of Adult-Born Interneurons

As in the developing cortex, the integration of interneurons in the olfactory bulb also seems under the influence of activity-dependent mechanisms. Migrating neuroblasts are sensitive to the action of neurotransmitters, although they seem to exert different effects than in the cortex. There are no specific studies on the expression of chloride transporters in adult-born interneurons, but analysis of their expression in early postnatal stages suggests that interneurons lack KCC2 when they arrive to the olfactory bulb (Mejia-Gervacio et al., 2011). Consequently, interneurons terminate their migration in the olfactory bulb in an environment with a high concentration of ambient GABA and under depolarizing conditions. Intriguingly, neuroblast migration is reduced by the tonic depolarizing action of GABA acting on $GABA_A$ receptors (Bolteus and Bordey, 2004; Mejia-Gervacio et al., 2011). These results, which contrast the proposed role for hyperpolarizing GABA as a stop signal for cortical interneurons, reveal that the function of ambient neurotransmitters in

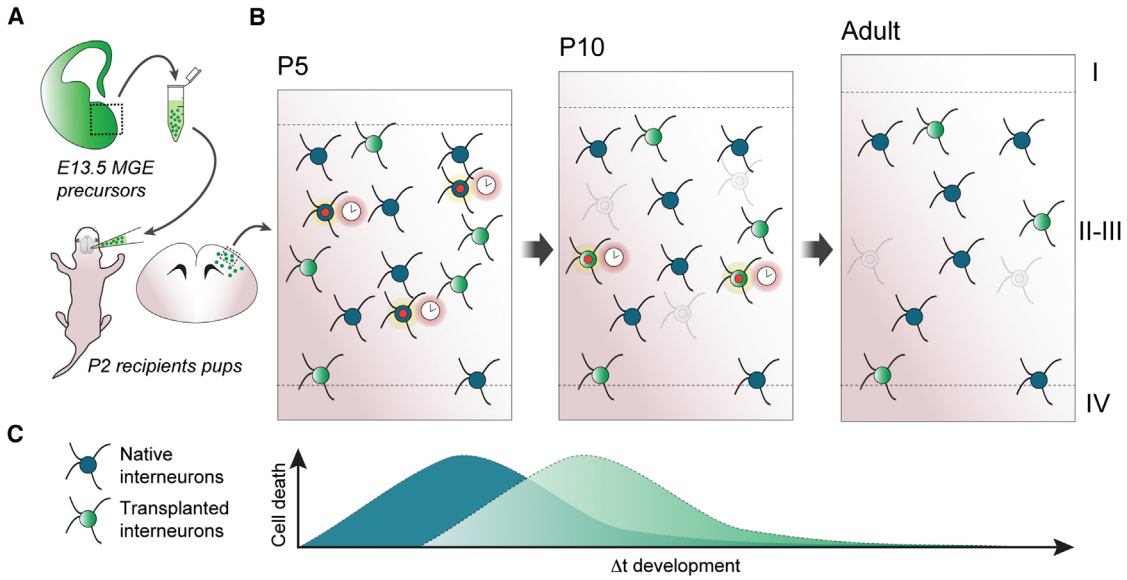

Figure 7. Intrinsic Developmental Cell Death of Cortical GABAergic Interneurons

(A) Schematic diagrams of the experimental paradigm used to study the programmed cell death of cortical interneurons (Southwell et al., 2012). MGE donor cells from GFP-expressing embryos were transplanted into the neocortex of early postnatal recipient mice, and their number and distribution were analyzed several days later, together with the native interneuron population.

(B) Schematic diagrams of coronal sections through the neocortex of transplanted mice at three different time points during postnatal development. Approximately 40% of interneurons (native and transplanted) undergo programmed cell death during early postnatal development. However, each population of interneurons undergoes cell death (red nucleus indicates active caspase-3) according to an internal clock that depends on the actual age of the interneurons, rather than according to environmental influence. Since transplanted interneurons (dark green) were moved forward in development, they undergo programmed cell death several days later than the native population (light green). The time window of cell death largely overlaps with the period of intense synaptogenesis, suggesting that the survival of interneurons might be linked to their recruitment into circuits.

(C) The temporal windows of neuronal cell death for the native (blue) and transplanted (green) interneurons are out of phase due to heterochronic transplantation. MGE, medial ganglionic eminence; I–IV, cortical layers I to IV.

the functional integration of GABAergic interneurons is more complex than previously thought.

Several studies have analyzed in detail the maturation and integration of adult-born interneurons into the olfactory bulb (Figure 6). The synaptic integration of newborn interneurons occurs over a period of approximately 3 weeks (Petreanu and Alvarez-Buylla, 2002), although newborn neurons already receive glutamatergic and GABAergic synapses within 24 hr after leaving the RMS (Katagiri et al., 2011; Panzanelli et al., 2009). As interneurons progressively settle into their final position, they acquire functional properties that make them indistinguishable from preexisting neurons (Belluzzi et al., 2003; Carleton et al., 2003). Interestingly, the majority of functional outputs from newborn interneurons at the end of their integration period and their characteristics do not seem to change over time (Bardy et al., 2010). In contrast, glutamatergic inputs onto newborn interneurons display enhanced plasticity during this period of maturation (Nissant et al., 2009), which may provide a basis for adult neurogenesis-dependent olfactory learning.

General Principles in the Integration of Embryonic and Adult GABAergic Interneurons

There are a number of emerging concepts that can be extracted from our current understanding of the mechanisms controlling the integration of GABAergic interneurons into the developing neocortex and in the mature olfactory bulb. In particular, it seems clear that many of the features that distinguish the different classes of GABAergic interneurons, such as their intrinsic properties and perhaps even their final allocation, are intrinsically determined.

Intrinsic Developmental Programs

Several stages in the development of GABAergic interneurons, both in the cerebral cortex and the olfactory bulb, seem to be regulated by the execution of a maturational program intrinsic to inhibitory neurons. In other words, the behavior of interneurons at any given time in development is better predicted by their cellular age than by changes in the local environment. Since interneurons are born asynchronously, this implies that the developing cerebral cortex contains a mixture of interneurons at diverse stages of maturation. These differences are obviously exaggerated in the olfactory bulb, where adult-born interneurons coexist with interneurons that were generated in the embryo.

The existence of an intrinsic maturational program in GABAergic interneurons predicts that interneurons born at different times would behave differently within the same environment. This has been observed, for example, in relation to the settlement of interneurons in the cortical plate. Birthdating analyses have shown that not all interneurons switch from tangential to radial migration simultaneously in response to a common trigger. Instead, interneurons invade the cortical plate when they are between 6 and 8 days old; therefore, early-born interneurons enter the cortical plate before late-born interneurons (López-Bendito

et al., 2008) (Figure 2). This indicates that the switch from tangential to radial migration is largely determined by the age of interneurons. Consistent with this idea, many late-born (embryonic day 15.5, E15.5) interneurons transplanted into E12.5 embryos settle in deep layers of the cortex instead of their normal superficial location (Pla et al., 2006), probably because under these circumstances they stop responding to the cues that support their tangential migration at the same time as early-born (12.5) interneurons, which settle in deep layers of the cortex. The intrinsic developmental program may therefore influence the settlement of interneurons in the cortex by regulating the responsiveness of each cohort of interneurons to cues present in the cortex.

Transplantation experiments have also revealed that the death of cortical interneurons in the early postnatal cortex might also be under intrinsic control (Figure 7). Southwell and colleagues (2012) observed that many cortical interneurons undergo programmed cell death in vivo between postnatal day 7 (P7) and P11 in vivo, when interneurons are between 11 and 18 days old. When transplanted into older cortices (P3), interneurons undergo programmed cell death later than normal (~P15), which demonstrates that this process is intrinsically linked to the cellular age of interneurons. Consistently, cortical interneurons undergo programmed cell death in vitro with the same temporal dynamics as in vivo (Southwell et al., 2012). In the adult olfactory bulb, interneurons also die within a well-defined temporal window, approximately 15–30 days after birth (Petreanu and Alvarez-Buylla, 2002).

Further evidence supporting the existence of an intrinsic clock that controls the maturation of these cells comes from the analysis of their modulation of ocular dominance plasticity. During a critical period in the postnatal development of the visual cortex, visual experience influences the organization of thalamocortical axon terminals to produce alternating ocular dominance domains (Hensch, 2005). Occlusion of one eye during this period triggers a rapid reorganization of thalamic terminals in the cortex, a process that is regulated by inhibitory neurotransmission. In mice, ocular dominance plasticity peaks between P26 and P28, when interneurons are roughly between 33 and 35 days of age. Transplantation of interneuron precursors into the postnatal cortex reopens the critical period of ocular dominance plasticity when transplanted interneurons reach a cellular age equivalent to that of endogenous inhibitory neurons during the normal critical period (Southwell et al., 2010).

Recent efforts to derive cortical interneurons from human pluripotent stem cells (hPSCs) or human-induced pluripotent stem cells (hiPSCs) have also emphasized the ability of these cells to differentiation according to an intrinsic program of maturation. Both in vitro and after transplantation into the rodent cortex, human GABAergic interneurons derived from hPSCs or hiPSCs mature following a protracted timeline of several months, thereby mimicking the endogenous human neural development (Maroof et al., 2013; Nicholas et al., 2013). Altogether, these findings suggest that multiple aspects of the integration of interneurons in cortical networks are regulated by the execution of a maturational program intrinsic to inhibitory neurons.

Adjusting Inhibition

Several mechanisms dynamically adjust the balance between excitation and inhibition in the adult brain (Haider et al., 2006; Turrigiano, 2011). However, it is likely that developmental programs are also coordinated to play an important role in this process. Indeed, the relative density of pyramidal cells and interneurons remains relatively constant from early stages of corticogenesis, when both classes of neurons are still migrating to their final destination (Sahara et al., 2012). One possibility is that the generation of both classes of neurons is coordinated through some kind of feedback mechanism that balances proliferation in the pallium and subpallium. Alternatively, the production of factors controlling the migration of GABAergic interneurons to the cortex might be proportional to the number of pyramidal cells generated. For example, it has been shown that cortical intermediate progenitor cells (IPCs) produce molecules that are required for the normal migration of interneurons (Tiveron et al., 2006), and mutants with reduced numbers of IPCs have a deficit in cortical interneurons (Sessa et al., 2010).

Cell death is another prominent factor regulating neuronal incorporation during development (Katz and Shatz, 1996; Voyvodic, 1996). It has long been appreciated that a sizable proportion of inhibitory neurons is eliminated from the cerebral cortex through apoptosis during the period of synaptogenesis (Miller, 1995), and recent work estimated that approximately 40% of the interneurons in the cortex perish around this time (Southwell et al., 2012). Similarly, only about half of the adult-born granule cells survive more than a few days after reaching the olfactory bulb (Petreanu and Alvarez-Buylla, 2002).

The mechanisms controlling the death of newborn olfactory bulb interneurons have been studied with some detail. There seems to exist a critical period during which sensory activity influences the survival of newborn interneurons (Kelsch et al., 2009; Yamaguchi and Mori, 2005), which largely overlaps with the period when interneurons become synaptically integrated into the olfactory bulb (15–30 days after birth). During this period, interneurons arriving to the olfactory bulb (i.e., roughly born at the same time) compete for survival, probably because newborn interneurons are more sensitive to the overall activity of nearby circuits than mature olfactory interneurons. In agreement with this idea, interneurons that survived this period tend to persist for life (Winner et al., 2002). Thus, both the synaptic integration and the survival of newborn interneurons seem to depend on sensory activity mechanisms, which are intrinsically linked to the cell excitability. Consistent with this, synaptic development and survival of newly generated neurons are dramatically impaired in anosmic mice (Corotto et al., 1994; Petreanu and Alvarez-Buylla, 2002), while sensory enrichment promotes the survival of newborn olfactory interneurons (Bovetti et al., 2009; Rochefort et al., 2002). Moreover, increasing cell-intrinsic excitability in maturing granule cells enhances their synaptic integration and partially rescues neuronal survival in a sensory-deprived olfactory bulb (Kelsch et al., 2009; Lin et al., 2010), while forced hyperpolarization decreases survival (Lin et al., 2010). Since most interneurons have already matured and received connections by the time they die, it has been hypothesized that only interneurons connected to active circuits would ultimately survive (Petreanu and Alvarez-Buylla, 2002), an idea that has

obtained experimental support in the adult dentate gyrus (Kee et al., 2007). Thus, the death of adult-born interneurons seems to be intimately linked to mechanisms of structural plasticity in the olfactory bulb.

It is presently unclear whether programmed cell death in developing cortical interneurons depends on similar mechanisms than in the olfactory bulb, but recent experiments pointed out an interesting parallel between both structures. Southwell and colleagues (2012) found that heterochronically transplanted interneurons do not influence cell death dynamics in the endogenous population (Figure 7). This seems to suggest that the competition for survival is normally restricted to cortical interneurons born roughly at the same time, as in the olfactory bulb. Thus, it is conceivable that cell death selectively eliminate inappropriately integrated cortical interneurons within specific lineages, although this hypothesis remains to be experimentally tested. In any case, these results reinforce the view that the integration of interneurons into cortical networks critically depends on a maturational program linked to their cellular age.

A Look Ahead

Much progress has been made over the past years regarding our understanding of the mechanisms regulating the migration of embryonic and adult-born GABAergic interneurons. However, our understanding of the integration of these cells into functional circuits in the cerebral cortex and olfactory bulb, respectively, is very limited. We know basically nothing about the mechanisms through which interneurons adopt their precise laminar distributions and how this process influences functional connectivity patterns between interneurons and pyramidal cells. Recent work has led to the suggestion that SST^+ and PV^+ interneurons connect promiscuously to nearby pyramidal cells (Fino and Yuste, 2011; Packer and Yuste, 2011); therefore, the connectivity maps of interneurons could simply result from the overlap of axonal and dendritic arborizations between both cell types (Packer et al., 2012). According to this principle, the laminar allocation of interneurons might be irrelevant for their functional integration into cortical networks, i.e., similar interneurons located in different layers might be interchangeable. On the other hand, it is well established that different classes of interneurons receive distinct excitatory and inhibitory laminar input patterns (Xu and Callaway, 2009; Yoshimura and Callaway, 2005). In agreement with this notion, a remarkable degree of specificity in the cellular selection of postsynaptic targets for at least some classes of interneurons seems to exist. For example, layer IV neurogliaform and SST^+ interneurons selectively target local PV^+ basket cells while largely avoiding pyramidal cells in this layer (Chittajallu et al., 2013; Xu et al., 2013). In contrast to the promiscuous view of cellular targeting by cortical interneurons (Packer et al., 2012), these observations suggest that the fine-scale connectivity of cortical networks might be directly influenced by the appropriate laminar allocation of interneurons. Future experiments should contribute to solve this apparent paradox.

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DISCUSSION

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Complex brain circuitries comprise hierarchical networks of excitatory and inhibitory neurons. GABAergic interneurons play an important role in modulating the excitatory output of pyramidal cells and they have a critical task in providing inhibition, synchronizing and shaping several types of cortical oscillations (McBain and Fisahn, 2001; Somogy and Klausberger, 2005). Understanding how GABAergic interneurons integrate into cortical networks is crucial to decipher the functioning of the cerebral cortex.

In mammals, cortical interneurons originate from a different progenitor region than pyramidal cells (Anderson et al 1997; De Carlos et al 1996; Fogarty et al 2007; Lavdas et al 1999; Sussel et al 1999; Tamamaki et al 1997; Wichterle et al 1999; Wichterle et al 2001). Thus, interneurons migrate a long distance before reaching their destination in the cortex, a process that involves a complex set of guidance cues. Disruption of this process leads to defects in the organization of the adult cortex and is likely linked with several neuropsychiatric disorders. Deciphering the mechanisms that control the guided migration of interneurons from their origin until their final location is therefore essential to understand how the cerebral cortex develops in health and disease.

Much progress has been made in understanding the development of cortical interneurons, in particular regarding their specification and migration (Marín and Rubenstein, 2003, Wonders and Anderson, 2006). Numerous studies have provided insights into the mechanisms controlling the tangential migration of interneurons to the cerebral cortex (Marín et al., 2001; Powell et al., 2001; Flames et al., 2004; Pozas and Ibañez, 2005; Zimmer et al., 2008; Hernández-Miranda et al., 2011, Lopez-Bendito et al., 2008, Sanchez-Alcañiz et al., 2011). However, little is known about the mechanisms regulating the integration of interneurons into the cortex and their final positioning in specific cortical layers.

In this Thesis we have attempted to shed some light into the mechanisms that regulate the intracortical migration of cortical interneurons, in particular as they colonize the cortical plate. We have also investigated the mechanisms controlling the laminar positioning of interneurons. Insights from our results are discussed in the following sections.

Nrg3 regulates interneuron invasion of the cortical plate

Previous studies have shown that Nrg1 influence multiple processes during neural development, including radial (Anton et. al, 1997, Schmid et al., 2003) and tangential neuronal migration (Yau et al., 2003, Flames et al. 2004). Here, we have found that Nrg3 regulates the migration of MGE-derived interneurons toward the CP. The function of Nrg3 is mediated by the ErbB4 receptor, which has been suggested to be the only receptor for this neuregulin (Zhang et al., 1997).

In agreement with previous studies (Li et al., 2012), we found that Nrg3 is expressed in the CP throughout development, from very early stages in cortical development to postnatal stages. Both in vitro and in vivo experiments are consistent with a model in which Nrg3 attracts MGE-derived interneurons into the developing CP. This is in sharp contrast with the conclusions of Li and colleagues (2012), who suggested that both Nrg1 and Nrg3 function as chemorepellent factors for migrating MGE interneurons. The explanation of these diverse results may derive from technical differences on how the experiments were performed by the two groups. In the co-culture experiments, for example, Li and colleagues transfected COS cells with a plasmid only encoding the EGF-like domain of Nrg3 and not the full-length protein. In our experiments, Nrg3 consistently induces chemoattractive responses both in vitro and in vivo, and these effects are mediated by ErbB4.

Analysis of Nrg3 and ErbB4 mutants revealed strikingly similar phenotypes in the distribution of MGE-derived interneurons, which suggest that Nrg3-ErbB4 signaling is responsible for these effects. In particular, it seems that the loss of ErbB4-Nrg3 signaling either delays interneuron maturation or their entry into the developing CP. As a consequence, interneuron positions tend to shift to progressively more superficial layers, where they accumulate.

It is interesting to note that the distribution defects in PV+ interneurons were subtler in *Nex-Cre;Nrg3* mutants than in *Lhx6-Cre; ErbB4* mutants. It is therefore possible than molecules other than Nrg3 may contribute to regulate this process by activating ErbB4 receptors. For example, type III Nrg1 is also present in the postnatal cortex, and may perhaps partially compensate Nrg3 function in *Nex-Cre;Nrg3* mutants. In addition to Nrg1 and Nrg3, three other neuregulins can bind ErbB4, Nrg2, Nrg4 and Nrg5 (Mei and Nave,

2014). Possibly, when Nrg3 is missing, one of these neuregulins could contribute to compensate its function.

The difference between the distribution in layers of ErbB4+ cells and PV+ cells in the *Nex-Cre; Nrg3* mice is most likely due to the fact that not all ErbB4+ cells are PV+, i.e., in the somatosensory cortex roughly 80% of ErbB4+ cells are PV+ (Fazzari et al., 2010). Thus, the defect we observed in ErbB4+ cells may reflect the contribution of other interneuron classes, such as Cr+, Sst+ and Cck+ interneurons (Vullhorst et al., 2009; Chen et al., 2010; Fazzari et al., 2010; Wen et al., 2010; Abe et al., 2011; Neddens et al., 2009; Ting et al., 2011).

Nrg3 functions as a membrane bound factor

Alternative promoter usage in the *Nrg1* gene gives rise to different Nrg1 isoforms with distinct trans-membrane topology and extracellular domains (Tan et al., 2007, Harrison et al., 2006). In particular, Nrg1 can exist in soluble or membrane-bound forms. Type I, II and IV Nrg1 isoforms are single-pass transmembrane proteins that lead to soluble fragments containing the active EGF-like domain. By contrast, type III Nrg1 contains an additional transmembrane domain that keeps the protein attached to the membrane even after is cleavaged (Meyer et al., 1997, Wang et al., 2001). It has been shown that *Nrg3* gene have many similarities with *Nrg1*, although there are also several differences between the two sequences (Zhang et al., 1997). For example, similarly to Nrg1, the C-terminal of Nrg3 acts as a transmembrane domain, while the N-terminal may act as an internal signal sequence. In contrast, the extra-cellular domain of Nrg3 is devoid of Ig-like domains, and, in addition, the EGF-like domain of Nrg3 is different from that of Nrg1 (Zhang et al., 1997).

Biochemical experiments have not clarified the structure of functional Nrg3 as a membrane bound or secreted molecule. Our experiments demonstrate that Nrg3 functions as a chemoattractant for interneurons at a short distance, mimicking the effect of type III Nrg1 (CRD-Nrg1), the membrane bound isoform of Nrg1. This led us to suggest that Nrg3 functions as a membrane bound protein in vivo, controlling cell migration over relatively short distances. It is worth noting, however, that COS cells may lack the enzymes required for the normal processing of Nrg3. This is unlikely, since similar assays were used to demonstrate the long-range effect of Nrg1 on MGE-derived cells (Flames et al., 2004; this Thesis).

A hierarchical organization of guidance cues

The final stages of intracortical dispersion of interneurons depend on the tangential to radial switch in the migratory mode. The mechanisms coordinating this switch remain largely unknown. A series of isochronic and heterochronic transplant experiments have demonstrated that interneurons with different birthdates remain within the tangential migration streams for a similar amount of time (Lopez-Bendito et al. 2008). The temporal regulation of the loss of responsiveness to Cxcl12 signaling seems to be critical for this process since interneurons that radially invade the CP no longer respond to Cxcl12 signaling (Li et al., 2008, Sanchez-Alcañiz et al., 2011). These observations suggest that interneurons might undergo time-dependent and synchronized maturation to coordinate the tangential to radial switch and their entry into the developing CP.

Experiments in this Thesis and previous studies have shown that the loss of Cxcl12 signaling is sufficient to induce interneurons to change their mode of migration from tangential to radial (Abe et al., 2015; Vogt et al., 2014; Liapi et al., 2006, Tiveron et al., 2006, Lopez-Bendito et al., 2008, Li et al. 2008, Tanaka et al., 2009, Lysko et al. 2011, Wang et al. 2011). Analysis of Cxcr4 and Cxcr7 mutants, in which interneurons accumulate prematurely in the CP (Tiveron et al., 2006; Li et al., 2008; Lopez-Bendito et al., 2008; Sanchez-Alcañiz et al., 2011; Wang et al., 2011), suggests that this area of the developing cortex contains a chemoattractive activity for interneurons because, in the absence of Cxcl12 signaling, interneurons enter in the CP as soon as they reach the cortex. Since interneurons normally tend to avoid the CP during embryonic stages, the different chemoattractive activities present in the embryonic cortex must be hierarchically organized. In fact, here we have shown that interneurons can in principle respond to both Cxcl12 and Nrg3 simultaneously but they prefer Cxcl12 over Nrg3 while both signals are present. These observations suggest a model by which interneurons first follow a gradient of Cxcl12 that is masking the expression of Nrg3 in the developing CP, until interneurons lose their responsiveness to the chemokine. At this point, they start to respond to Nrg3 and occupy the CP. This is consistent with the cellular function attributed to Cxcl12, which minimizes the potential of interneurons to sense cues outside the tangential streams by reducing their branching frequency (Lysko et al., 2011).

How do interneurons stop responding to Cxcl12 is still unknown. Here we have shown that although Cxcl12 is still expressed in the MZ even postnatally (Stumm & Höllt 2007), its receptor Cxcr4 downregulate its expression during development. This suggests

that interneurons may turn off *Cxcr4* expression within 24-48 hour in the cortex, perhaps in response to a signal present in the cortex or as part of an internal program. The fact that cortical interneurons might follow an intrinsically determined developmental program is supported by findings on the mechanisms controlling the maturation and death of interneurons (Southwell et al., 2010, 2012), but additional experiments would be required to directly address this possibility. Another mechanism that would explain the loss of Cxcl12 responsiveness is that interneurons that invade the cortex block their response to the chemokine through an internal switch, i.e., without downregulating the expression of Cxcr4. In the cerebellum it has been shown that granule cells terminate their tangential migration by blocking the Cxcl12/Cxcr4 signaling pathway through a mechanisms that involves EphrinB/Eph function (Lu et al 2001). It remains to be explored whether this or a similar mechanism may regulate the tangential to radial switch in the migration of interneurons in the cerebral cortex.

How do interneurons reach their final location in the cortex?

One of the objectives of this Thesis was to elucidate the mechanisms that regulate the layering of interneurons in the cerebral cortex. This process occurs during the first postnatal days (Hevner et al., 2004; Pla et al., 2006; Miyoshi & Fishell, 2011), and it is likely to be regulated by mechanisms different from those that recruit interneurons within the CP.

In the last years several studies have clarified some aspects about the regulation of the final steps of interneuron migration. Thus, it is well established that MGE-derived interneurons occupy cortical layers following an inside-out pattern of migration in a similar way to that used by pyramidal cells, with early-born cells populating lower layers and late-born cells populating upper layers of the cortex (Miller, 1985; Fairen et al., 1986; Valcanis & Tan, 2003; Pla et al., 2006). This observation suggests that the laminar allocation of cortical interneurons might be linked to their birthdate, and that interneurons may use similar mechanisms than pyramidal cells to adopt their final position (Kriegstein & Noctor, 2004). However, CGE-derived interneurons tend to populate supragranular layers of the cortex irrespective of their birthdate (Miyoshi et al., 2010). This seems to indicate that the time of neurogenesis is not always linked with the process of laminar acquisition, at least for some classes of interneurons.

One hypothesis that would explain how coetaneous MGE-derived interneurons and pyramidal cells end up in the same layers of the cortex is that interneurons follow specific classes of pyramidal cells to their final destination (Hevner et al., 2004; Pla et al., 2006; Lodato et al., 2011). In this model, interneurons would express genes encoding proteins that allow them to respond to cues provided by pyramidal cells in a complementary receptor-ligand pattern, so that interneurons would follow factors expressed by pyramidal cells to end up their journey. According to this idea, early-born MGE-derived interneurons would follow cues provided by infragranular pyramidal cells, whereas late-born interneurons would preferentially interact with supragranular pyramidal cells. Two experimental observations are consistent with this hypothesis. First, interneurons start to allocate in their final laminar position after coetaneous pyramidal cells (Pla et al., 2006, Miyoshi & Fishell, 2011). Second, disrupting the layering of pyramidal cells disrupts the laminar allocation of MGE-derived interneurons (Pla et al., 2006, Ramos et al., 2006, Lodato et al., 2011).

Mechanisms regulating the laminar allocation of MGE interneurons: Cx3cl1

In this thesis, we search for candidate genes differentially expressed by late born MGE interneurons when they begin migrating into the corresponding superficial layers of the cortex. To this end, we used an unbiased approach to identify genes that are differentially expressed in late born interneurons before and after CP entry. Among those genes upregulated by interneurons at early postnatal stages, we focused our attention on the chemokine Cx3cl1, also known as Fractalkine.

Cx3cr1 is the receptor for Cx3cl1, and it has been shown to be expressed by microglia, which suggests a role for neuronal-microglia interactions in this process (Nishiyori et al., 1998). Previous studies have shown that microglia regulate the laminar positioning of cortical interneurons (Squarzoni et al., 2014). Our study reveals for the first time that Cx3cl1 is expressed in MGE interneurons, and, particularly, that is more abundantly expressed at the time when interneurons adopt their final laminar position. Previous studies have shown that Cx3cl1 is expressed in the brain, in particular in the olfactory bulb, cerebral cortex, hippocampus, amygdala and basal ganglia (Tarozzo et al., 2003).

Several functions have been attributed to Cx3cl1/Cxrcr1signaling. For example, it is well known that microglial cells regulate neuronal cell death during CNS development (Bessis

et al., 2007). However, microglia can also promote neuronal survival in the postnatal forebrain. So, microglial cells have been shown to promote survival of layer 5 pyramidal neurons of the motor cortex during the first postnatal week (Ueno et al., 2013). In addition, it has been suggested that Cx3cl1/Cx3cr1 signaling also promotes adult neurogenesis of the hippocampus. In fact, it has been shown that genetic disruption of Cx3cr1 reduces cellular proliferation in the subgranular zone of the dentate gyrus (Bachstetter et al., Rogers et al., 2011). Moreover, in the adult mouse hippocampus, microglia regulate the number of immature neurons maintained in the subventricular zone by phagocytosis (Sierra et al., 2010, 2013).

Besides their roles on neuronal cell death, survival and adult neurogenesis, microglia have been shown to contribute to synaptogenesis and synaptic remodeling. In fact, microglia, through the production of BDNF, promotes synapse formation (Parkhurst et al., 2013). Microglia also contributes to postnatal synaptic pruning (Paolicelli et al., 2011; Schafer et al., 2012, 2013; Kettenmann et al., 2013) and synaptic refinement in the hippocampus (Paolicelli et al., 2011; Zhan et al., 2014). Finally, microglia function have been linked to the maturation of thalamocortical synapses. In fact, reduced density of microglia cells in the somatosensory neocortex of *Cx3cr1* mutant mice has been shown to impact on the normal maturation of thalamocortical synapses (Hoshiko et al., 2012).

The function of Cx3cl1 in MGE interneurons has never been studied before. In this Thesis we analyzed the distribution of cortical interneurons in Cx3cl1 mutant mice in two different backgrounds, C57BL/6 and FVB. Cx3cl1 mutants do not seem to have any particular defect in the C57BL/6 genetic background (Cook et al., 2001), whereas mutant mice developed serious seizures and aggressive behavior at approximately two months old of age FVB background (unpublished observations). We did not observe any abnormality in the laminar distribution of interneurons in the cortex in any of the two backgrounds.

Additional experiments should be performed to discard a role for Cx3cl1 in the development of cortical interneurons. First, both pyramidal cells and interneurons express Cx3cl1 (data not shown). Thus, it would be ideal to perform conditional loss of function experiments in which Cx3cl1 is deleted from interneurons only. To this end, we could use a conditional mouse or another genetic tool, such as shRNAs or microRNAs designed against Cx3cl1 and targeted specifically for MGE interneurons. The genetic deletion of Cx3cl1 from both pyramidal cells and interneurons in *Cx3cl1* mutant mice may mask the functions that this chemokine might exert in these different cell populations. Moreover, in

confrontations experiments we observed that Cx3cl1 has a long-range chemoattractant function for interneurons (data not shown). We cannot predict if interneurons attract other subtypes of interneurons through the expression of Cx3cl1, or if pyramidal cells expressing Cx3cl1 attract interneurons that express the same chemokine. In both cases (interneurons vs. interneurons or pyramidal cells vs. interneurons) this process might be regulated by homophilic interactions between chemokines. We exclude an interaction between pyramidal cells and interneurons through the Cx3cl1 receptor because Cx3cr1 is exclusively expressed by microglia (Nishiyori et al., 1998, Jung et al., 2011).

It is worth noting that Cx3cl1 exists in two forms, soluble and membrane-bound. Unlike classic small peptide chemokines, Cx3cl1 is synthesized as a transmembrane protein (Bazan et al., Pan et al., 1997) that can promote integrin-independent adhesion in brain inflammation (Fong et al., 1998, Haskell et al., 1999). Subsequent cleavage by metalloproteases such as ADAM10 and ADAM17/ TACE (Hundhausen et al., 2003, Garton et al., Tsou et al., 2001), either constitutive or induced, generates soluble Cx3cl1 with potential chemoattractive activity. However, the specific contribution of the membrane-bound versus soluble Cx3cl1 isoforms remain to be analyzed. For example, we could take advantage of the BAC transgenic mice generated by Kim and colleagues (2011) that express either normal or an obligatory secreted chemokine variant in a Cx3cl1-deficient genetic background.

Mechanisms regulating the laminar allocation of MGE interneurons

The analysis of the microarrays experiments revealed a set of candidate genes whose expression is either up- or downregulated in MGE-derived interneurons as they enter the CP. Some of these genes are logical candidates to regulate the lamination of cortical interneurons, but functional experiments should be performed to identify their specific function in this process.

We identified several cadherins and protocadherins to be expressed in early postnatal MGE interneurons. For instance, we found Cdh7 and Cdh9 to be more expressed in MGE interneurons at the time of CP invasion than during their tangential spread. Cadherins have been linked to many functions in the developing brain. While many classic cadherins promote axon extension, others appear to confine axon growth and targeting. In particular, Cdh7 has been shown to regulate the wiring of the cerebellum by controlling two sequential steps, axonal growth termination and synaptic specificity (Kuwako et al., 2014).

Also, it has been shown that Cdh9 is required for selective targeting and synapse formation between hippocampal dentate gyrus (DG) granule neurons and CA3 pyramidal neurons (Williams et al., 2011). The molecular basis for such selective synaptic partnering involves Cdh9, as loss of this cadherin from either DG neurons or CA3 neurons disrupted mossy fiber targeting and synapse formation. In addition to affecting axon outgrowth, classic cadherins can also act as a "stop signal" to direct ingrowing axons to their correct terminal target layer or region (Yamagata, Herman and Sanes, 1995, Inoue and Sanes, 1997). For example, in the mammalian visual system, RGC axons in Cdh6-deficient mice fail to innervate their appropriate visual targets, but instead project to inappropriate visual nuclei (Osterhout et al., 2011). It has also been shown that dynamic trafficking of N-cadherin regulates migration of neocortical glutamatergic neurons along radial glial guides (Kawauchi et al., 2010). Additionally, it has been suggested that cortical GABAergic interneurons require N-cadherin for both tangential migration to the neocortex and for the subsequent radial migration (Luccardini et al., 2013). In fact, it has been shown that N-cadherin ablation in postmitotic MGE derived interneurons delays tangential migration and CP invasion. Altogether, these observations suggest that Cdh7 and Cdh9 may play important roles in the cerebral cortex, in particular, in the regulation of the final steps of interneurons migration.

Some members of the Pcdh family have also been implicated in axon targeting. For example, deletion of the constant region of the α-Pcdh gene cluster in mice leads to abnormal targeting of olfactory axons to their appropriate glomeruli in the olfactory bulb (Hasegawa et al., 2008). Other studies in mice have shown that genetic deletion of Pcdh-10 (a δ-Pcdh) impairs striatal axon outgrowth and impedes the thalamocortical projection through the ventral telencephalon (Uemura et al., 2007). One of the genes that we found in our screening is Pcdh11x, but its function in brain development is still controversial. It has been mainly linked to the late onset of Alzheimer disease (Carrasquillo et al., 2009; Miar et al., 2011) and as a determinant of cerebral asymmetry (Priddle at al., 2013), but it is unclear if it is plays others roles in brain development.

Others genes that we have been found in our list are Robo2 and Slit2, which have been described to be crucial for many functions in brain formation, including proliferation (Borrell et al., 2012) and axon guidance (Thompson et al., 2009; Ricaño Cornejo et al., 2011). These genes show an interesting pattern of expression in the cerebral cortex and are

upregulated in interneurons at the time of layer allocation. Therefore it would be interesting to explore whether they play a role in this process.

Finally, two additional candidate genes are Rxfp1 and Lgi2. Rxfp1 belongs to the relaxin family peptide receptor, and it exists as a receptor but also as a truncated form due to alternative spliced isoforms (Scott et al. 2006). Even if the function of this molecule is unknown it seems that the truncated form acts as a functional antagonist of the receptor (Scott et al. 2006). Rxfp1 has been previously shown to be expressed in the brain (Bathgate et al., 2006), but it has never been reported in interneurons, and its function in the cerebral cortex remains completely unknown. It has been mainly related to brain cancer (Thanasupawat et al., 2015) and metastasis (Binder et al., 2014), and its expression has been found in the brain parenchymal arterioles (Lung et al., 2011 and 2013). Finally, Lgi2 is another interesting gene. A member of the leuchin-rich family of genes that has been linked to epilepsy in dogs, (Seppälä et al., 2011), its function in cortical development remains unexplored.

CONCLUSIONS

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Conclusions

1) Blocking chemokine signaling is sufficient to elicit a switch in interneurons migration from tangential to radial, probably unleashing an attractive signal from the cortical plate.

2) Nrg3 is highly expressed by pyramidal cells in the developing cortical plate since its inception. Its expression is maintained in pyramidal cells as they mature and form the cortical layers.

3) Nrg3 acts as a potent short-range chemoattractant for interneurons, similar to the membrane-bound form of Nrg1, CRD-Nrg1. Nrg3 exerts its chemoattractive function through the tyrosine kinase receptor ErbB4.

4) Nrg3 contributes to the normal allocation of interneurons in the cortex. This has been shown through gain and loss of function experiments that reinforce the notion that the timed entry of interneurons in the developing cortical plate is required for their normal lamination.

5) Interneurons show a preference for Cxcl12 over Nrg3 in vitro, which suggests a hierarchical organization of guidance cues controlling intracortical migration. In vivo, interneurons migrate close to Cxcl12 sources – thereby avoiding the cortical plate – until they lose responsiveness to the chemokine.

6) *Nkx2.1-CreER* mice represent a useful tool to analyze the temporal dynamics of cortical layering for MGE-derived interneurons, as shown through the analysis of the distribution of GFP+ interneurons at different stages of development.

7) There are important changes in the transcriptome of cortical interneurons during their transition from tangential to radial migration, as shown by gene profiling analyses using Affimetrix® arrays.

8) The chemokine Cx3cl1 is upregulated by late born interneurons as they invade the cortical plate, but this factor does not seem to play a major role in the regulation of this process.

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Conclusiones

1) El bloqueo de la señal de las quimioquinas es suficiente para provocar un cambio en la migración de las interneuronas de tangencial a radial, revelando de esta manera una señal atractiva desde la placa cortical.

2) Nrg3 se expresa a muy altos niveles en las células piramidales durante el desarrollo de la placa cortical. Su expresión se mantiene en las células piramidales durante sus maduración y mientras que forman las capas corticales.

3) Nrg3 actúa como una potente molécula quimioatractiva para las interneuronas. Este efecto es similar al de isoforma de Nrg1 unida a la membrana, CRD-Nrg1. Nrg3 ejerce su función a través del receptor tirosina quinasa ErbB4 .

4) Nrg3 contribuye a la correcta distribución de las interneuronas en la corteza. Esto se ha demostrado a través de experimentos de ganancia y pérdida de función de *Nrg3*, que refuerzan la idea de que la entrada temporalmente organizada de las interneuronas en la placa cortical es fundamental para sus correcta laminación.

5) Las interneuronas muestran una preferencia por Cxcl12 sobre Nrg3 in vitro, lo que sugiere una organización jerárquica de las señales que controlan la migración cortical. In vivo, las interneuronas migran cerca de fuentes de Cxcl12 - evitando de este modo la placa cortical - hasta que pierden su capacidad de respuesta a esta quimioquina.

6) Los ratones *Nkx2.1-CreER* representan una herramienta útil para analizar la dinámica temporal de la distribución en las capas corticales de las interneuronas originadas en la eminencia ganglionar medial.

7) Hay cambios importantes en el transcriptoma de las interneuronas corticales durante la transición de migración tangencial a radial, tal y como hemos demostrado a través del análisis de los perfiles transcripcionales de estas células utilizando Affimetrix *microarrays*.

8) La quimioquina Cx3cl1 se expresa en las interneuronas que nacen relativamente tarde en la eminencia ganglionar medial mientras éstas invaden la placa cortical, pero no parece jugar un papel importante en la regulación de este proceso.

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