Role of Robo1 receptor in semaphorin signalling system and cortical interneuron migration

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by

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I, Luis Rodrigo Hernández-Miranda, confirm that the
work presented here in this thesis is my own.
Where information has been derived from other sources,
I confirm that it has been indicated clearly in the thesis.

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Abstract

En route to the cerebral cortex, interneurons encounter the developing striatum and avoid it. It has been shown that these cells express neuropilin (Nrp) as well as PlexinA receptors, which allow these cells to respond to Sema3A and Sema3F chemorepulsive cues expressed in the developing striatum and as consequence they migrate around it and into their proper tangential migratory paths. Robo proteins (receptors for the chemorepulsive family of ligands Slit) have also been observed in cortical interneurons, and they are thought to modulate the morphology of migrating interneurons as well as to play a role in their migration.

In the present work, I found that Robo1, but not Robo2 or Slit1/Slit2, deficient (*Robo1*$^{-/-}$) mice contain a significant number of cortical interneurons migrating aberrantly through their developing striatum. *In vitro* experiments showed that dissociated cells taken from the medial ganglionic eminence (MGE, major source of cortical interneurons) of *Robo1*$^{-/-}$ mice do not respond to either Sema3A or Sema3F induced chemorepulsion. Moreover, I observed significant down regulation of *Nrp* and *PlexinA* receptors, as well as reduced levels of *Sema3F* expression and of some intracellular effectors activated by Sema3A and Sema3F in *Robo1*$^{-/-}$ cortical interneurons. Using a cell line as an *in vitro* model, I confirmed that perturbation of Robo1 signalling results in loss of responsiveness to Sema3A and Sema3F, as well as down regulation of their receptors. Additionally, I found that Robo1 can bind directly to Nrp and PlexinA proteins.

Taken together, the data presented here suggest a novel role for Robo1 receptor in controlling the expression of distinct components of the class 3 semaphorin signalling system and thus, the migration of cortical interneurons. They also suggest that the migration of cortical interneurons around the striatum might result from the collaborative effort of Robo1receptors and the class 3 semaphorin signalling system.

Dedication

For my loving and supportive Sarah Meier

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Publications

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Abbreviations

ACSF: artificial cerebral spinal fluid

ANOVA: analysis of variance

AT: anneal temperature

BDNF: brain derived neurotrophic factor

bp: base pairs

BSNP: burst-spiking non-pyramidal cells

CAM: cell adhesion molecules

Cat #: catalogue number

CB: Calbindin

CB^+ cells: CB immunoreactive cells

Cb: cerebellum

CCK: cholecystokinin

cells/mm^2: number of cells per square millimetre

CGE: caudal ganglionic eminence

ChAT: choline acetyltransferase

CM: Conditioned media

CM-FLAG: condition media from *p3xFLAG-CMV-8* transfected COS-7 cells

CM-myc: condition media from *pCDNA3.1-myc* transfected COS-7 cells

CM-SEMA3A: condition media from *semaphorin3A-myc* transfected COS-7 cells

CM-SEMA3F: condition media from *semaphorin3F-FLAG* transfected COS-7 cells

CM-Slit1: condition media from *Slit1-myc* transfected COS-7 cells

CM-Slit2: condition media from *Slit2-myc* transfected COS-7 cells

CP: cortical plate

CR: calretinin

CR^+ cells: CR immunoreactive cells

CRF: corticotrophin realising factor

CSN: central nervous system

CXCR4: CXC chemokine receptor 4

DAB: 3,3'-Diaminobenzidine

DAPI: 4'-6-Diamidino-2-Phenyllindole

DARPP-32: dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa

DEPC: Diethly Pyrocarbonate

DIV: days *in vitro*

Dlx: distal-less homeobox transcriptor factor

***Dlx1*$^{-/-}$*/Dlx2*$^{-/-}$ mice**: *Dlx1*/*Dlx2* double deficient mice

DN: dominant negative

E: embryonic day

Eph: Eph-receptor tyrosine kinases

ErB: receptor tyrosine kinases of the ErbB family

F: forward primer

FACS: Fluorescent Activated Cell Sorter

FL: full length

FOXP2: forkhead homeobox P2 transcription factor

FOXP2^{+}: FOXP2 immunoreactive cells

FS: fast spiking cells

GABA: γ-Animobutyric acid

GABARs: GABA receptors

GAD: acid decarboxylase

GEs: the ganglionic eminences

GFP: green fluorescent protein

Gp: globus pallidus

hem: cortical hem

HGF/SF: hepatocyte growth factor/scatter factor

hp: hippocampus

IF: immunofluorescence

Ig: immunoglobulin

IHC: immunohistochemistry

IP: immunoprecipitation

IS: irregular-spiking cells

IZ: intermediate zone

KCC2: potassium-chloride co-transporter

LGE: lateral ganglionic eminence

LS: late-spiking cells

LTS: low-threshold-spiking cells

MGE: medial ganglionic eminence

MTZ: mantle zone

MZ: marginal zone

NCx: neocortex

NGRs: neuregulin growth factors

NK: neuromedin K

***Nkx2.1*$^{-/-}$ mice**: *Nkx2.1* deficient mice

Nrp: neuropilin

NPY: neuropeptide Y

P: postnatal day

PBS: phosphate buffered saline

PBST: PBS containing 0.1% Triton X-100

Pc: piriform cortex

PCPA: DL-P-chlorophenylalanine inhibitor

PCR: polymerase chain reaction

POA: preoptic area

PP: preplate

PS: product size

PV: parvalbumin

PV^{+} cells: PV inmunoreactive cells

QPCR: quantitative real-time polymerase chain reaction

R: reverse primer

Ref: reference

Robo: roundabout receptor

***Robo1*$^{-/-}$ mice**: Robo1 deficient Dulox mice

***Robo2*$^{-/-}$ mice**: Robo2 deficient mice

***Robo3*$^{-/-}$ mice**: Robo3 deficient mice

RSNP: regular-spiking non-pyramidal cells

Rt: room temperature

RT-PCR: reverse transcription polymerase chain reaction

SbK: substance K

SbP: substance P

SDF-1: chemokine stromal-derived factor 1

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: standard error of the mean

Sema3A: semaphorin3A

Sema3F: semaphorin3F

***Slit1*$^{-/-}$*/Slit2*$^{-/-}$ mice**: Slit1/Slit2 double deficient mice

SOM: somatostatin

SP: subplate

Sp: septum

St: striatum

SVZ: subventricular zone

Th: thalamus

TrkB: tyrosine kinase receptors B

***TrkB*$^{-/-}$ mice**: TrkB deficient mice

TrkC: tyrosine kinase receptors C

V: voltage

VEGF: vascular endothelial growth factor

VIP: vasoactive intestinal peptide

VZ: ventricular zone

WB: Western blot

WM: white matter

3V: third ventricule

4% PFA: 4% paraformaldehyde

5-HT: serotonin

By the deficits, we may know the talents, by the exceptions, we may discern the rules, and by studying pathology we may construct a model of health. And –most important- from this model may evolve the insights and tools we need to affect our own lives, mold our own destinies, change ourselves and our society in ways that, as yet, we can only imagine.

Laurence Miller

Chapter 1: Introduction

1.1 Basic organisation of the mature neocortex

The outermost part of the forebrain, the cerebral cortex (also referred as pallidum), is a fine sheet of nervous tissue responsible for processing most of the high-order functions carried out within the mammalian nervous system (Mountcastle, 1997, 1998; Karlen and Krubitzer, 2006; Medina and Abellan, 2009). As in the rest of the nervous system, the cellular component of the cerebral cortex includes neuronal (projection neurons and interneurons) and non-neuronal cells (mainly protoplasmic astrocytes and microglia). Anatomical observation on the cerebral cortex have identified that this structure presents different laminae varying from two to six layers (Mountcastle, 1997, 1998; Karlen and Krubitzer, 2006; Medina and Abellan, 2009). Thus, three distinct parts of the cerebral cortex have been documented; archicortex (a two-layered structure that forms most of the hippocampus), paleocortex (a three-layered structure that forms the olfactory cortex) and neocortex (a six-layered structure and represents the largest part of the cerebral cortex). Phylogenetically, the neocortex is the most recent acquisition of the cerebral cortex and it is thought to develop from parts of the paleocortex and archicortex (Karten, 1991; Mountcastle, 1998; Medina and Abellan, 2009). Unlike the archicortex and paleocortex, the neocortex is exclusively found in mammalian species. Functionally, the archicortex has been associated with memory and spatial navigation, whilst the paleocortex is crucial for the sense of smell (Mountcastle, 1998). The neocortex, on the other hand, has been related to complex processes as thinking, consciousness, perception, control of coordinated movement, memory and learning (Karten, 1991; Karlen and Krubitzer, 2006; Medina and Abellan, 2009).

1.1.1 Cell types in the neocortex

Projection neurons account for approximate 70-85% of all neurons of the neocortex and for the neocortical output (DeFelipe, 1993; Hevner, 2006). They are commonly pyramidal cells in shape and vary in size through the neocortex (Spruston, 2008). Projection neurons are excitatory and utilise L-Glutamate as their principal neurotransmitter (Spruston,

2008). In addition to having a pyramidal soma, they project in most cases a single apical dendrite towards the pia surface from the apex of their soma and several dendrites from the base of it (Spruston, 2008). Their dendrites also contain numerous protrusions or spines that increase their synaptic surface. These cells project axons towards the white matter that targets other cortical or subcortical structures (Spruston, 2008). Interneurons, on the other hand, represent less than 15-30% of all the neurons population. The majority of cortical interneurons (also known as non-pyramidal cells) are inhibitory cells and utilise γ-Animobutyric acid (GABA) as their main neurotransmitter (see below). However, there exist a number of excitatory non-pyramidal cells (mostly stellate neurons), which use L-Glutamate as main neurotransmitter (DeFelipe, 1993). These cells are considered as interneurons because their axons do not ever leave the neocortex (DeFelipe, 1993). For the sake of simplicity, I will consider only inhibitory cells as cortical interneurons throughout the text and, since my work aimed to study the migration of these cells, they will receive special attention from section 1.3.

Glial cells are a robust component of the neocortex. They do not participate directly in the processing and transmission of information, but they are crucial for maintaining the internal homeostasis of the nervous system as well as providing support and protection to neurons (Levison et al., 2005; Vaccarino et al., 2007). Glial cells in the neocortex are divided mainly into two groups: microglial cells and protoplasmic astrocytes. Microglial cells are high specialised macrophages, which protect the neocortex by processing damaged tissue via phagocytosis. Protoplasmic astrocytes are the most abundant glia type in the neocortex, they participate in the maintenance of the extracellular environment of neurons. Astrocytes project a number of processes end-feet that reach the blood vessels and create a barrier called the blood-brain barrier.

1.1.2 Lamination of the neocortex

The arrangement of the neocortex into horizontal layers (or laminae) emerges from the distinct distribution and density of the cells that compose it (Mountcastle, 1998; Medina and Abellan, 2009). Histologists have numbered the six layers that make up the neocortex from the pia to the white matter, as layers I to VI (Mountcastle, 1997, 1998). Layer I (or molecular layer) is located immediately below the pia mater. It contains mostly neuropil (axons and apical dendrites from pyramidal neurons) and only few cells (mainly Cajal-Retzius cells and interneurons). Layer II (or external granular layer) consists of small pyramidal neurons and numerous stellate neurons. Layer III (or external pyramidal layer)

contains small and medium size pyramidal neurons. Layer IV (or internal granular layer) is composed of different types of stellate neurons and relatively few medium size pyramidal neurons. Layer V (or internal pyramidal layer) contains large pyramidal cells. Layer VI (or multiform layer) is made up of small spindle-like pyramidal cells and multiform neurons (Mountcastle, 1997, 1998).

The lamination of the neocortex is also related to the bidirectional connectivity that cortical neurons establish with other cortical and subcortical structures (Mountcastle, 1997, 1998; Lopez-Bendito and Molnár, 2003). Thus, layer IV is the main target of subcortical afferents and it distributes the incoming information to the upper cortical layer (particularly layers II and III). Layer II and III establish intra or inter hemispheric connections through cortico-cortical efferents. Layers II and III are also specialised in receiving cortico-cortical afferents. Layer V and VI are the main output of the neocortex. Layer V mainly project to distant subcortical structures, whereas layer VI project mainly to the thalamus (Mountcastle, 1997, 1998; Lopez-Bendito and Molnár, 2003).

1.1.3 Neocortical areas

In addition to showing a high organisation into horizontal layers, the neocortex is also divided into areas, each of them characterised by a peculiar cellular composition and laminar organisation (Mountcastle, 1998; O'Leary and Nakagawa, 2002; O'Leary et al., 2007). In mammals, three main groups of areas have been observed: association, motor and sensory areas. Depending on their function, these three main groups of areas can be further subdivided in primary, secondary or accessory areas (Mountcastle, 1998; O'Leary and Nakagawa, 2002; O'Leary et al., 2007). All functional areas are bilateral, but they seem not to have the same functional performance, as certain areas in one hemisphere dominate functionally over their counterparts in the other hemisphere, in a phenomenon called lateralization.

1.2 Neocortical development

The precise organisation of the mature neocortex emerges from the development of the anterior part of the central nervous system (CSN). Upon the formation of the neural tube a series of continuous and dynamic steps take place in a process known as neurulation, where the neural tube folds and form several vesicles (Greene and Copp, 2009; Harrington et al., 2009). The rostal-most vesicle, the prosencephalon, expands further and

develops into the forebrain that will give rise to the neocortex from its dorsal part (Rubenstein et al., 1998). At early stages of neocortical development, this structure is mainly composed of a single layer of cells that exhibit typical traits of epithelial cells (better known as neuroepithelial cell), as they have a highly polarised morphology along their apical-basal axis such as tight and adherens junctions in the apical end of their lateral membranes (Götz and Huttner , 2005; Vieira et al., 2010).. The apical end of neuroepithelial cells contacts the ventricular surface, whereas the basal end of these cells resides beneath the pial membrane (Bystron et al., 2008). This single-cell layer is also named the ventricular zone (VZ) due to its close proximity to the developing lateral ventricles (Bystron et al., 2008).

During the cell cycle of the neuroepithelial cells, their nuclei move up and down the apical-basal axis in a process called interkinetic nuclear migration (Hayes and Nowakowski, 2000). Since at any given time their nuclei are located at varying distances from their apical-basal axis, they look like a pseudostratified tissue (also called as speudostratified neuroepithelium) (Götz and Huttner , 2005; Bystron et al., 2008; Vieira et al., 2010). In forebrain development, the neuroepithelium generates several other layers in a process termed corticogenesis. At the early phases of corticogenesis, neuroepithelial cells down regulate certain epithelial features (e.i. tight junctions) and develop an astroglial appearance and thus, these transformed neuroepithelial cells become a new cell type named radial glial cells (Pinto and Götz, 2007).

Radial glial cells are also polarised cells which project apical and basal processes that reach the ventricular and pial surface, respectively (Götz and Huttner , 2005; Pinto and Götz, 2007). Symmetrical mitotic divisions of radial glial cells expand the pool of these cells and contribute to replace neuroepithelial cells from the VZ (Götz et al., 2002; Götz and Huttner , 2005). Therefore, most neurons in the brain are generated either directly or indirectly from radial glial cells (Noctor et al., 2001, 2002). As development proceeds, these cells divide asymmetrically and generate a second pool of proliferative cells called basal progenitors (also referred as intermediate progenitors), which will form the subventricular zone (SVZ) underneath the VZ (Götz and Huttner , 2005; Martínez-Cerdeño et al., 2006; Pontious et al., 2008). Unlike radial glial cells, basal progenitors do not project cells processes to either the ventricular or pial surface, but most important they do not undergo interkinetic nuclear migration during their cell cycle (Götz and Huttner , 2005; Martínez-Cerdeño et al., 2006; Pontious et al., 2008). Both groups of proliferative cells divide symmetrically (to generate identical progeny) or asymmetrically (to generate non-identical offspring) (Fishell and Kriegstein, 2003; Farkas and Huttner, 2008). At later

stages of forebrain development, the basal progenitors become more numerous than radial glial cells and as consequence, the SVZ expands in size. As the SVZ expands, the VZ diminishes and develops into the mature ependymal layer that resides in the ventricular surface (Tramontin et al., 2003). At late stages of development, the SVZ generates most of the glial cells that populate the neocortex (Levinson and Goldman, 1993).

The first cohorts of postmitotic cells generated from the dorsal VZ migrate radially by translocating their soma to the margins of the cortical wall and form a plexiform layer that is called the preplate (PP) (De Carlos and O'Leary, 1992; Super et al., 1998; Super and Uylings, 2001; Nadarajah and Parnavelas, 2002; Molnár et al., 2006; Bystron et al., 2008). The morphology of those cells that translocate their soma to the pia surface is typically characterized by a long and radially oriented basal cell process that reaches the pial surface (Nadarajah et al., 2001). This process is essential for the translocation of these cells, as it allows them to have fast and continuous advancements (Nadarajah et al., 2001). Late-born postmitotic cells generated from the dorsal VZ and SVZ also adopt also radial movements towards the pial surface, but these cells utilise the radially oriented cell processes of radial glial cells as a scaffold for their locomotion (Sidman and Rakic, 1973; Nadarajah and Parnavelas, 2002; Kriegstein and Noctor, 2004; Bystron et al., 2008). Morphologically, these migrating cells have a short leading cell process (oriented to the pial surface) and a trailing cell process (Sidman and Rakic, 1973; Nadarajah and Parnavelas, 2002). These cells exhibit slow saltatory patterns of locomotion with short and fast movements interspersed with stationary periods (Nadarajah et al., 2001; Nadarajah and Parnavelas, 2002). Once the leading process of these cells reaches the PP, they switch the mode of migration to soma translocation movement (Nadarajah and Parnavelas, 2002). Shortly after the formation of the PP, subsequent cohorts of postmitotic cells (projection neurons) split this layer into a superficial one named the marginal zone (MZ or cortical layer I) underneath the pial surface and a deep layer termed the subplate (SP) (Marin-Padilla, 1978; Super et al., 1998; Bystron et al., 2008) The late-born postmitotic cells located between the MZ and SP form the cortical plate (CP), which eventually develops into the neocortical layers II-VI. The CP is assembled in an “inside-out” sequence, with newly arriving cells migrating through existing neurons of the CP before stopping their migration underneath the MZ (Super et al., 1998; Bystron et al., 2008). Upon the splitting of the PP and the formation of the CP, another layer of sparse cells emerges between the SP and the SVZ, which is called the intermediate zone (IZ) characterized mainly by axonal processes and migrating postmitotic cells (Bystron et al., 2008).

The postmitotic cells located in the MZ are manly Cajal-Retzius cells and tangentially migrating interneurons, whilst those making up the SP are known merely as subplate cells (Marin-Padilla, 1998; Sarnat and Flores-Sarnat, 2002; Soriano and Del Río, 2005). Both groups of cells are transient during development, and during early postnatal development they diminish in number, although few can be found in the adult neocortex. The function of these cells is crucial in neocortical development, as they participate in the correct migration and integration of cortical projection neurons into the CP and the establishment of thalamo-cortical and cortico-thalamic connections (De Carlos and O'Leary DD, 1992; Super et al., 1998; Lopez-Bendito and Molnár, 2003; Soriano and Del Río, 2005; Molnár et al., 2006). The growth of cortico-thalamic projections is concomitant with the formation of thalamo-cortical projections in rodents (Lopez-Bendito and Molnár, 2003; Vanderhaeghen and Polleux, 2004; Price, et al., 2006). Upon their arrival to the developing neocortex, thalamo-cortical axons form transient contacts with SP cells before entering to the neocortex in early postnatal life of rodents (firstly they reach and arborise in layer VI and subsequently, they arrive at layer IV and densely arborise within it) (Agmon et al., 1993; Lopez-Bendito and Molnár, 2003).

The lamination of the neocortex starts at about birth in rodents and finishes around the end of the first postnatal week of life (see Fig. 2 in Agmon et al., 1993). The first layer to be segregated from the CP is layer VI and, as postnatal development progresses, the other layers segregate from the CP starting from layer V to layer II (Bear et al., 2001). From the second to about the end of the third week of postnatal life, extensive elongation and arborisation of dendrites by projection- and inter- neurons is observed in the neocortex of the mouse (Chien, 2005). Synaptogenesis and axonal arborisations in the neocortex also occur simultaneously to the development of dendritic trees (Patton and Burgess, 2005). During neocortical development neurons, dendrites, axons and synapses are produced in excess, however, during late postnatal development (early fourth week of postnatal life in rodents) a significant number of these cortical component are refined and eliminated in order to establish the mature neocortex, which is achieved at about the end of the first month of life in rodents (Luo and O'Leary, 2005; Low and Cheng, 2006).

The development of the neocortex and the migration of cortical projection neurons have been extensively studied for more than one hundred year. However, the origin and migration of cortical interneurons has been investigated primarly during the past fifteen years. As this thesis focuses on the migration of cortical interneurons, a description of our current understanding on the diversity, origin and migration of cortical interneurons is provided below.

1.3 Cortical interneurons

Cortical interneurons represent about 15-30% of the total number of neurons in the neocortex (DeFelipe, 1993; Kawaguchi and Kubota, 1997; Gupta et al., 2000). They share a number of features that allow them to be grouped together. Firstly, the vast majority of them utilise GABA as their main neurotransmitter, and form symmetric (inhibitory) synapses. Secondly, mature cortical interneurons commonly have smooth (aspiny) dendrites and somata. Thirdly, they receive symmetric or asymmetric (excitatory) synapses onto their somata. Fourthly, they have usually short axons which arborise within the cerebral cortex. Finally, cortical interneurons project axonal terminals laterally or vertically, but they never project down into the white matter. In spite of their relatively small number in the cerebral cortex and the characteristics they share, cortical interneurons are an astonishing diverse group of neurons. Several classes of cortical interneurons have been documented on basis of the protein contents, axonal arborisations and electrophysical properties they exhibit (Fig. 1.1; DeFelipe, 1993; Markram et al., 2004).

1.3.1 Classification of cortical interneurons according to their protein contents

A number of calcium-binding proteins co-localise with GABA in the majority of interneurons. Calbindin (CB), parvalbumin (PV) and calretinin (CR) are three members of the EF-hand family of calcium-binding proteins (EF-hand is a stretch of amino acids forming a helix-loop-helix structure), which are broadly expressed in the neocortex and other areas of the nervous system (Hof et al., 1999). The main function of these proteins is to buffer the intracellular concentration of calcium, and thus they control the amplitude of calcium signals (Hof et al., 1999). The distinct expression of these proteins in cortical interneurons has been used to separate them into distinct classes of cells (Kubota et al., 1994; DeFelipe, 1997).

CB immunoreactive cells (CB^+ cells) are located in virtually all neocortical layers. CB^+ cells in layer II and III have multipolar cell morphology and seem to belong to a subclass of double bouquet interneurons (Fig. 1.1A; DeFelipe et al., 1989a), whereas cells in layers V and VI have elongated or multipolar shape, and have some features of Martinotti cells with an ascending axon that extends into layer I (Fig. 1.1G; DeFelipe et al., 1989a). A third group of CB^+ cells is identified in layers II-VI, they are characterised by having small cell bodies and high elaborated dendritic trees similar to neurogliaform

cells (Fig.1.1E). A fourth class of CB^+ cells is found in layer I; they have round, oval or triangular somata and resemble an adult version of Cajal-Retzius cells (Fig. 1.1J; Hof et al., 1999). Besides the specific expression of CB in cortical interneurons, weak CB immunoreactivity can also be found in some classes of projection neurons, especially in primates (Hayes and Lewis, 1992; Kondo et al., 1994).

PV inmunoreactive cells (PV^+ cells) are found mainly in cortical layers III to V. Two classes of PV^+ cells have been described in the neocortex (Van Brederode et al., 1990; Lewis and Lund, 1990; Conde et al., 1994). The first class of PV^+ cells are present in layer III-V, they are documented as cells with large round bodies and multipolar shape, which are similar to those of large basket cells (Fig. 1.1H; Akil and Lewis, 1992). The second class of PV^+ cells are mostly located in layers II and III, and show small to medium size multipolar cell bodies and a very thin axon; these cells have been associated with chandelier interneurons (Fig. 1.1I; DeFelipe et al., 1989b; Akil and Lewis, 1992).

CR immunoreactive cells (CR^+ cells) are mainly situated in all neocortical layers, but high densities of these cells can be observed in layers II and III. Bitufted cells with both dendritic trees and axonic projections orientated vertically are the most common type of CR^+ cells. They have been classified as double bouquet or bipolar interneurons (Fig. 1.1A,F). Commonly, CB^+ cells in layer I (Cajal-Retzius cells) may also show CR inmmunoreactivity (Fig. 1.1J; Hof et al., 1999).

As mentioned above, calcium-binding proteins have been utilised as markers for non-overlapping classes of interneurons. Nevertheless, there exists a certain degree of co-expression between these proteins in interneurons and they can be found in distinct classes of cortical interneurons (Wang et al., 2002). Special is the case of CB and CR, which co-localise in bitufted, bipolar, double bouquet and Cajal-Retzius cells; or CB and PV in large basket cells and chandelier cells (Kubota et al., 1994; Cauli et al., 1997; del Rio and DeFelipe, 1997a,b; Wang et al., 2002). Since calcium binding proteins regulate the intracellular concentration of calcium, it is very likely that the presence of two or more calcium-binding proteins in a given class of interneurons might indicate high metabolic needs.

Apart from the expression of calcium-binding proteins, GABAergic cells also express neuropeptides such as somatostatin (SOM)-14 amino acids, vasoactive intestinal peptide (VIP)-28 amino acids, cholecystokinin (CCK)-8 amino acids, substance P (SbP)-11 amino acids, substance K (SbK) and neuromedin K (NK)-10 amino acids each, corticotrophin realising factor (CRF)-40 amino acids and neuropeptide Y (NPY)-36 amino acids (Hendry et al., 1984a,b; Morrison et al., 1984; Rogers, 1992). Similar to

calcium-binding proteins, specific classes of cortical interneurons tend to co-express distinct combination of neuropeptides. Indeed, it has been documented that some classes of cortical interneurons can co-express up to seven or more neuropeptides and calcium binding proteins (Morrison et al., 1984; Wahle, 1993; Wang et al., 2002). Moreover, anatomical data suggest that some neuropeptides are more likely to be expressed in some classes of interneurons rather than others (see below). Interestingly, it has also been suggested that some classes of cortical interneuron express these peptides transiently during development (Parnavelas and Cavanagh, 1988).

1.3.2 Morphological classification of cortical interneurons

Dendritic trees are possibly the most inconsistent traits of cortical interneurons and they have not been used to classify them. Nonetheless, the axonic arborisations of cortical interneuron tend to reveal precisely their morphological identity. Thus, eight classes of cortical interneurons have been identify, which include: basket cells, chandelier cells, martinotti cells, bipolar cells, double bouquet cells, bitufted cells, neurogliaform cells and layer I cells (Mountcastle, 1998; Markram et al., 2004; see below).

Basket cells represent about 50% of all cortical interneurons within the cerebral cortex. The term 'basket cell' comes from the basket-like appearance of their axonic terminals around the pyramidal cell somata, which result from convergent innervations by several basket cells. They specialise in targeting the somata and proximal dendrites of projection neurons and other cortical interneurons. Three types of basket cells have been described: large basket cells, small basket cells and nest basket cells. *Large basket cells* (cell diameter of 20-30 μm) have large, aspiny and multipolar dendrites (Fig. 1.1H). These cells possess expansive axonal arborisations and they are the primary source of lateral inhibition across columns within the layers that contain their somata. The somato-dendritic morphology is frequently multipolar, but in some cases it can be bitufted, pyramidal or bipolar. These cells express CB, PV, NPY, CCK and occasionally SOM and CR, but they never express VIP. *Small basket cells* (cell diameter of 12-22 μm) have small, aspiny and multipolar dendrites (Fig. 1.1D). Their somato-dendritic morphology can be multipolar, bitufted or bipolar. They are distinguished from large basket cells by their frequently branching and 'curvy' axons around their somata. Unlike other basket cells, they express VIP. *Nest basket cells* (cell diameter of 12-22 μm) seem to be a hybrid of large and small basket cells (Fig. 1.1B). They adopt their name because their axonic arborisations resemble a birds' nest. Nest basket cells do not express CR or VIP.

Chandelier cells (cell diameter of 10-15 μm) can have multipolar or bitufted dendritic morphology (Fig. 1.1I). These interneurons are axon-targeting and typically express CB or PV and is some cells both proteins are co-expressed. They also express CCK, NPY and VIP, but never CR. Their local axonal arborisations form highly branched processes, often ramifying around, above or below their somata with a high bouton density. The characteristic terminal portions of the axon form short vertical rows of boutons, resembling a chandelier.

Martinotti cells (cell diameter of 12-22 μm) have bitufted morphology with more elaborated dendritic trees than other interneurons (Fig. 1.1G). They project axons specifically towards layer I, where they inhibit the tufts of apical dendrites of projection neurons. Their axons can also project horizontally in layer I for long distances to inhibit apical dendrites in neighbouring and distant areas. These cells not only target the most distal dendrites, but also proximal dendrites and the somata of pyramidal neurons. These cells always express SOM and never PV or VIP.

Bipolar cells (cell diameter of 10-18 μm) are small cells with spindle or ovoid somata and narrow bipolar or bitufted dendrites that extend vertically towards layer I and down to layer VI (Fig. 1.1F). Their axons normally emerge from one of the primary dendrites and form a band that crosses all layers. Their bouton density is low and they contact only a few cells compared to other interneurons; these cells tend to target the basal dendrites of pyramidal neurons. They typically express CR, NPY, CCK, SOM and VIP.

Double bouquet cells (cell diameter of 10-18 μm) usually have bitufted dendritic morphology (Fig. 1.1A). They are characterised for possessing a tight fascicular axonal cylinder that resembles a 'horse tail'. These axonal cylinders protrude very thick varicose collaterals that may extend across all layers. These cells establish synaptic contacts basically on dendrites co-express CB and CR, although they can also express VIP or CCK, but not PV, SOM or NPY.

Bitufted cells (cell diameter of 12-22 μm) share morphological characteristics with bipolar and double bouquet cells such as ovoid somata and give rise to primary dendrites from opposite poles to form tufts. Nevertheless, bitufted cells, unlike the narrow vertical axonal band of bipolar cells or the 'horse tail' of double bouquet cells, have wider horizontal axonal spans. Their vertical projections are less extensive and cross only neighbouring layers. These cells are dendrite-targeting cells. They express CB, CR, NPY, VIP, SOM and CCK, but not PV.

Neurogliaform cells (cell diameter of 10-12 μm) are small, button-type cells with many radiating dendrites that are short, aspiny and rarely branched (Fig. 1.1E). Their dendritic arborisations are symmetrical and spherical. Their axons arise from any part of the soma or from the base of a dendrite and, shortly after their origin, they break up into dense intertwined trees of ultra-thin processes highly branched.

Layer I interneurons fall into two categories. The first comprises large neurons with horizontal processes, and are known as mature Cajal-Retzius cells. The axons of these cells are confined to layer I and extend horizontally; from their axons emerge a number of ascending or descending terminal processes (Fig. 1.1J). The second category corresponds to a number of medium size interneurons that project dendrites and axons largely constrained to layer I (Fig. 1.1C).

1.3.3 Electrophysiological classification of cortical interneurons

Early electrophysiological studies identified cortical interneurons as fast spiking (FS) cells (McCormick et al., 1985; Connors and Gutnick, 1990). Subsequent examinations revealed that there exist other discharge patterns such as low-threshold-spiking (LTS), burst-spiking non-pyramidal (BSNP), regular-spiking non-pyramidal (RSNP), late-spiking (LS) and irregular-spiking (IS) cells (Kawaguchi, 1993; Kawaguchi and Kubota, 1993, 1996, 1997, 1998; Porter et al., 1998). BSNP cells exhibit typical burst-like discharges and are manly found in cortical layer V; some of these cells have the morphology of Martinotti cells and double bouquet cells (Kawaguchi, 1993; Kawaguchi and Kubota 1993, 1997). RSNP cells discharge similar to regular-spiking projection neurons, they are maily located in layers II, III and V, and their morphology resembles that of Martinotti cell, double bouquet cells and bipolar cells (Kawaguchi, 1993; Kawaguchi and Kubota 1996, 1998). LS cells delay their discharge after being depolarised, and they are found in layers II, III and V. Morphologically, these cells are defined as neurogliaform cells (Kawaguchi, 1993; Kawaguchi and Kubota 1996). IS cells display an initial burst of action potentials which are followed by irregulary and spaced spikes. These cells are less numerous than those described above and they are normally found in layers II, III and V (Porter et al., 1998).

1.4 Origin of cortical interneurons: the ganglionic eminences

Cortical interneurons emerge largely from ventral proliferative zones within the developing subpallium, the ganglionic eminences (GEs). Anatomically, the GEs are transient embryonic telencephalic structures situated ventrally in the developing forebrain and in proximity to the lateral ventricles. These areas become evident at embryonic day 11 (E11 in the mouse brain) as structures that protrude ventro-laterally into the walls of the lateral and third ventricles (Smart and Sturrock, 1979; Bhide, 1996). A day later, a second elevation emerges at the dorso-lateral part of the lateral ventricular wall (Smart and Sturrock, 1979). These elevations are the medial and lateral ganglionic eminences (MGE and LGE, respectively; Fig. 1.2A,B). Between E11 and E14, the MGE represents the most conspicuous protuberance in the basal forebrain (Wichterle et al., 2001) but, as development proceeds, the LGE becomes larger than the MGE and circumscribes it rostrally, medially and caudally (Smart and Struck, 1979). Between E12 and E14, a deep and narrow sulcus separates the MGE from the LGE at the most rostral-medial levels of the brain (Fig. 1.2B); caudally, the LGE and MGE fuse and, they are continued by the caudal ganglionic eminence (CGE; Smart and Sturrock, 1979; Bhide, 1996, Nery et al., 2002; Fig. 1.2C). By E14, the sulcus between MGE and LGE begins to disappear and by E15 is barely distinguishable (Smart and Sturrock, 1979; Wichterle et al., 2001; Fig. 1.2D-F). The maximum surface area of the GEs is reached by E15 to E16, after which there is a steady diminution of their size that ends prenatally with a restricted area just underneath the ependimal layer (Smart and Sturrock, 1979).

Cytoarchitectonically, the GEs possess three distinct layers: two are proliferative (VZ and SVZ) and one is postmitotic, the mantle zone (MTZ). The proliferative zones of the GEs are fairly homogenous and resemble their cortical counterparts (Bhide, 1996; Brazel et al., 2003). Sturrock and Smart (1980) characterised the SVZ cells as having small, dark and irregular nuclei with moderate cytoplasm; and the VZ as a pseudo-stratified neuroepithelium containing radial glial cells which protrude radial processes that span the entire width of the ventral telencephalon. In spite of the morphological similarities among the proliferative zones of the GEs, they differ significantly in both the genes they express and the progeny they produce (see below).

Molecularly, it has been reported that the proliferative zones of the GEs are distinguished from their cortical counterparts by the expression of specific transcription factors (see Table 1.1). Members of the distal-less homeobox transcriptor factor (Dlx) family, namely Dlx -1,- 2,- 5 and -6 have been widely described to play an essential role in the patterning of the ventral forebrain (see Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Bulfone et al., 1993; Liu et al., 1997; Eisenstat et al., 1999; Puelles et al., 2000; Perera et al., 2004). Specifically, it has been reported that *Dlx1* and *Dlx2* are expressed in subsets of VZ and SVZ cells (Porteus et al., 1991; Price et al., 1991; Bulfone et al., 1993; Anderson et al 1997b; Puelles et al., 2000), whereas *Dlx5* is expressed in the SVZ and MTZ, and *Dlx6* is mainly expressed in the MTZ of the GEs (Eisenstat et al., 1999; Liu et al., 1997; Perera et al., 2004). Other transcription factors expressed in the VZ, SVZ and MTZ of the GEs include *Meis1* and *Tshz2* (Toresson et al., 2000; Long et al., 2009). *Brn4*, *Six3* and *Sp9* are transcription factors that show restricted expression within the VZ and SVZ (Long et al., 2009). *Gsh2*, *Mash1* (also known as Ascl1) and *Vax1* are transcription factors exclusively expressed in the VZ (Casarosa et al., 1999; Horton et al., 1999; Corbin et al., 2000; Marin et al., 2000; Toresson and Campbell, 2001; Taglialatela et al., 2004). The anatomic subdivision of the GE can also be distinguished by the expression of specific transcription factors. In this regard, it has been reported that the transcription factors *Nkx2.1*, *Nkx6.2*, *Lhx6*, *Lhx8* and *Gsh1* are specifically expressed in the MGE (Bulfone et al., 1993; Sussel et al., 1999; Marin et al. 2000, Toresson and Campbell, 2001; Asbreuk et al., 2002; Zhao et al., 2003; Alifragis et al., 2004; Liodis et al., 2007; Du et al., 2008; Zhao et al., 2008, Fragkouli et al., 2009); *Meis2*, *Nolz-1*, *Er81* and *IsL1* are expressed in the LGE (Toresson et al., 2000; Sckogh et al., 2003; Chang et al., 2004); and *COUP-TFII* (also known as *Nr2f2*; Kanatani et al., 2008) is preferentially expressed in the CGE.

1.5 The three subdivisions of the GEs give rise to the vast majority of cortical interneurons in rodents

Different experimental approaches including dye tracing labelling, *in utero* transplantation and *in vivo* Cre-lox technology, among others, suggest that the vast majority of cortical interneurons, in rodents, are generated in the three subdivisions of the GEs (Anderson et al., 1997a, 2001; Lavdas et al., 1999; Wichterle et al., 2001; Nery et al., 2002). In fact, the diversity of cortical interneurons is thought to emerge, at least in part, from the local characteristics that exist within their place of origin.

Recent genetic studies have pointed out that the GEs can be further subdivided into well-defined areas or subdomains by the combination of transcription factors that they express, and it is thought that these subdomains are responsible for the distinct progeny of cortical interneurons (Butt et al., 2005; Flames et al., 2007; Fogarty et al., 2007). This is the case for the MGE, where it has been reported that SOM^+, PV^+ and CB^+ cortical interneurons originate from *NKx2.1/Lhx6* expressing precursors in the MGE, whilst cortical interneurons co-expressing CR^+ and SOM^+ are generated from precursor that express *Nkx6.2/Lhx6* (Xu et al., 2004, 2008; Fogarty et al., 2007). The CGE produces cortical interneurons co-expressing NPY and CR from the *COUP-TFII*$^+$ domain (Nery et al., 2002; Fogarty et al., 2007). The role of the LGE in the generation of cortical interneurons had been the subject of debate given that *en route* to the neocortex, a significant proportion of MGE-derived cortical interneurons migrates through it, posing the question as to whether the LGE actually possess endogenous precursors for the neocortex (Tamamaki et al., 1997; Wichterle et al., 2001). Nevertheless, in a series of elegant experiments carried out on *Dlx1* and *Dlx2* double deficient (*Dlx1*$^{-/-}$/*Dlx2*$^{-/-}$) and *Nkx2.1* deficient (*Nkx2.1*$^{-/-}$) mice, Anderson and colleagues (2001) showed that the LGE contributes with late born interneurons to the neocortex. Interestingly, the LGE also contains two molecular subdomains established by the combination of *Dlx1*, *IsL1* and *Er81*, but they generate subcortical rather than cortical GABAergic cells. Thus, *Dlx1*$^+$/*IsL1*$^+$ precursors in the LGE generate striatal projection neurons, whilst LGE precursor cells expressing *Dlx1/Er81* give rise to olfactory bulb interneurons (Stenman et al., 2003).

Among the GEs, the MGE has been reported to be the major source of cortical interneurons (Lavdas et al., 1999; Wichterle et al., 2001; Corbin et al., 2001). Indeed, MGE-derived cortical interneurons account for nearly 70% of the GABAergic cells in the neocortex of rodents, whereas the CGE contributes with about 15% of cortical interneurons (Xu et al., 2003; Butt et al., 2005; Flames et al., 2007; Fogarty et al., 2007; Wonders et al., 2008). Thus, the remaining number of cortical interneurons might be generated in the LGE (Anderson et al., 2001; Jimenez et al., 2002). However, recent reports suggest that there are other brain regions that contribute with interneurons to the neocortex (Gelman et al., 2009). This is particularly the case of the embryonic preoptic area (POA, Marin and Rubenstain, 2001). The POA in the ventral-most portion of the forebrain is a substantial part of the telencephalic stalk, also known as the non-evaginated telencephalon (Puelles et al., 2000). Anatomically, the embryonic POA is described as a region rostral to the optic indentation that limits the telencephalon and diencephalon, it is

also adjacent to the MGE (Fig. 1.2C,F). Cytoarchitectonically, the POA contains a VZ, but it lacks of an evident SVZ (Gelman et al., 2009). Molecularly, it has been described that the POA shares a number of transcription factor with the GEs, principally with the MGE, some of these factors include: *Nkx2.1*, *Nkx6.2*, *Vax1* and others; but it has also been observed that the POA has its own molecular identity (see Table 1.1). Traditionally, the POA was thought to give rise to postmitotic cells that populate the adult POA, olygodendrocytes and cholinergic neurons; however, recent data also suggests that at least a small fraction of cortical interneurons derive from the embryonic POA (Gelman et al., 2009).

Table 1.1 Transcription factors express in the ventral proliferative zones

Gene	Official name	Compartment	Zone	Ref
Dlx1	Distal-less homeobox 1	LGE, MGE, CGE	VZ, SVZ,	1-5
Dlx2	Distal-less homeobox 2	LGE, MGE, CGE	VZ, SVZ,	1-5
Dlx5	Distal-less homeobox 5	LGE, MGE, CGE	SVZ, MTZ	6-8
Dlx6	Distal-less homeobox 6	LGE, MGE, CGE	MTZ	7
Meis1	Meis homeobox 1	LGE, MGE, CGE	VZ, SVZ, MTZ	9
Tshz2	Teashirt Zinc Finger member 2	LGE, MGE, CGE	VZ, SVZ, MTZ	10
Brn4	POU domain, class 3, TF 4	LGE, MGE, CGE	VZ, SVZ	10
Six3	Sine Oculis-related homeobox 3	LGE, MGE, CGE	VZ, SVZ	10
Sp9	Sp9 transcription factor	LGE, MGE, CGE	VZ, SVZ	10
Gsh2	GS homeobox 2	LGE, MGE, CGE	VZ	11-12
Mash1	Achaete-Scute Complex Homolog 1	LGE, MGE, CGE	VZ	13-14
Vax1	Ventral Anterior Homeobox gene 1	LGE, MGE, POA	VZ	15
Lhx6	LIM homeobox protein 6	MGE	VZ, SVZ	16-19
Lhx7	LIM homeobox protein 8	MGE	VZ, SVZ	20-21
Gsh1	GS homeobox 1	MGE	VZ, SVZ	12
Meis2	Meis homeobox 2	LGE	VZ, SVZ	9
Nolz1	Zinc Finger protein 503	LGE	VZ, SVZ	22
Er81	Ets variant gene 1	LGE	VZ, SVZ	23
IsL1	ISL1 transcription factor	LGE	VZ, SVZ	23
CouptfII	Nuclear Receptor Subfamily 2, group F, member 2	CGE	VZ, SVZ	24
Nkx2.1	NK2 homeobox 1	MGE, POA	mainly VZ	3, 25
Nkx6.2	NK6 homeobox 2	MGE, POA	VZ, SVZ	26
NKx5.1	NK5 homeobox 1	POA	VZ	27

1) Porteus et al., 1991; 2) Price et al., 1991; 3) Bulfone et al., 1993; 4) Anderson et al., 1997b; 5) Puelles et al., 2000; 6) Eisenstat et al., 1999; 7) Liu et al., 1997; 8) Perera et al., 2004; 9) Toresson et al., 2000; 10) Long et al., 2009; 11) Corbin et al., 2000; 12) Toresson and Campbell, 2001; 13) Casarosa et al., 1999; 14) Horton et al., 1999; 15) Taglialatela et al., 2004; 16) Marin et al., 2000; 17) Alifragis et al., 2004; 18)Liodis et al., 2007; 19) Zhao et al., 2008; 20) Asbreuk et al., 2002; 21) Zhao et al., 2003; 22) Chang et al., 2004; 23) Sckogh et al., 2003; 24) Kanatani et al., 2008; 25) Sussel et al., 1999; 26) Fogarty et al., 2007; 27) Gelman et al., 2009. Abbreviations: CGE, caudal ganglionic eminence; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MTZ, mantle zone; POA, preoptic area; Ref, reference; SVZ, subventricular zone; VZ, ventricular zone.

1.6 Migratory paths of cortical interneurons

In order to populate the neocortex, cortical interneurons migrate along highly directed and temporally regulated tangential routes from their origins in the subpallium (Anderson et al., 1997a; Tamamaki et al., 1997; Lavdas et al., 1999; Wichterle et al., 1999, 2001; see Fig. 1.3A-C). In contrast to radial migration of cortical projection neurons that employ radial glia as scaffold to allocate themselves in the neocortex, it is presently unclear if migrating cortical interneurons utilise any cellular substrate to reach the neocortex.

Early observations suggested that radial glia do not provide such substrate for their migration, in view of the fact that virtually no radial glial process bends or runs parallel to the pia surface (O'Rourke et al., 1995). However, in a recent study that used a combination of *in vivo* and *in vitro* imaging, Yokota et al. (2007) have shown that some interneurons, upon contacting radial glial end-feet in the cortical wall, modify their tangential trajectories and adopt radial movements to ascend or descend within the cortical wall, suggesting that radial glia might provide a structural matrix for allocating interneurons within the developing cerebral cortex. A number of studies have suggested that TAG-1 expressing corticofugal axons might be a putative substrate for migrating interneurons (Metin and Godement, 1996; Parnavelas 2000; Denaxa et al., 2001; Morante-Oria et al., 2003; McManus et al., 2004). In support of this notion, it has been shown that disruption of TAG-1 signalling *in vitro* results in a significant reduction in the migration of cortical interneurons (Denaxa et al., 2001). However, extensive analysis of TAG-1 deficient mice showed no defects in the number of cortical interneuron within the neocortex, suggesting that axons might not be used as substrata for their migration (Denaxa et al., 2005). Nevertheless, it is likely that other members of the family of adhesion molecules may compensate for the loss of TAG-1 function in TAG-1 deficient mice.

The tangential migratory paths (or streams) that cortical interneurons follow to reach the neocortex are established in a chronological order (Metin et al., 2006). Early-born MGE-derived cortical interneurons (E11.5-E12 in mouse) migrate superficially to the developing striatum, setting the first migratory path. In the cortical wall, these cells follow a superficial route within the PP (Lavdas et al., 1999; Fig. 1.3A). A day later, other MGE-derived cortical interneurons follow a deeper route between the LGE and striatum, establishing a second migratory path. In the cortical wall, these cells migrate along the boundary between the lower IZ and the emerging cortical SVZ (DeDiego et al., 1994; De

Carlos et al., 1996; Tamamaki et al., 1997; Anderson et al., 1997a; Fig. 1.3B). At later stages of development (E13.5-E14), the PP is split by the arrival of postmitotic cells that form the CP into the MZ and the SP underneath the CP. Thus, the superficial stream of migrating cells also splits into the MZ and SP layers (Fig. 1.3C). Late-born LGE-derived cortical interneurons join the deep stream of migrating MGE-derived cortical interneurons and enter into the cortex through the IZ/SVZ border (Fig. 1.3C). A caudal stream has also been described for the migration of CGE-derived cortical interneurons and, together with the MGE, is one of the most conspicuous streams of migrating cortical interneurons (Nery et al., 2002; Yozu et al., 2005; Fig. 1.3E). A recent study, using transgenic mice expressing green fluorescent protein (GFP) under the control of 5-HT_{3A} promoter and time-lapse imaging, has documented a new stream of cortical interneurons that is established in postnatal life (Inta et al., 2008). This stream is formed by migrating 5-HT_{3A}^{+} cells that are generated in the dorsal SVZ underneath the hippocampus. This area was first described as the sub-callosal zone and thought to generate mainly oligodendrocytes (Seri et al., 2006). However, it was later suggested that it also produces interneurons in postnatal life (Merkle et al., 2007; Ventura and Goldman, 2007). Thus, time-lapse imaging has shown that cortical interneurons originating in this area can migrate tangentially towards the occipital cortex following a deep path underneath the hippocampus (Inta et al., 2008; Fig. 1.3F).

Time-lapse imaging in slice cultures has revealed that, upon arriving to the developing neocortex, migrating interneurons are not completely constrained to their migratory paths (Nadarajah et al., 2002b; Fig. 1.3D). Specifically, Nadarajah et al. (2002a) have shown that cortical interneurons leave their migratory streams in the MZ, SP or IZ/SVZ and reach the CP through radial movement, a finding confirmed by Tanaka et al. (2003) and others (Yokota et al., 2007). Nadarajah et al. (2002a) also showed that, upon reaching the cortical wall, a substantial proportion of cortical interneurons actively seek the VZ, a mode of movement termed "ventricle-directed migration". After pausing in the VZ for an extended period of time, they migrate radially towards the pia to take up their positions in the CP. Focusing on the developing MZ, Ang et al. (2003) and, more recently, Tanaka et al. (2006, 2009) described multidirectional, long-distance and often prolonged movement of interneurons in this layer prior to descending radially to their positions in the CP. It has been speculated that this migratory behaviour in the MZ may contribute to their dispersion of cortical interneurons throughout the neocortex (Tanaka et al., 2009). Thus, it appears that, once interneurons reach the cortex through their confined streams, they adopt radial trajectories to find their right place in the CP.

1.7 Molecular mechanisms involved in the migration of cortical interneuron

1.7.1 Motogenic factors

After leaving the cell cycle, postmitotic cortical interneurons migrate away from their proliferative zones towards their final destination in the neocortex. It has been reported that GE-derived cells exhibit an intrinsic migratory capacity *in vitro* (Wichterle et al., 1999; Nery et al., 2002). Additional soluble factors have been thought to play a role in the initial movements of GE-derived cells from the GEs by acting as motogenic factors *in vivo*.

Hepatocyte Growth Factor/Scatter Factor (HGF/SF) has been largely documented as a promoter of cell motility for different cell lines (Birchmeier and Gherardi, 1998; Stella and Comoglio, 1999). Expression of HGF/SF and its receptor MET is present in the GEs and along the migratory routes of cortical interneurons in the developing brain (Powell et al., 2001). Experiments *in vitro* have shown HGF/SF enhances the migration of cortical interneurons (Powell et al., 2001). Analysis of urokinase-type plasminogen activator receptor deficient mice (where the inactive pro-form of HGF/SF is not cleaved to its active form) has also revealed a reduction in the number of interneurons in the neocortex, and also an accumulation of these cells in the corticostriatal border, demonstrating a essential function of HGF/SF in the motility of migrating cortical interneurons (Powell et al., 2001).

Several lines of research suggest that members of the neurotrophin family can act as motogenic factors for the migration of cortical interneurons. First, neurotrophins are widely expressed in the developing neocortex (Maisonpierre et al., 1990; Timmusk et al., 1993; Friedman et al., 1998; Fukumitsu et al., 1998) and have been proposed to be pivotal in neuronal migration (Behar et al., 1997, 2000; Brunstrom et al., 1997). Second, tyrosine kinase receptors B and C (TrkB and TrkC, respectively), the cognate receptors for neurotrophins, are expressed in cortical interneurons (Klein et al., 1990; Gorba and Wahle, 1999). Third, brain derived neurotrophic factor (BDNF) and neurotrophin 4 stimulate interneuron migration *in vitro* (Polleux et al., 2002). Fourth, analysis of TrkB deficient (*TrkB*$^{-/-}$) mice revealed a significant reduction in the number of cortical interneuron, emphasising the role of these molecules as motogenic factors (Polleux et al., 2002). However, it has been documented that disruption of BDNF signalling leads to down regulation of CB and neuropeptides expressed in interneurons (Jones et al., 1994; Arenas et al., 1996; Fiumelli et al., 2000), casting some doubt as to whether the reduction

of interneurons in the *TrkB*$^{-/-}$ animals reflects an actual defect in their migration or simply a disruption of intracellular proteins in interneurons. New evidence suggests that neurotrophins are indeed required for the migration of cortical interneurons. Examination of nestin-BDNF transgenic mice has revealed that over-expression of BDNF leads to an inappropriate integration of interneurons within the CP, as well as abnormal segregation of Cajal-Retzius cells and interneurons in the MZ (Alcantara et al., 2006). Therefore, it seems that BDNF signalling regulates the distribution of both Cajal-Retzius cells and interneurons in the MZ and participates in the allocation of cortical interneuron within the CP.

Neurotransmitters such as GABA or serotonin (5-HT) have an active role in controlling the migration of cortical neurons, including interneurons (Behar et al., 1996, 1998, 1999, 2000; Manet et al., 2006; Heng et al., 2007). Firstly, chemotaxis and pharmacological experiments have demonstrated that cortical neurons respond to GABA in a concentration-dependent manner (Benhar et al., 1996) Thus, low concentration of GABA promotes cell migration, whereas higher concentrations induce random movements (Behar et al., 1996). Secondly, disruption of GABA receptors (GABARs) leads to alterations in the migratory dynamics of cortical neurons *in vitro* (Behar et al., 1996, 1998). Thirdly, GABA expression is present in the migratory paths of cortical interneurons and, furthermore, these cells express GABARs (Lopez-Bendito et al., 2003; Cuzon et al., 2006). Fourthly, transplantation studies revealed that type A GABARs signalling is necessary for cortical interneurons to traverse the cortical-striatal notch en route to the neocortex (Cuzon et al., 2006). Finally, type B GABARs signalling is required for the correct navigation of interneurons within the developing cortex (Lopez-Bendito et al., 2003). A number of investigators have also shown that L-Glutamate receptors, such as NMDA, kinate and AMPA, are expressed in cortical interneurons, and these receptors are thought to participate in their migration, possibly through the increase of intracellular calcium (Metin et al., 2000; Poluch et al., 2001; Soria and Valdeomillos, 2002). How cortical interneurons cease their journey to take up their correct positions within the developing neocortex is largely unknown. However, early data indicated that GABA plays a significant role in the cessation of their movement (Behar et al., 1996). Moreover, it has been recently shown that interneurons up regulate the potassium-chloride co-transporter (KCC2), after reaching the neocortex. Up regulation of KCC2 results in reduction of cortical interneuron motility through the activation of GABARs and the diminution of their membrane potential (Bortone and Polleux, 2009).

5-HT is another neurotransmitter that is thought to affect cortical interneuron migration (Vitalis et al., 2007; Riccio et al., 2009). Pharmacological treatment of rodent embryos, using the specific DL-P-chlorophenylalanine inhibitor (PCPA) of 5-HT synthesis, has revealed alterations in the cytoarchitecture of the neocortex (Vitalis et al., 2007). Interestingly, 5-HT depletion after treatment with PCPA results in altered incorporation of cortical interneurons into the CP and it affects the differentiation of interneuron expressing CR and/ or CCK (Vitalis et al., 2007). In addition, recent work by Riccio and colleagues (2009) also suggests that 5-HT, through the activation of 5-HT6 receptors (expressed by cortical interneurons), can significantly reduce the migration of interneurons in the developing brain, which confirms the important role of 5HT in interneuron motility.

1.7.2 Chemotactic molecules

Once cortical interneurons have initiated their migration, extracellular guidance systems (chemotactic factors) impart directionality throughout their migration towards the neocortex. A combination of chemoattractive and chemorepulsive cues expressed within the pallium and subpallium, respectively, is thought to direct cortical interneurons from the GEs to the cerebral cortex (Marin and Rubenstein, 2003; Metin et al., 2006; Hernandez-Miranda et al., 2010; see Fig. 1.4). Indeed, *in vitro* evidence from slice culture assays illustrates that the neocortex does provide attractive cues, whilst the subpallial areas produce repulsive factors for migrating cortical interneurons (Polleux et al., 2002; Marin et al., 2003; Wichterle et al., 2003).

The chemokine stromal-derived factor 1 (SDF-1, also known as CXCL12) is a well-known chemoattractant for leukocytes, germ cells and neurons (Tashiro et al., 1993; Bleul et al., 1996; Lazarini et al., 2000; Klein et al., 2001; Doitsidou et al., 2002; Li et al., 2008; Liapi et al., 2008). SDF-1 is highly expressed in the leptomeninx and the IZ/SVZ in the developing neocortex (Tham et al., 2001; Zhu et al., 2002; Tiveron et al., 2006). Stumm and colleagues (2003, 2007) identified that the CXC chemokine receptor 4 (CXCR4, receptor for SDF-1) is expressed in Cajal-Retzus cells and tangentially migrating interneurons within the developing neocortex, and showed that SDF-1 serves as a chemoattractant for migrating interneurons on their way to the CP. In addition, more recent evidence illustrates that SDF-1 signalling is essential for both radial (projection neurons) and tangential (interneurons) migration within the cortical wall (Liapi et al., 2008). Interestingly, it has been suggested that this chemokine is not required for the

migration of interneurons from the subpallium to the cortex, but is crucial to maintain interneurons migrating tangentially once they enter the cortical wall (Stumm et al., 2003; Tiveron et al., 2006; Li et al., 2008; Liapi et al., 2008; Lopez-Bendito et al., 2008). Interestingly, it seems that the effect of SDF-1 on migrating interneurons is time-dependent since late-born, but not early-born, interneurons failed to integrate into their appropriate cortical layer in the absence of SDF-1 signalling (Stumm et al., 2003). Thus, several studies indicate that SDF-1 is also expressed by projection neuron progenitors in the cortical SVZ and this expression has been speculated to be crucial for the recognition of the IZ/SVZ path by migrating interneurons (Tiveron et al., 2006; Stumm et al., 2007).

The family of neuregulin growth factors (NGRs) contains four structurally related genes and a number of chemotactic cues that result from the alternative splicing of the genes that encode for them (Birchmeier, 2009). NRGs are ligands for receptor tyrosine kinases of the ErbB (ErB) family and activate a wide spectrum of intracellular signalling cascades, resulting in the induction of distinct cellular responses in different organs (Buonanno and Fischbach, 2001; Falls, 2003a,b; Anton et al., 2004; Britsch, 2007). NGRs have been related to a large number of important events in the developing nervous system (Falls, 2003a; Anton et al., 2004). Specifically, several lines of evidence suggest that NRG-1 acts as a chemoattractant for migrating cortical interneurons (Yau et al., 2003; Flames et al., 2004). Firstly, ErB4 is expressed in tangentially migrating neurons and co-localise with the interneuron marker Dlx2 (Yau et al., 2003). Secondly, soluble NGR1 is expressed in the cortical proliferative zones, and has been hypothesised to attract migrating interneurons to the IZ/SVZ path (Flames et al., 2004; Ghashghaei et al., 2006). Thirdly, secreted NGR1 is a potent chemoattractant for MGE-derived cells *in vitro* (Flames, 2004). Fourthly, loss-of-function assays have demonstrated that the migration of cortical interneurons depends on ErB4 signalling as their numbers in the cerebral cortex are significantly decreased in conditional ErB4 mutants (Flames et al., 2004).

To date, the chemorepulsive molecules expressed in the subpallium remain largely unknown. Nonetheless, the membrane-bound Ephrin family of ligands and their Eph-receptor tyrosine kinases (Eph) have been recently proposed as chemorepellants for migrating cortical interneurons (Zimmer et al., 2008). Experimental evidence shows that Ephrins can direct migration and enhance the motility of neurons *in vitro* and *in vivo* (Santiago and Erickson, 2002; Klein, 2004; Nomura et al., 2006; Zimmer et al., 2007). Zimmer and colleagues (2008) have reported that CB^{+} cells isolated from the MGE express EphA4 receptor. In addition, these authors showed that EphrinA5 and its receptor EphA4 are complementarily expressed in the VZ and SVZ of the GEs, respectively

(Zimmer et al., 2008). Moreover, *in vitro* stripe assays have further demonstrated that EphrinA5 is a potent chemorepellent for MGE-derived cells (Zimmer et al, 2008).

1.7.3 Channelling cortical interneurons to their proper migratory paths

GE-derived cortical interneurons *en route* to the neocortex encounter the developing striatum and avoid entering within it. It is thought that repulsive cues expressed within the developing striatum create an exclusion zone for cortical interneurons and participate in channelling them into their appropriate adjacent paths (Marin et al., 2001; Metin et al., 2006). The first molecules to be proposed as directly involved in maintaining cortical interneurons away from the striatum were two members of the family of class 3 semaphorins, semaphorin3A (Sema3A) and semaphorin3F (Sema3F) which are abundantly expressed in the developing striatum (Marin et al., 2001). Class 3 semaphorins are mediated by neuropilin (Nrp) and PlexinA receptors (Neufeld and Kessler, 2008; Roth et al., 2009). Together, class 3 semaphorins and the receptors and intracellular effectors activated by them, form the class 3 semaphorin signalling system (Tamagnone and Comoglio, 2000). *In vitro* and *in vivo* studies have shown that cortical interneurons contain Nrp1 and Nrp2 receptors and they respond to the chemorepulsive activity induced by Sema3A and Sema3F (Marin et al., 2001; Tamamaki et al., 2003a). Marin et al. (2001) showed that loss of Nrp function leads to increased number of cortical interneurons within the striatum, emphasising the relevance of class 3 semaphorins in maintaining the developing striatum clear of cortical interneurons, in addition to channelling these cells in the appropriate migratory paths (Marin et al., 2001). Recent analysis of postnatal Nrp2 deficient mice showed a significantly reduced number of interneurons within their hippocampus and neocortex when compared to control counterparts (Gant et al., 2009), indicating again that class 3 semaphorin signalling system plays a significant role in cortical interneurons migration. In addition, recent evidence suggests proteoglycans might play as well a substantial role in maintaining cortical interneurons away from the developing striatum by stabilising the diffusion of class 3 semaphorins within this area (Zimmer et al., 2010). This is the case of chondroitin-4-sulfate, which is co-expressed with Sema3A (Zimmer et al., 2010). Moreover, chondroitin-4-sulfate can bind directly to sema3A and prevent the diffusion of of it from the developing striatum (Zimmer et al., 2010). Additionally, *in vitro* studies by Zimmer and colleagues (2010) have demonstrated that chondroitin-4-sulfate has an intrinsic chemorepulsive action on cortical interneurons.

1.7.4 Role of Slit-Robo signalling system in cortical interneuron migration

Slit proteins are large secreted chemotactic molecules, which are evolutionary conserved (Rothberg et al. 1990). To date, three members of this family have been recognised, Slit1, Slit2 and Slit3 (Holmes et al., 1998; Itoh et al., 1998; Brose et al., 1999; Li et al., 1999). Slit ligands were first identified as factors involved in the development of midline glial cells and were subsequently found to be midline axon repellents (Battye et al., 1999; Kidd et al., 1999). The chemorepulsive activity of the Slit family is mediated by members of the Roundabout (Robo) receptor family (Kidd et al., 1998a).

The Robo receptor family belongs to the immunoglobulin (Ig) super family of cell adhesion molecules (CAM), which are conserved throughout evolution (Sundaresan et al., 1998a,b; Bashaw et al., 2000). Four members of the Robo family have been identified in vertebrates: Robo1, Robo2, Robo3 (also known as Rig1) and Robo4 (also known as magic roundabout) (Kidd et al., 1998a,b; Yuan et al., 1999a; Huminiecki et al., 2002). Robo1 and Robo2 are widely expressed during development and in adult life (Holmes et al. 1998), while Robo3 expression is restricted to the developing CNS, and Robo4 is specifically found in endothelial cells (Huminiecki et al., 2002; Park et al., 2003). All four Robo receptors have been shown to bind similarly to Slit proteins (Park et al., 2003; Liu et al., 2004; Mambetisaeva et al., 2005; Cammuri et al., 2005).

During brain development, *Slit1* is strongly expressed throughout the VZ and SVZ of the GEs, as well as at the ventral midline and in other basal regions of the forebrain (Yuan et al., 1999b; Bagri et al., 2002; Marillat et al., 2002; Whitford et al., 2002). Robo (Robo1, Robo2 and Robo3) receptors show distinct, but complementary expression patterns to *Slit1* expression (Yuan et al., 1999b; Bagri et al., 2002; Marillat et al., 2002; Whitford et al., 2002). *Robo1* and *Robo2* expression corresponds to subpallial regions through which cortical interneurons migrate and overlaps with their migratory paths at the level of MZ and IZ/SVZ layers in the developing neocortex (Andrews et al., 2006, 2007, 2008). Robo3 is expressed mainly in the GEs and the cortical MZ during early development, but it is down regulated by E13 (Cammuri et al., 2005; Barber et al., 2009).

Evidence that Slit plays a role in the migration of GE-derived interneurons came from *in vitro* studies carried by Zhu et al. (1999), which show that cell aggregates expressing Slit proteins do repel co-explants of GE-derived interneurons. Moreover, it has also been shown that Robo1, Robo2 and Robo3 are expressed in cortical interneurons

(Andrews et al., 2006, 2007, 2008; Barber et al., 2009), suggesting that Slit-Robo signalling might play a pivotal role in their migration.

Surprisingly, cell tracing studies carried out on slice cultures prepared from Slit1/Slit2 double deficient mice (*Slit1*$^{-/-}$/ *Slit2*$^{-/-}$ mice) have shown no defects in the tangential migration of cortical interneurons (Marin et al., 2003). Furthermore, no differences in the number or distribution of GABAergic interneurons (GABA^{+}, Lhx6^{+} or Dlx2^{+} cells) were detected in the cortices of *Slit1*$^{-/-}$/*Slit2*$^{-/-}$ mice when compared to control littermates (Marín et al., 2003), suggesting then that Slit signalling does not play an essential role in cortical interneuron migration. Nevertheless, analysis of Robo1 deficient (exon 5 deleted) transgenic mice has illustrated that there exists a significant increase in CB^{+} cells within the embryonic cortex of these animals when compared to control littermates (Andrews et al., 2006). This result has been subsequently confirmed and extended in a new line of Robo1 deficient Dulox mice (hereafter *Robo1*$^{-/-}$ mice) in which the full Robo1 receptor gene (exons 1-22 inclusive) has been floxed and deleted (Andrews et al., 2008). Moreover, Andrews and colleagues (2008) have documented that this increase in interneuron numbers persists into adulthood. Interestingly, this effect has only been observed within the rostral-middle, but not in the caudal portions of the cerebral cortex (Andrews et al., 2006), suggesting that deletion of Robo1 receptor alters the migration and/or generation of MGE-derived cortical interneurons, but not those cortical interneurons generated in the CGE or LGE. Analysis of Robo2 or Robo3 deficient mice (*Robo2*$^{-/-}$ and *Robo3*$^{-/-}$ mice, respectively) showed no differences in the number or position of CB^{+} cell in their cortices when compared to their control litermates, suggesting that Robo2 and Robo3 receptors might not be involved in the migration of cortical interneurons. Interestingly, a detailed inspection of *Robo1*$^{-/-}$, *Robo2*$^{-/-}$ and *Robo3*$^{-/-}$ mice has shown marked alterations in the morphology of migrating cortical interneurons during development, indicating that Robo signalling participates in the elaboration of neurites by these cells (Andrews et al., 2006, 2008; Barber et al., 2009). In addition to the increased number of CB^{+} cells observed in the developing cortex of Robo1 deficient (exon 5 deleted) mice, Andrews and colleagues (2006) reported increased staining of CB in the striatum of these animals when compared to control littermates, suggesting that the absence of Robo1 receptor results in aberrant migration of cortical interneurons through this structure. However, CB is also expressed by the vast majority of striatal projection neurons (Ouimet et al., 1988; Liu and Gaybriel, 1992) and thus, the exact nature of the increased CB^{+} cells in the developing striatum is presently unknown.

1.8 Aims

Given that the striatum of *Robo1*$^{-/-}$ mice shows increased CB staining than control littermates, it seems that deletion of Robo1 receptor may augment striatal cells in these animals. Alternatively, deletion of the receptor may result in aberrant migration of cortical interneurons through the striatum of *Robo1*$^{-/-}$ mice. Thus, it is the main goal of the present work to elucidate what occurs in the developing striatum of *Robo1*$^{-/-}$ mice.

Specifically, I will investigate whether the striatum of *Robo1*$^{-/-}$ mice contains more striatal projection neurons. If this is the case, I will investigate how Robo1 receptor regulates the cell cycle of LGE progenitor (precursors of these striatal cells). Alternatively, if I find no difference in the number of striatal cells (either projection – or inter –neurons), I will explore whether migration of cortical interneurons occurs through the striatum of *Robo1*$^{-/-}$ mice. If this is the case, I will investigate the responsiveness of *Robo1*$^{-/-}$ cortical interneurons to Sema3A and Sema3F cues. If these cells show loss of responsiveness to these cues, I will then study the expression of Nrp or PlexinA receptors as well of the intracellular effectors activated by Sema3A and Sema3F in *Robo1*$^{-/-}$ cortical interneurons.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were obtained from Gibco (Paisley, Scotland, UK), Invitrogene Ltd (Carlsbad, CA, USA), Millipore (Temecula, CA, USA), Promega (Madison, WI, USA) Sigma (St. Louis, MO, USA), Vector Laboratories (Burlingame, CA, USA) and VWR International Ltd (Leicestershire, England, UK) unless otherwise stated.

2.1.2 Animals

Experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and institutional guidelines. *Robo1*$^{-/-}$, *Robo2*$^{-/-}$, and *Slit1*$^{-/-}$/ *Slit2*$^{-/-}$ transgenic mice were generated as described previously (Andrews et al., 2008; Lu et al., 2007; Plump et al., 2002, respectively). *GAD67-GFP*$^{neo/-}$ mice (Tamamaki et al., 2003b) were also used in this study. Adult *Robo2*$^{nestin/cre}$ mice were kindly provided by Dr. J.F. Cloutier (Cho et al., 2007), for simplicity I will name these animals merely as adult *Robo2*$^{-/-}$ mice. All mouse strains were maintained in a C57/bl6J background obtained from Charles River Ltd. The day the vaginal plug was found was considered as E0.5, and day of birth was considered as postnatal day (P) 0.

2.1.3 Cell lines

GN11 cell were generously provided by Drs. A. Cariboni and R. Maggi (University of Milan, Milan, Italy), and COS-7 cells were obtained from American Type Culture Collection (Manassas, VA, USA).

2.1.4 Bacteria

DH5α (Invitrogen) and XL10gold (Stratagene, England, UK) chemically competent strains of *Escherichia coli* bacteria were used for the standard propagation of DNA plasmids.

2.1.5 Primers

2.1.5.1 Primers for genotyping

Table 2.1 Panel of primers used in the present study for Genotyping

Gene	Condition	Secuence (5'->3')	AT/PS	Reference
Robo1	Wild type **(F)**	CTTTCCCATTGAGCCATAAG	59°C/	Andrews et al.,
Robo1	Wild type **(R)**	GATACCCCAGGAATAGAACA	450 bp	2008
Robo1	Knockout **(F)**	CTTTCCCATTGAGCCATAAG	59°C/	Andrews et al.,
Robo1	Knockout **(R)**	GCATCTTGTCCATACATGGA	450 bp	2008
Robo2	Wild type **(F)**	AAATGAAATATCCCCAAATTAGAGC	59°C/	Lu et al., 2007
Robo2	Wild type **(R)**	TCTTTTTCTGCTTTGAACAACAA	1000 bp	
Robo2	Knockout **(F)**	AAATGAAATATCCCCAAATTAGAGC	59°C/	Lu et al., 2007
Robo2	Knockout **(R)**	ATTTGCTGAGAGCAGGCATT	1500 bp	
Slit1	Wild type **(F)**	AAGATGCCTCCTCTGACTTC	60°C/	Plump et al.,
Slit1	Wild type **(R)**	ACCCTTAGCTTCTACCAACC	470 bp	2002
Slit1	Knockout **(F)**	TCTCCTTTGATCT GAGACCG	60°C/	Plump et al.,
Slit1	Knockout **(R)**	AGGTTTCTCGAGCGTCATAG	550 bp	2002
Slit2	Wild type **(F)**	AAGACCTGTCGCTTCTGTCAG	60°C/	Plump et al.,
Slit2	Wild type **(R)**	AAACAGGTTTCTACCGCACG	470 bp	2002
Slit2	Knockout **(F)**	AAGACCTGTCGCTTCTGTCAG	60°C/	Plump et al.,
Slit2	Knockout **(R)**	AAGTCTAGTAGAGTCGAGCG	470 bp	2002

Abbreviation: AT, anneal temperature; bp, base pairs; F, forward primer; PS, product size; R, reverse primer.

2.1.5.2 Primers for Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Table 2.2 Panel of primers used in the present study for RT-PCR

Gene	Primer set	Sequence (5'->3')	AT/PS
Robo1	Primer A **(F)**	GCTCCTCGGTTTAGGCTCTT	60°C/
Robo1	Primer A **(R)**	TCATTGTCCTCGGGTAGGTC	162 bp
Robo1	Primer B **(F)**	TGGAAACATCTTCCTCTTCTGG	60°C/
Robo1	Primer B **(R)**	GTAGCCCAGCGAATTGTCAT	240 bp
Robo2	Primer A **(F)**	AACGAAGGAGGAGTGCTCTG	60°C/
Robo2	Primer A **(R)**	CGAAGACGAGATCCTTGACC	318 bp
Robo2	Primer B **(F)**	TTGCTCTTTGGATTTCTCTGC	60°C/
Robo2	Primer B **(R)**	CACCCTCTCACCATCCTTGT	154 bp
Robo3	Primer A **(F)**	AAGAACCAGCGAAGGAGGAC	60°C/
Robo3	Primer A **(R)**	GAGGGATCTCCAGGAGGAAG	416 bp
Robo3	Primer B **(F)**	TCACCAAAGCATGCTCAGTC	60°C/
Robo3	Primer B **(R)**	GGAAGACCAGGTTTGCTCTG	290 bp

Abbreviation: AT, anneal temperature; bp, base pairs; F, forward primer; PS, product size; R, reverse primer; RT-PCR, reverse transcription polymerase chain reaction.

2.1.5.3 Primers for quantitative real-time Polymerase Chain Reaction (QPCR)

Table 2.3 Panel of primers used in the present study for QPCR

Gene	Primer	Secuence (5'->3')	AT
Akt	Forward	ATGAACGACGTAGCCATTGTG	60°C
Akt	Reverse	TTGTAGCCAATAAAGGTGCCAT	60°C
Cdc42	Forward	AAAAGTGGGTGCCTGAGATAAC	60°C
Cdc42	Reverse	GGCTCTTCTTCGGTTCTGGAG	60°C
Crmp1	Forward	GCATCCCGCACATCACCAG	60°C
Crmp1	Reverse	TTCACTCCACCAGGAACAATCAG	60°C
Dlx1	Forward	ATGCCAGAAAGTCTCAACAGC	60°C
Dlx1	Reverse	AACAGTGCATGGAGTAGTGCC	60°C
Dlx2	Forward	GTGGCTGATATGCACTCGACC	60°C
Dlx2	Reverse	GCTGGTTGGTGTAGTAGCTGC	60°C
Farp2	Forward	GTTATTTGACATCGAGCCCGAAGT	60°C
Farp2	Reverse	GAACTCCAGACCAAAGTAGTCAC	60°C
Flt1	Forward	TGGCTCTACGACCTTAGACTG	60°C
Flt1	Reverse	CAGGTTTGACTTGTCTGAGGTT	60°C
Gapdh	Forward	AGGGCATCTTGGGCTACAC	60°C
Gapdh	Reverse	CATACCAGGAAATGACGTTGA	60°C
Kdr	Forward	TTTGGCAAATACAACCCTTCAGA	60°C
Kdr	Reverse	GCAGAAGATACTGTCACCACC	60°C
Lhx6	Forward	GCCGCATCCATTACGACACC	60°C
Lhx6	Reverse	TGGCTGGCTTGGGCTGAC	60°C
Lhx8	Forward	TCAGAGAGTGGTTACGGTCAC	60°C
Lhx8	Reverse	CTGCTCGTCACATACCAGCTC	60°C
Nkx2.1	Forward	CGAGCGGCATGAATATGAG	60°C
Nkx2.1	Reverse	GACCTGCGTGGGTGTCAG	60°C
Nrp1	Forward	GGATGGATTCCCTGAAGTTG	60°C
Nrp1	Reverse	TGGATAGAACGCCTGAAGAG	60°C
Nrp2	Forward	GCTGGCTACATCACTTCCCC	60°C
Nrp2	Reverse	CAATCCACTCACAGTTCTGGTG	60°C
PlexinA1	Forward	CAGCACAGACAACGTCAACAA	60°C
PlexinA1	Reverse	GCTTGAAGAGATCGTCCAACC	60°C
PlexinA2	Forward	AACCTGTCTGTGGTTCTGCTC	60°C
PlexinA2	Reverse	TCCAGTCACGATTCTCAGAGT	60°C
PlexinA3	Forward	CAGATACCACTCTGACTCACCT	60°C
PlexinA3	Reverse	GGCCCGTAGCTCAGTTAGG	60°C
PlexinA4	Forward	TGAGGACAACCCCAAGTGTTA	60°C
PlexinA4	Reverse	ACGCGATCAGCCTGTTTTCT	60°C
Rac1	Forward	CAAGTGTGTGGTGGTGGGAGAC	60°C
Rac1	Reverse	CATAACATTGGCAGAATAGTTGTCAAAGAC	60°C
Rnd1	Forward	CAGTTGGGCGCAGAAATCTAC	60°C
Rnd1	Reverse	TGGGCTAGACTTGTTCAGACA	60°C
Robo1	Forward	GACCTGATCGTCTCCAAAGGA	60°C
Robo1	Reverse	TTGTCGGTCTCCACTCTTTCC	60°C
Sema3A	Forward	CCAAGACTGAAATTATCGTACAAAGAAATG	60°C
Sema3A	Reverse	AGAAGGAAGGTGTGGTAACTGGAG	60°C
Sema3F	Forward	CATCTGCCTCAACGATGACG	60°C
Sema3F	Reverse	AGAGCCTGAAGAGGTAAAGACA	60°C
VegfR3	Forward	GGTTGTGCATGACTGTGAAGG	60°C
VegfR3	Reverse	GCGTGTCAGGTTTGTTGATGAA	60°C

Abbreviation: AT, anneal temperature; bp, base pairs; QPCR, quantitative real-time polymerase chain reaction.

2.1.6 Antibodies

2.1.6.1 Primary antibodies

Table 2.4 Panel of primary antibodies used in the present study

Antibody	Host	Source	Cat #	Use/ Dilution	Reference
anti-CB	rabbit polyclonal	Swant	CB-38	IHC 1:2000 IF: 1:1000	Andrews et al., 2008
anti-ChAT	goat polyclonal	Millipore	AB144P	IHC 1:250	
anti-CR	rabbit polyclonal	Swant	7699/4	IHC 1:2000	
anti-DARPP-32	rabbit polyclonal	Millipore	AB1656	IHC 1:500	
anti-FLAG	mouse monoclonal	Sigma	F3165	IF 1:250 WB 1:5000	
anti-FOXP2	rabbit polyclonal	Abcam	Ab16046	IHC 1:750 IF 1:200	
anti-myc	mouse monoclonal	Sigma	M5546	IF 1:250 WB 1:2000	
anti-Nrp1	rabbit polyclonal	Santa Cruz	H-286	IF 1:100 WB 1:1000 IP 1:1000	Cariboni et al., 2007
anti-Nrp2	rabbit polyclonal	Santa Cruz	H-300	IF 1:100 WB 1:1000 IP 1:1000	Cariboni et al., 2007
anti-PlexinA1	rabbit polyclonal	Santa Cruz	H-60	WB 1:1000 IP 1:1000	
anti-PV	mouse monoclonal	Swant	235	IHC 1:250	
anti-Robo1	rabbit polyclonal	Prof F. Murakami		IF 1:5000 WB 1:1000 IP 1:1000	Andrews et al., 2006
anti-Robo2	rabbit polyclonal	Prof F. Murakami		IF 1:5000	Andrews et al., 2007
anti-Robo3	rabbit polyclonal	Prof F. Murakami		IF 1:1000	Barber et al., 2009
anti-SOM	rabbit polyclonal	Millipore	AB5494	IHC 1:100	
ß-Tubulin	mouse monoclonal	Sigma	T-4026	IP 1:1000	

Abbreviations: Cat #, catalogue number; CB, calbindin; ChAT, choline acetyltransferase; CR, calretinin; DARPP-32, dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa; FOXP2, forkhead homeobox P2; GFP, green fluorescent protein; IF, immunofluorescence; IHC, immunohistochemistry**;** IP, immunoprecipitation; Nrp1, neuropilin 1; Nrp2, neuropilin 2 and PV, parvalbumin; SOM, somatostatin; WB, Western blot.

2.1.6.2 Secondary antibodies

Table 2.5 Panel of secondary antibodies used in the present study

Antibody	Host	Source	Cat #	Dilution	Use
Biotinylated anti-goat IgG	rabbit	Vector Laboratories	BA-5000	1:250	IHC
Biotinylated anti-mouse IgG	horse	Vector Laboratories	BA-2080	1:250	IHC
Biotinylated anti-rabbit IgG	goat	Vector Laboratories	BA-1000	1:250	IHC
Peroxidase anti-rabbit IgG	goat	Vector Laboratories	PI-1000	1:250	WB/IP
Peroxidase anti-mouse IgG	horse	Vector Laboratories	PI-2000	1:250	WB/IP
Alexa 568 anti-rabbit IgG	goat	Invitrogen	A-11008	1:250	IF

Abbreviations: Cat #, catalogue number; IF, immunofluorescence; IHC, immunohistochemistry IP, immunoprecipitation; WB, Western blot.

2.1.7 Plasmids

Table 2.6 Panel of plasmids used in the present study

Plasmid name	Host	Gene inserted	Use	Source	Reference
CAG-IRES-EGFP		*GFP tag*	Control plasmid	Dr. M. Hoshimo	Friocourt et al., 2007
p3xFLAG-CMV-8		*Flag tag*	Control plasmid	Invitrogen	Cariboni et al., 2007
pCDNA3.1-myc		*Myc tagged*	Control plasmid	Invitrogen	Patel et al., 2001
Sema 3A-myc	chicken	*FL-Semaphorin 3A*	Chemorepulsion	Dr. A. Cariboni	Cariboni et al., 2007
Sema 3F-flag	mouse	*FL-Semaphorin 3F*	Chemorepulsion	Dr. A. Cariboni	Cariboni et al., 2007
Slit1-myc	human	*FL-Slit1*	Chemorepulsion	Dr. V. Sundaresan	Patel et al., 2001
Slit2-myc	human	*FL-Slit2*	Chemorepulsion	Dr. V. Sundaresan	Patel et al., 2001
Robo1-myc	human	*FL-Robo1*	IP	Dr. V. Sundaresan	Hivert et al., 2002
Nrp1-myc	human	*FL-Nrp1*	IP	Dr. A. Cariboni	Cariboni et al., 2007
Nrp2-myc	human	*FL-Nrp2*	IP	Dr. A. Cariboni	Cariboni et al., 2007
DN Robo1-GFP	human	*Truncated-Robo1*	Functional blocking	Dr. V. Sundaresan	Hammond et al., 2005

Abbreviations: DN, dominant negative; FL, full length; IP, immunoprecipitation.

2.1.8 RNA probes for in situ hybridisation

Table 2.7 Panel of RNA probes for in situ hybridisation used in the present study

Probe	Type	Linearisation	Transcription	Source	Institution
Dlx1	Sense Antisense	HindIII EcoRI	T7 T3	Dr. N Kessaris Dr. N Kessaris	Wolfson Institute, UCL, UK
Lhx6	Sense Antisense	EcoR1 NotI	T7 T3	Dr. N Kessaris Dr. N Kessaris	Wolfson Institute, UCL, UK
Lhx8	Sense Antisense	EcoRI NotI	Sp6 T3	Dr. N Kessaris Dr. N Kessaris	Wolfson Institute, UCL, UK
Nkx2.1	Sense Antisense	HindIII EcoRI	T3 T7	Dr. N Kessaris Dr. N Kessaris	Wolfson Institute, UCL, UK
Nrp1	Sense Antisense	HindIII NotI	Sp6 T7	Dr. C Ruhrberg Dr. C Ruhrberg	Institute of Ophthalmology, UCL, UK
Nrp2	Sense Antisense	HindIII EcoRI	T3 T7	Dr. C Ruhrberg Dr. C Ruhrberg	Institute of Ophthalmology, UCL, UK
PlexinA1	Sense Antisense	NotI XhotI	T7 T3	Dr. N Perälä Dr. N Perälä	University of Helsinki, Finland
PlexinA2	Sense Antisense	NotI BamHI	T3 T7	Dr. N Perälä Dr. N Perälä	University of Helsinki, Finland

2.2 Methods

2.2.1 Genotyping

Genotypes of transgenic animals were determined by PCR on genomic tail DNA. Tails were treated with 500 μg/ml of Proteinase K in lysis buffer (200 mM NaCl, 100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS; Sigma) at 55^{o}C overnight. Genomic DNA was extracted by phenol-chloroform method and eluted in water. The primers used in this study for genotyping are shown in Table 2.1. The PCR conditions for genotyping were as follow: one cycle of 94^{o}C, 2 minutes; followed by 35 cycles of: 94^{o}C, 1 minute; 60^{o}C, 1 minute; 72^{o}C, 2 minutes; and a final extension 72^{o}C for 7 minutes. PCR products were identified by 1-2% agarose gel electrophoresis.

2.2.2 Histological techniques

2.2.2.1 Tissue processing for immunohistochemistry and *in situ* hybridisation

Embryonic mice were harvested from sacrificed dams and their brains were carefully dissected out in cold artificial cerebral spinal fluid (ACSF, 25 mM KCl, 2 mM KH_2PO_4, 25 mM HEPES, 37 mM D-glucose, 10 mM $MgSO_4$, 175 mM sucrose, 0.5 mM $CaCl_2$ and 5000 units penicillin/5000 μg streptomycin solution in 1000 ml of distilled water, pH 7.4; Sigma) and immersion fixed in 4% paraformaldehyde made (4% PFA, pH 7.4) in sodium phosphate buffered saline (PBS, 0.15 M NaCl, 0.1 M sodium phosphate, pH 7.4; Sigma) overnight at 4^{o}C. Adult mice were perfused transcardially with 4% PFA-PBS, subsequently, their brains were removed from the skull and post-fixed in the same fixative at 4^{o}C overnight. Following fixation, embryonic and postnatal brains were cryoprotected in 30% sucrose made in PBS for two days at 4^{o}C. After cryoprotection, brains were embedded in a mixture of 15% sucrose made in PBS/50% Tissue-Tek OCT (Zoeterwoude, The Netherlands; Sakura Finetek Europe), frozen in 2-methylbutane (VWR International Ltd), and kept at -80^{o}C until needed.

2.2.2.2 Immunohistochemical techniques

Frozen brains were sectioned in the coronal plane at 10 or 20 μm [for immunofluorescence or 3,3'-Diaminobenzidine (DAB; Sigma) immunohistochemical detection, respectively] using a Cryostat (Bright Instruments, Huntingdon, UK). Sections were washed in PBS, blocked in blocking solution [3% albumin bovine serum (Sigma) made in PBS containing 0.1% Triton X-100 (PBST; Sigma)] at room temperature (Rt) for 2 hours. They were then incubated in primary antibodies (see Table 2.4) made in blocking solution for 2 hours at Rt, followed by overnight at 4^{o}C. Following incubation in primary antibodies sections were washed in PBST and incubated in appropriate secondary anti-species antibodies (see Table 2.5) for 3 hours at Rt. For immunofluorescence detection, after incubation in appropriate fluorescent antibodies, sections were washed in PBS and counterstained with 4'-6-Diamidino-2-Phenyllindole (DAPI, 1:20,000; D-9542: Sigma) made in PBS. For DAB immunohistochemical detection, after incubation in biotinilated antibodies, sections were washed in PBS, incubated in ABC complex solution (Vector laboratories) for 2 hours at Rt and processed immediately for DAB immunohistochemical detection.

2.2.2.3 Quantification of immuoreactive cells in the striatum

All morphometric analyses were conducted separately for the rostral, medial and caudal levels of the striatum based on the following anatomical landmarks. In embryonic tissue: the rostral level was considered where the septum was clearly identifiable, the medial level was selected where the intraventricular foramen and the anterior-dorsal thalamus were present, and the caudal level was chosen where the telo-diencephalic junction was distinguishable and the CGE was present. In postnatal tissue: the rostral level was chosen where the septum was clearly visible; the medial level was selected where the anterior commissure crosses bilaterally, and the caudal level was considered where the hippocampus was present.

To determine striatal area, sections were stained with 0.025% thionin solution for 2 minutes and rinsed through a series of alcohols (70-100%). Striatal area was estimated using the Image J free software (ImageJ; NIH, version 1.3). To assess the total number of immunoreactive cells throughout the rostral-caudal extend of the striatum, a minimum of 3 non-consecutive sections were stained for each marker per animal, age and condition.

2.2.2.4 *In situ* hybridisation

In situ hybridisation was performed as described elsewhere (Faux et al., 2010). Briefly, embryonic brains were dissected in sterile PBS and fixed in 4% PFA-PBS overnight, followed by cryoprotection in 30% sucrose treated with 0.01% Diethly Pyrocarbonate (DEPC; Sigma) made in PBS at 4^{o}C for two days. Then, brains were frozen in 15% sucrose made in DEPC-PBS/50%Tissue-Tek OCT and sectioned in the coronal plane at 12 μm. Subsequently, sections were dried for 2 hours at Rt before incubation in hybridisation buffer [1X DEPC treated 'Salts' (200 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.5, 5 mM $NaH_2PO_4.2H_2O$, 5 mM Na_2HPO4; Sigma); 50% deionized formamide (Ambion, CA, USA); 0.1 mg/ml RNAse-free yeast tRNA (Invitrogen); 1x Denhardts (RNase/DNase free; Invitrogen); 10% dextran sulphate (Sigma)] containing 100-500 ng/ml DIG labeled-RNA probes at 65^{o}C overnight. DIG tagged sense and antisense RNA probes were generated by linearisation and reverse transcription of plasmids with their appropriate enzymes (see Table 2.7 and section 2.2.4.7). Following hybridisation, sections were washed 3 times in 50% formamide 1XSSC (Ambion) and 0.1% tween-20 (Sigma) at 65^{o}C and 2 times at Rt in 1X MABT (20 mM Maleic acid, 30 mM NaCl, 0.1% tween-20; Sigma) before incubating in blocking solution [2% blocking reagent (Roche, Mannheim, Germany), 10% normal goat serum (Vector Laboratories) in 1X MABT], followed by overnight incubation in alkaline phosphatase conjugated anti-DIG antibody (1:1500; Roche). Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche) diluted 1:1000 in 1X MABT with 5% polyvinyl alcohol (VWR International Ltd) was used for the colorimetric detection for 6-16 hours at Rt. Sections were mounted using Glycergel Mounting Medium (Dako, CA, USA).

2.2.3 *In vitro* cell culture experiments

2.2.3.1 Cell cultures of COS-7 and GN11 cells

COS-7 and GN11 cells were cultured as a monolayer on 10 cm Petri dishes in cell culture medium (DMEM-F12 with 10% FBS and 2 mM L-glutamine and 2500 units penicillin/2500 μg streptomycin) in a humidified incubator (95%Air/5%CO_2) at 37^{o}C. The cell culture medium was replaced at 2 days *in vitro* (DIV) intervals. At 80% of confluence, cells were passaged by trypsination as described below for dissociated cell

cultures (see section 2.2.3.4) after a single wash with sterile PBS at Rt. Cells within a minimum of six passages were used in all experiments.

2.2.3.2 Transfection of COS-7 and GN11 cells

COS-7 and GN11 cells (at 80% of confluence) were grown in cell culture medium in the absence of antibiotics for 24 hours in the humidified incubator at 37^{o}C. Afterwards, cells were incubated for 3 hours at 37^{o}C with the selected plasmid (1 μg/ml; see Table 2.6) in the presence of the Lipofectamine-2000 (Invitrogen) according to the manufacturer's instructions.

2.2.3.3 Production of semaphorin and Slit-conditioned media

Conditioned media (CM) from *semaphorin3A-myc* plasmid (CM-SEMA3A), *semaphorin3F-FLAG* plasmid (CM-SEMA3F), *Slit1-myc* plasmid (CM-SLIT1), *Slit2-myc* plasmid (CM-SLIT2), *pCDNA3.1-myc* plasmid (CM-myc), *p3xFLAG-CMV-8* plasmid (CM-FLAG) transfected COS-7 cells were obtained by leaving transfected COS-7 cells in serum-free medium for 24 hours in the humidified incubator at 37^{o}C. Cell supernatants were collected in ice-cold tubes, centrifuged at 3000 g for 5 minutes at Rt, and immediately used for chemotactic assays (see below). Secretion of chemotactic cues was confirmed by immunohistochemistry and Western blot (WB) analysis using anti-myc, or anti-FLAG antibodies (see table 2.4).

2.2.3.4 Dissociated MGE cell cultures and morphological assessment

Dissociated cell cultures were prepared from E13.5 mouse brains according to the method of Cavanagh et al. (1997). Briefly, MGE tissues were dissected out in cold ACSF under a stereo microscope. They were incubated in trypsinisation medium [0.05% trypsin (Sigma) with 100 μg/ml DNaseI (Roche) in Neurobasal medium (Invitrogen)] for 15 minutes at 37^{o}C. Trypsinisation was quenched with neutralisation medium [10% of FBS (Gibco) in Neurobasal medium] for 5 minutes at 37^{o}C. MGE tissues were then triturated with a fire-polished Pasteur pipette until no cellular aggregates were visible. The homogenous cell suspensions were subsequently pelleted by centrifugation at 1,000 x g for 3 minutes. A concentration of 300 cells/μl was plated out on 13mm coverslips coated with 10 μg/ml poly-L-lysine and 10 μg/ml laminin and left for 30 minutes at 37^{o}C to allow them to set

on the coverslips. Afterwards, cells were overlaid in dissociated culture medium [Neurobasal media containing B27 supplement, 100 µg/ml penicillin/streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen)] and incubated in the humidified incubator at 37^{o}C. Dissociated culture medium was changed daily, and after 2-3DIV, cell cultures were used for collapse assays or WB analysis (see below).

2.2.3.5 Collapse assay

Dissociated MGE cells or Robo1-DN-transfected GN11 cells (after 2DIV) were treated with control-CM (CM-myc; CM-FLAG) or CM-chemotactic cues (CM-Sema3A, CM-Sema3F) for 1 hour at 37^{o}C. Subsequently, cells were fixed in pre-warmed 1% PFA-PBS for 20 min, rinsed in PBS and preceded for CB immunofluorescence staining (for MGE dissociated cells) or alexa fluor 546 phalloidin staining (for GN11 cells) according to manufacturer's protocols (Invitrogen). 200 random cells per animal and condition were photographed and qualitative analysed for growth cone collapse or neurite retraction.

2.2.3.6 Tissue processing for Matrigel explants

E13.5 mouse embryos were harvested from sacrificed dams and their brains were carefully dissected out in ACSF under a stereo microscope. Small pieces of MGE (approximately 250-300 µm across) were cut and placed onto 13 mm coverslips coated with 10 µg/ml poly-L-lysine and 10 µg/ml laminin in 24-well plates. Explants were embedded in a mixture of 75% Matrigel® solution (BD Biosciences, CA, USA) and 25% control-CM or CM-chemotactic cues supplemented with B27 (Gibco). After a period of 30 minutes, to allow the Matrigel® solution to set, 1ml of control-CM or CM-chemotactic cues supplemented with B27 was added to different set of experiments. Explants were cultured for 2DIV in the humidified incubator at 37^{o}C. Cell migration from MGE explants was semi-quantified by the Sholl method (Sholl, 1953). Briefly, 10 concentric circles of gradually increasing radius (spaced every 30 µm from the edge of the explants) were drawn on digitalised pictures of MGE explants. Cell migration from the explants was estimated by counting the number of concentric circles that migrating cells reached after 2 DIV.

2.2.3.7 Microchemotaxis assays (Boyden's chamber)

Microchemotaxis was performed on dissociated MGE or GN11 cells using a 48-well Boyden's chamber (Neuro Probe, MD, USA) as described previously (Maggi et al., 2000; Cariboni et al., 2005). Briefly, dissociated MGE or GN11 cells were suspended (10^5 cells/50 μl) in serum-free medium and placed in the open-bottom wells of the upper compartment of the chamber. The upper and lower compartments were separated by a polycarbonate porous membrane (8 μm pores; Neuro Probe) pre-coated with 10 μg/ml of poly-L-lysine and 10 μg/ml of laminin in distilled water (for MGE cells) or 0.2 mg/ml of gelatine (Sigma) made in sterile PBS (for GN11 cells). 27 μl of CM-chemotactic cues were placed into the lower compartment of the chamber. The chambers were kept in the humidified incubator at 37^{o}C for 18 hours (MGE cells) or 4 hours (GN11 cells). After incubation, the migrated cells that adhered to the underside of the membrane were fixed and stained using the Diff-Quick kit (Reagena, Toivala, Finland). For quantitative analysis, the membranes were observed using an Olympus light microscope with a 20x objective adapted with a 500 x 500μm grid. Four random fields of stained cells were counted for each well, and the number of cells per square millimetre (cells/mm^2) was calculated.

2.2.4 RNA and DNA methods

2.2.4.1 RNA extraction and cDNA preparation from FACS-sorted MGE cells

E15.5 *Robo1$^{-/-}$;GAD67-GFP$^{neo/-}$* and *Robo1$^{+/-}$;GAD67-GFP$^{neo/-}$* embryos were harvested from sacrificed dams and their brains were carefully dissected out in 0.01% DEPC-treated ACSF under a stereo microscope. MGEs from these animals were dissected out in cold DEPC-treated ACSF and incubated in trypsinisation medium for 10 minutes at 37^{o}C. Trypsinisation was quenched with neutralisation medium for 5 minutes at 37^{o}C. MGE tissues were then triturated with a fire-polished Pasteur pipette until no cellular aggregates were visible. The homogenous cellular suspensions were subsequently pelleted by centrifugation at 1,000 x g for 3 minutes and re-suspended in 5 ml of dissociated cell culture medium. Cell suspensions were then segregated by Fluorescent Activated Cell Sorter apparatus (FACS). FACS-sorted GFP$^+$ and non GFP$^+$ cell suspensions were collected in separate sterile 1.5 ml eppendorf tubes. Total RNA was extracted from the cell suspensions using the Qiagen RNeasy Plus kit (Qiagen, Hilden, Germany). RNA was

treated with DNase I to remove any remaining trace amounts of DNA. The quality of the RNA was assessed using an Agilent bioanalyzer nanochip (Agilent, CA, USA). cDNA was generated with 25 ng of RNA using the Qiagen Whole Transcriptome Amplification Kit (Qiagen) as described in the manufacturer's protocol.

2.2.4.2 RNA extraction and cDNA preparation from MGE tissue

E15.5 mouse embryos were harvested as described above. MGE tissues were dissected out in cold DEPC-treated ACSF under a stereo microscope. MGE tissues were incubated in trypsinisation medium for 15 minutes at 37^{o}C. Trypsinisation was quenched with neutralisation medium for 5 minutes at 37^{o}C. MGE tissues were then triturated with a fire-polished Pasteur pipette until no cellular aggregates were visible. The homogenous cellular suspensions were subsequently pelleted by centrifugation at 1,000 x g for 3 minutes. Total RNA was extracted from pelleted cells using the Qiagen RNeasy Plus kit, and with DNaseI and assessed as described in section 2.2.4.1. cDNA was generated with 1 µg of RNA using SuperScript^{TM} kit (Invitrogene) as described in the manufacturer's protocol.

2.2.4.3 RNA extraction and cDNA preparation from GN11 cells

GN11 cells were cultured as a monolayer on 10 cm Petri dishes in cell culture medium at 37^{o}C. At 80% of confluence, GN11 cells were incubated in trypsinisation medium for 15 minutes at 37^{o}C. Trypsinisation was quenched with neutralisation medium for 5 minutes at 37^{o}C. GN11 cells were then triturated with a fire-polished Pasteur pipette until no cellular aggregates were visible. The homogenous cellular suspensions were subsequently pelleted by centrifugation at 1,000 x g for 3 minutes. Total RNA was extracted from pelleted cells, treated with DNaseI and assessed as described in section 2.2.4.1. cDNA was generated with 1 µg of RNA using SuperScript^{TM} kit as described above.

2.2.4.4 Polymerase chain reaction (PCR) on GN11 cells

Primers used for PCR are listed in Table 2.2. PCR amplification of cDNA was performed using a total reaction volume of 50 µl in a 0.5 ml thin-walled eppendorf tube. cDNA taken from GN11 cells was used as template for PCR reactions. Products were amplified with non-proof-reading DNA polymerase, *Taq* DNA polymarase (Qiagen), as follows:

template DNA, 1 μM Forward Primer, 1 μM Reverse Primer, 500 μM dNTPs, 1x $MgCl_2$-free *Taq* buffer, 2 mM $MgCl_2$, 2 units *Taq* DNA polymerase. Polymerase chain reaction was performed in a Thermalcycler (Helena BioSciences; Newcastle, England, UK). PCR conditions were 94^{o}C for 2 min, followed by 40 three-step cycles of 94^{o}C for 15 seconds, 60^{o}C for 30 seconds, 72^{o}C for 30 seconds.

2.2.4.5 Quantitative real time PCR (QPCR) on FACS-sorted MGE cells or GN11 cells

Primers for QPCR were designed by Sigma (see Table 2.3). The QPCR reaction was performed using Sybr Green reagent (Sigma) on a Chromo4 PTC-200 Real-Time PCR Detector system (Biorad, CA, USA). PCR conditions were 94^{o}C for 2 minutes, followed by 40 three-step cycles of 94^{0}C for 15 seconds, 60^{o}C for 30 seconds, 72^{o}C for 30 seconds. GAPDH and/or Lhx6 were used for endogenous reference gene controls. Each primer set amplified a single PCR product of predicted size as determined by melt-curve analysis following PCR and by agarose gel electrophoresis (see below), and had approximately equal amplification efficiencies when validated using a serial dilution of representative cDNA. Each QPCR was performed in triplicate and relative quantification was determined according to the delta-delta c(t) method (Livak and Schmittgen, 2001; Faux et al., 2010).

2.2.4.6 Agarose gel electrophoresis

1% or 2% agarose-TAE gel was used in resolving DNA samples. 1% or 2% w/v agarose was made up with 1x TAE buffer (0.4 M Tris, 0.2 M sodium acetate, 20 mM EDTA; pH8.3; Sigma) in a Duran bottle, boiled, and left to cool to approximately 50^{o}C before ethidium bromide (Sigma) was added to a final concentration of 0.2 μg/ml. The solution was poured to an appropriate depth in a gel tray containing a comb with suitably sized teeth, and left for 45 minutes to set. DNA samples were mixed with 10x loading buffer (water; 50% v/v glycerol and 3% bromophenol blue) immediately prior to well-loading. For fragment size comparison, samples were run against 20 μl of 1 kb or 100bp DNA ladders (Gibco).

2.2.4.7 RNA probe synthesis

DH5α or XL10gold chemically competent strains of *Escherichia coli* bacteria were transformed by heat-shock (30 seconds at 42^{o}C followed by 10 minutes in ice) with plasmids containing the RNA probes used in this study (see Table 2.7). Transformed bacteria were plated in 10 cm plates containing (2% of LB medium, 1.5% of Agar and 100 µg/ml of ampiciline in 1000 ml of water; Sigma and Gibco) overnight at 37^{o}C. Purification of plasmids was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to manufacturer's instructions. Linearisation of plasmids was carried out by incubating 10 µl (1µg/µl) of purified DNA plasmids, 5 µl of corresponding enzyme (see Table 2.7), 10 µl of 10X appropriate buffer (Promega), 1µl of BSA (Promega) and 74 µl of ultra pure water (Ambion) at 37°C for 3 hours. To confirm linearisation of plasmids, 1 µl of linearised plasmid was visualized in 1% of Agarose gels. Extraction of DNA was performed by the phenol-chloroform method. Briefly, 1 µl of protease K (Invitrogen) was added to 99 µl linearised plasmid solution, equal volume of phenol/chlorophorm and centrifuged at 5000 x g for 5 minutes. The aqueous phase was collected and placed into a fresh sterile 1.5 ml eppendorf tube. 1X volume of chloroform was added to aqueous phase and centrifuged at 5000 x g for 5 minutes, the upper layer was collected and placed into a fresh sterile 1.5 ml eppendorf tube. Precipitation of DNA was carried out using 1 in tenth volume of 3 M sodium acetate and 2 volumes of 100% ethanol and incubated in dry ice for 1 hour, centrifuged 13000 x g for 5 minutes. The pellet was then washed in 70% ethanol and centrifuged 13000 x g, and subsequently dried out and resuspended in 10 µl of ultra pure water. *In vitro* transcription was carried as follows: 5 µl of 5X transcription buffer (Promega), 1 µg of linearised DNA, 2 µl of DIG labeling mix (Roche), 2 µl of 100mM DTT (Invitrogen), 0.5 µl of RNAasin (Invitrogen), 1 µl of corresponding RNA polymerase (see Table 2.7) and made up to 20 µl with ultrapure water. The reaction was incubated at 37°C for 2 hours. Subsequently, 3 µl of DNase (Invitrogen) were added to the transcription reaction, incubated at 37°C for 30 minutes and stoped with 2 µl of 0.5M EDTA (Promega). RNA probes were cleaned using MiniQuick Spin RNA columns (Roche). RNA was precipitated using 1 in tenth of 4 M lithium chloride and 2.5 volumes of 100% ethanol overnight. To confirm RNA synthesis, 1µl of RNA was run in 2% agarose gels.

2.2.5 Western blotting

2.2.5.1 Protein isolation

Robo1-myc-, Nrp1-myc-, Nrp2-myc-; pCDNA3.1-myc-transfected-GN11 cells or E15.5 MGE dissociated cells were lysed in pre-chilled lysis buffer on ice for 20 minutes. The cells were then scraped into pre-chilled eppendorf tubes. Two different lysis buffers were used; one for immunobloting (for MGE) (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40 and protease/phosphatase inhibitors; Boehringer, Mannheim, Germany) and the other for immunoprecipitation (IP) (for MGE or GN11 cells) (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 8.0, 1% Triton X-100 and 10% glycerol and protease/phosphatase inhibitors). The lysates obtained from MGE dissociated cells or GN11 cells were centrifuged at 13,000 rpm for 8 minutes at 4°C. Supernatant fractions were collected into a fresh 1.5 ml eppendorf tube, and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad).

2.2.5.2 Bio-Rad protein assay

Protein concentration from the lysates was estimated using Bio-Rad Protein Assay based on the Bradford method (Bio-Rad). 1µl of each lysate was added to 800 µl of water and 200 µl of Bio-Rad reagent. The absorbance readings for these samples were observed at a wavelength of 595 nm. These values were then compared to a curve drawn from a set of standard curves. 20 µg of sample protein was used for all blottings.

2.2.5.3 IP

E15.5 MGE or transfected-GN11 cell lysates were immunoprecipitated with antibody-Protein A agarose beads (Invitrogen) complexes overnight at 4°C. Antibody- Protein A agarose beads complexes were prepared as follows: Protein A agarose beads were washed thoroughly in IP lysis buffer (see above) containing 1% bovine serum albumin (BSA) an protein inhibitors. Afterwards, protein A agarose beads were incubated with selected antibodies [for MGE (anti-PlexinA1; anti-Nrp1; anti-Nrp2; anti-Robo1); for transfected-GN11 cell (anti-myc)] for 15 minutes at 37°C. Subsequently, immunoprecipitated lysates were processed for protein detection as indicated in sections 2.2.5.4-2.2.5.7 (see below).

2.2.5.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was essentially performed as described by (Sambrook and Russel, 2001). Polyacrylamide gels were prepared in a vertical gel electrophoresis system (Mini Protean III^{TM} apparatus, Bio-Rad), with resolving gel layer containing acrylamide, Tris pH 8.8, SDS, ammonium persulphate, and TEMED. The percentage of acrylamide was dependent on the size of the protein of interest. Stacking gels contained 5% acrylamide in Tris pH 6.8, SDS, ammonium persulfate, and TEMED. Protein samples were mixed with sample buffer, denaturised at 95^{o}C for 5 minutes, and resolved at a constant voltage (V) in 1x running buffer (25 mM Tris, 250 mM glycine, 0.1% w/v SDS; pH8.3; Sigma).

2.2.5.5 Staining of Polyacrylamide gels with Coomassie R250

Polyacrylamide gels were carefully removed from the electrophoresis system. Resolving gel was immersed in Coomassie blue solution containing 0.24% w/v Coomassie Brilliant Blue R250 (Bio-Rad), 50% v/v methanol, 10% v/v glacial acetic acid, and left under gentle agitation for 1 hour at Rt. Afterwards, stained gels were transferred to a de-staining solution (25% methanol, 7% glacial acetic acid) to remove the unbound dye.

2.2.5.6 Transfer of proteins to PVDF membranes

Resolved proteins were transferred from SDS-PAGE gels to PVDF membranes (Bio-Rad) using the wet transfer method, originally described by Towbin et al. (1992). The SDS-PAGE gel, a PVDF membrane and six appropriately sized pieces of 3 mm Whatman paper (Whatman International Ltd; Kent, UK) were pre-soaked in transfer buffer (48 mM Tris-Base, 39 mM glycine, 0.075% w/v SDS and 20% v/v methanol) and assembled in the Mini Protean III^{TM} apparatus (Bio-Rad), as instructed by the manufacturer. The transfer was conducted overnight at 30V, 4^{o}C.

2.2.5.7 Protein detection

PVDF membranes were incubated for 1 hour in blocking solution containing 5% w/v Marvel in PBST. PVDF membranes were incubated with primary antibodies (see Table 2.4) made in blocking solution for 6 hours at Rt. Subsequently, PVDF membranes were washed in PBST, before incubation with horse-radish peroxidase conjugated secondary

antibodies (see Table 2.5) made in blocking solution for 2 hours at Rt. After incubation, membranes were washed in PBST. Detection of proteins was performed using the Enhanced chemiluminescence reagent (GE-Healthcare; Buckinghamshire, UK), following manufacturer's instructions. The membrane was quickly wrapped in Sarawrap and placed in a cassette under Kodak X-OMAT film (Rochester, NY, USA). Films were developed after varying exposure times to visualise immunodetected proteins.

2.2.6 Digital image acquisition and processing

Optical and fluorescent images were collected using a Leica light microscope (DM5000B; Leica Microsystems, Wetzlar, Germany). Images were reconstructed and digitised with Photoshop CS4 software (Adobe Systems Incorporated, CA, USA).

2.2.7 Statistics

Statistical analyses were performed by GraphPad 3 software (GraphPad Software**,** Inc., CA, USA). All data is reported as mean number and standard error of the mean (SEM). The statistical significance between group means was tested via one-way analysis of variance (one-way ANOVA), followed by Bonferroni's post hoc test (for multiple comparison tests). Significance was set at a p value of ≤ 0.05.

Chapter 3: Deletion of Robo1, but not Robo2 or Slit, results in aberrant migration of cortical interneurons through the developing striatum

3.1 Background

The MGE in rodents has been reported as the major source of interneurons that populate the neocortex (Lavdas et al., 1999; Wichterle et al., 2001). After leaving the proliferative zone, these neurons start an active migration towards their final destination in the neocortex (Marin and Rubenstein, 2003; Métin et al., 2006). The lack of a cellular scaffold for the migration of cortical interneurons has led to hypothesise that a combination of chemoattractive and chemorepellant cues, in the neocortex and subpallium, respectively, plays an important role in the migration of these cells (reviewed in Hernandez-Miranda et al., 2010). The chemorepulsive cues that 'push' cortical interneurons away from the MGE are unknown. Nevertheless, several lines of evidence suggest that the family of Slit ligands might participate in repelling interneurons from their proliferative zones. First, it has been shown that Slit1is expressed in the proliferative zones of the GEs (Marillat et al., 2002). Second, cortical interneurons express Robo1, Robo2 and Robo3 proteins, the cognate receptors for Slit ligands (Andrews et al., 2006, 2008; Barber et al., 2009). Third, *in vitro* experiments have demonstrated that Slit ligands actually repel GE-derived cells (Hu, 1999; Zhu et al., 1999). Thus, attempts to clarify the role of Slit-Robo signalling system in cortical interneuron migration have resulted in the generation of deficient mice for specific members of the Slit and Robo families.

Cell tracing studies, carried out in slice cultures prepared from *Slit1*$^{-/-}$/*Slit2*$^{-/-}$ mice, have shown no defects in the tangential migration of t cortical interneurons (Marín et al., 2003). Moreover, no differences were found in the number or distribution of GABAergic interneurons (GABA^{+}, Lhx6^{+} or Dlx2^{+} cells) within the cerebral cortex of *Slit1*$^{-/-}$/*Slit2*$^{-/-}$ animals when compared to control littermates (Marín et al., 2003), suggesting that Slit signalling might not be required for the correct migration and positioning of cortical interneurons during development. Interestingly, analysis of deficient mice for each Robo receptor (*Robo1*$^{-/-}$, *Robo2*$^{-/-}$ *and Robo3*$^{-/-}$ mice) has revealed that only *Robo1*$^{-/-}$, but not *Robo2*$^{-/-}$ or *Robo3*$^{-/-}$, animals contain significantly more CB^{+} cells in their cortices than control littermates, indicating that more interneurons might be present in the neocortex of

Robo1$^{-/-}$ animals (Andrews et al., 2006, 2008; Barber et al., 2009). Andrews et al. (2006, 2008) also reported increased staining of CB in the striatum of Robo1 deficient (exon 5 deleted) mice, suggesting that the absence of this receptor results in aberrant migration of cortical interneurons through this structure. However, CB is also expressed by the vast majority of striatal projection neurons (Ouimet et al., 1988; Liu and Gaybriel, 1992) and thus, the exact nature of the increased CB^+ cells in the developing striatum of Robo1 deficient mice is presently unknown.

The aim of the present chapter is to investigate whether deletion of Robo1 receptor alters the migration of cortical interneurons or, alternatively, whether it results in an increased number of striatal projection neurons.

3.2 Results

3.2.1 Increased number of GABAergic cells in the neocortex and striatum of embryonic *Robo1*$^{-/-}$ mice

To explore whether the increased CB staining observed in the striatum of Robo1 deficient (exon 5 deleted) mice is actually due to an increase of GABAergic cells present in that area, I cross-mated *Robo1*$^{-/-}$ mice with *GAD67-GFP*$^{neo/-}$ animals to generate *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ and subsequently *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice (Robo1 control and deficient animals, respectively). GAD67 and GAD65 are the two isoforms of the glutamic acid decarboxylase (GAD) enzyme, which catalyse the decarboxylation of L-Glutamate to GABA and CO_2, and they are expressed in interneurons (Feldblum et al., 1993).

Examination of E15.5 *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice demonstrated that the absence of Robo1 receptors results in an actual increase of GABAergic ($GAD67^+$) cells in the neocortex of these animals when compared to control littermates (Fig. 3.1A,B). Specifically, the increase in GABAergic cells was observed in the MZ, CP, IZ and SVZ layers of *Robo1*$^{-/-}$ mice (Fig. 3.1C,D). Interestingly, the developing striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ animals also presented a greater number of GABAergic cells compared to *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ mice (see boxes in Fig. 3.1A,B,E,F). This result is in agreement with the previous finding of increased CB staining in the striatum of Robo1 deficient (exon 5 deleted) mice (Andrews, 2006), indicating that deletion of Robo1 receptors does not only affect the number of GABAergic cells in the neocortex, but also within the striatum.

3.2.2 Robo1 and Robo2, but not Robo3, immunoreactivity in the developing striatum

The fact that more GABAergic cells were found in the developing striatum of *Robo1*$^{-/-}$ mice clearly suggests that Robo1 receptors might play a significant role in the development of this structure. In support of this notion, it has been shown by in situ hybridisation (Marillat et al., 2002) and imunohistochemistry (Andrews et al., 2007) that Robo1 and Robo2 receptors are expressed within the developing striatum. However, it is not clear whether these proteins are actually present in striatal cells or in the cortico-striatal axons that innervate the developing striatum. In addition, it is not known whether Robo3 receptor is also present in this structure.

To clarify whether Robo receptors are expressed in cells that populate the developing striatum, I took coronal sections from *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ mice at E13.5 and E15.5 and immunostained them with specific antibodies for Robo1, Robo2 and Robo3 receptors (Fig. 3.2-3.4).

At E13.5 and E15.5, Robo1 inmunoreactivity was observed in the neocortex, cortical hem, piriform cortex, septum (only at E15.5) and developing striatum (Fig. 3.2B,H). Co-localisation of Robo1 and GAD67^{+} cells was observed in the neocortex (data not shown), the developing striatum (Fig. 3.2D-F, J-L), piriform cortex and septum at both ages (Fig. 3.2A-C, G-I).

At E13.5 and E15.5, Robo2 inmunoreactivity was observed in the neocortex, cortical hem and developing striatum (Fig. 3.3B,H). Co-localisation of Robo2 and GAD67^{+} cells was observed in the neocortex (data not shown) and the developing striatum at both ages (Fig. 3.3D-F, J-L).

At E13.5 and E15.5, Robo3 inmunoreactivity was observed weakly in the cerebral cortex and cortical hem, but it was not detected in the developing striatum (Fig. 3.4B,H). Co-localisation of Robo3 and GAD67^{+} cells was not observed in any area analysed (data not shown; Fig. 3.4).

Taken together, this data show that GABAergic cells in the striatum contain Robo1 and Robo2, but not Robo3, receptors. Therefore, it is likely that Robo1 and Robo2 signalling contributes to the normal development of the striatal area. Since it has been shown that Robo3 receptor is down regulated by E13 in the developing forebrain (Barber et al., 2009), and I found no immunoreactivity for Robo3 in the developing striatum, I will not study this receptor further. To explore the putative role of Robo1 and Robo2 receptors in the development of this structure, I will analyse distinct cell population that make up the developing and mature striatum in *Robo1*$^{-/-}$ and *Robo2*$^{-/-}$ mice.

3.2.3 No changes in the number of differentiating striatal projection- or inter-neurons in *Robo1*$^{-/-}$ mice

The striatum is a structure in which both projection- and inter- neurons express GABA (Gerfen, 1992). Projection neurons represent 90-95% of the nerve cells in the adult striatum, whilst striatal interneurons account for about 5-10% of the total number of striatal cells (Gerfen, 1992). The fact that more GAD67^{+} cells were observed in the developing striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice suggested that an increase in the number of these cell types might occur in the absence of Robo1 receptor.

To assess whether the increased number of GAD67^{+} cells in the developing striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice were striatal projection neurons, I prepared coronal sections from *Robo1*$^{-/-}$ mice and *Robo1*$^{+/-}$ littermates at E15.5 and E18.5, and immunostained them for Forkhead box protein P2 transcription factor (FOXP2, a marker of developing striatal projection neurons; Takahashi et al., 2003). I first confirmed that FOXP2 immuoreactivity was actually restricted to the developing striatum by immunostaining coronal sections of E15.5 *Robo1*$^{+/-}$;*GAD67-GFP*$^{neo/-}$ mice (Fig. 3.5). FOXP2 immuoreactivity was also restricted to the developing striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ at the same age (Fig. 3.5).

Quantifications of FOXP2 immunoreactive (FOXP2^{+}) cells throughout the rostral-caudal extend of the developing striatum of *Robo1*$^{-/-}$ mice and *Robo1*$^{+/-}$ littermates at E15.5 and E18.5 (n=4 animals per age and condition), showed no difference between both groups of animals at the two ages examined (Fig. 3.6), indicating that deletion of Robo1 receptors does not alter the generation and/or positioning of striatal projection neurons during development.

To evaluate whether the increased number of cells in the developing striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice were developing striatal interneurons, I took coronal sections of *Robo1*$^{-/-}$ mice (n=3) and *Robo1*$^{+/-}$ littermates (n=3) and processed them for *in situ* hybridisation with RNA probes against *Nkx2.1* (unlike cortical interneurons, striatal counterparts maintain the expression of this transcription factor, see Nobrega-Pareira et al., 2008) and *Lhx8* (marker of striatal cholinergic cells; Asbreuk et al., 2002; Zhao et al., 2003; Fragkouli et al., 2009) at E15.5. Quantification of the total number of *Nkx2.1*$^{+}$ or *Lhx8*$^{+}$ cells showed no differences between *Robo1*$^{-/-}$ and control littermates (Fig. 3.7), suggesting that deletion of Robo1 receptors does not affect the generation and/or positioning of striatal interneurons during development.

3.2.4 No differences in the number of mature striatal projection- or inter- neurons in *Robo1*$^{-/-}$ mice

In contrast to the neocortex, the adult striatum is not organised in layers. However, structurally it shows a complex organisation involving the segregation of striatal projection neurons into two well-defined compartments, the matrix and patch compartments (Gerfen, 1992). Similar to the neocortex, neurons in these compartments are generated in a precise sequence, with the majority of patch neurons being produced prior to those in the matrix compartment (Van der Kooy and Fishell, 1987; Song and Harlan, 1994). Interestingly, the segregation of striatal projection neurons is not even into both compartments, as 85% of the projection neurons are allocated within the matrix compartment and the remaining cells reside in the patch compartment (Gerfen, 1992). The fact that more GABAergic cells, but not more developing striatal projection- or inter-neurons, are observed in the striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ led me to hypothesise that the increased number of cells in these animals could reflect an imbalance in the mature populations of striatal projection neurons.

To clarify this idea, I also took coronal sections from adult (P90) *Robo1*$^{-/-}$ (n=3) and *Robo1*$^{+/-}$ (n=3) mice, and immunostained them for CB (marker of mature projection neurons belonging to the matrix compartment) and DARPP-32 (marker of both mature matrix and patch projection neurons) (Ouimet et al., 1988; Liu and Gaybriel, 1992). Quantification of CB^{+} (Fig. 3.8A-C) or DARPP32^{+} cells (Fig. 3.8D-F) through the rostral-caudal extend of the mature striatum of *Robo1*$^{-/-}$ mice and *Robo1*$^{+/-}$ littermates revealed no differences in the number of matrix or patch projection neurons between both groups of animals.

To rule out that deletion of Robo1 receptors results in an increased number of mature striatal interneurons, I prepared coronal sections of adult *Robo1*$^{-/-}$ (n=3) and *Robo1*$^{+/-}$ (n=3) mice, and immunostained them for three known markers of non-overlapping subpopulations of striatal interneurons such as PV, SOM and ChAT (Kawaguchi et al., 1995). Counts of the three cell type markers also showed no differences between the two groups of animals (Fig. 3.9C,F,I).

Taken together, my examination of developing and adult *Robo1*$^{-/-}$ animals shows that deletion of Robo1 receptor does not impair the establishment of projection- or inter-neurons within the striatum.

3.2.5 Deletion of Robo1 receptor results in aberrant migration of cortical interneurons throughout the developing striatum

The fact that no differences in either developing or mature striatal cells (projection- and inter- neurons) were observed in the striatum of *Robo1*$^{-/-}$ mice (Fig 3.6-3.9) led me to hypothesise that deletion of Robo1 receptors results in aberrant migration of cortical interneurons through the developing striatal area. The finding of excess GAD67-GFP^{+} cells in the developing striatum of *Robo1*$^{-/-}$ mice seems to support this notion (Fig. 3.1), as previous studies have shown that striatal neurons preferentially express GAD65, whereas cortical interneurons express higher levels of GAD67 proteins (Greif et al., 1992; Mercugliano et al., 1992; Feldblum et al., 1993; Hendrickson et al., 1994).

To confirm that deletion of Robo1 receptor leads to aberrant migration of cortical interneurons through the developing striatum, I prepared coronal sections from *Robo1*$^{-/-}$ mice and *Robo1*$^{+/-}$ littermates at E15.5, E18.5 and P0, and immunostained them for CB. The marker CB has been widely used to label migrating cortical interneurons during development (Anderson et al., 1997a; Andrews et al., 2006). Additionally, it was precisely an increase of CB staining what was qualitatively observed in the developing striatum of Robo1 deficient (exon 5 deleted) mice (Andrews et al., 2006).

Quantification of CB^{+} cells present throughout the rostral-caudal extend of the developing striatum of *Robo1*$^{-/-}$ mice and *Robo1*$^{+/-}$ littermates at E15.5, E18.5 and P0 (n=4 pear age and condition), revealed a significant increase of these cells in *Robo1*$^{-/-}$ mice when compared to control littermates at all three ages analysed (Fig. 3.10). At E15.5, the increase was approximately 23% ($p < 0.01$, Fig. 3.10A-C); at E18.5, about 39% ($p < 0.01$, Fig. 3.10D-F); and at P0, approximately 16% ($p < 0.05$, Fig. 3.10G-I).

Thus, these observations suggest that deletion of Robo1 receptors indeed results in aberrant migration of cortical interneurons through the striatum. Further support for this suggestion comes from the absence of co-localisation between FOXP2^{+} and GAD67-GFP^{+} cells in *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo-/-}$ mice (Fig. 3.11), which strongly supports the idea that the greater number of GAD67^{+} cells or CB^{+} cells observed in the developing striatum of *Robo1*$^{-/-}$ mice are not striatal projection neurons, but actually cortical interneurons that migrate aberrantly through that area *en route* to the neocortex. Interestingly, it seems that these cortical interneurons form a wave of migrating cells that apparently reach a peak at E18.5 and diminishes by the time of birth (Fig. 3.10).

3.2.6 Deletion of Robo2 receptor does not alter the number of striatal cells or cortical interneurons

It has been shown previously that cortical interneurons also express Robo2 receptors (Andrews et al., 2007). Examination of *Robo2*$^{-/-}$ mice revealed no significant differences in the number of interneurons in their neocortices compared to *Robo2*$^{+/-}$ littermates, suggesting that this receptor is not involved in their migration (Andrews et al., 2008). Interestingly, Robo2 receptors are abundantly expressed in cells that populate the developing striatum (see Fig. 3.3; Marillat et al., 2002; Andrews et al., 2007). Nonetheless, no analysis about the role of these receptors in the development of the striatum has yet been carried out.

To explore whether deletion of Robo2 receptors alters the development of striatal projection neurons, I took coronal sections from E15.5 *Robo2*$^{-/-}$ (n=5) and control animals (n=4), and imunostained them for FOXP2. Quantification of FOXP2^{+} cells in the striatum of *Robo2*$^{-/-}$ animals revealed no significant differences when compared to control littermates (Fig. 3.12A-C). Furthermore, analysis of E15.5 *Robo2*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ (n=3) mice revealed no obvious differences in the number of GAD67^{+} present in their striatum when compared to *Robo2*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ (n=3) littermates (Fig. 3.12G,H). To examine whether deletion of Robo2 receptors could lead to aberrant migration of cortical interneurons through the developing striatum, I immunostained coronal sections of E15.5 *Robo2*$^{-/-}$ mice (n=5) and *Robo2*$^{+/-}$ littermates (n=4) for CB. Interestingly, I found only a significant increase in the number of CB^{+} cells rostrally in the striatum of *Robo2*$^{-/-}$ (rostral, *Robo2*$^{+/-}$ 96.8±2.4 cells/x10^5 μm^2, *Robo2*$^{-/-}$ 127.5±2.3 cells/x10^5 μm^2, $p<0.01$), but not medially or caudally when compared with *Robo2*$^{+/-}$ (n=4) mice at E15.5 (Fig 3.12D-F). To confirm that no alterations in mature striatal projection neurons occur in the absence of Robo2 receptors, I immunostained coronal sections from adult *Robo2*$^{-/-}$ animals (n=2) and *Robo2*$^{+/-}$ littermates (n=3) for CB and DARPP32. No significant differences in the number of these cell types were detected in both groups of animals (Fig. 3.13). Moreover, analysis of PV^{+}, SOM^{+} and ChAT^{+} cells also showed no differences in the number of adult striatal interneurons between *Robo2*$^{-/-}$ animals (n=2) and control counterparts (n=2) (Fig. 3.14). Thus, these and previous observations (Andrews et al., 2008) suggest that, unlike Robo1, Robo2 receptors do not participate in the migration and/or positioning of GABAergic cells either within the neocortex or striatum.

3.2.7 Deletion of Slit1 and Slit2 ligands does not affect the number of striatal projection neurons

Evidence suggests that absence of Slit1 and Slit2 signalling does not affect eitheir the tangential migration or the correct targeting of migrating cortical interneurons (Marin et al., 2003). Interestingly, cells within the developing striatum express Robo1 and Robo2 receptors (Fig. 3.2, 3.3), suggesting that Slit signalling might be involved in the development of this structure.

To clarify whether the absence of Slit1 and Slit2 signalling affects the development of striatal projection neurons, I took coronal sections from E15.5 *Slit1*$^{-/-}$/ *Slit2*$^{-/-}$ embryos (n=2) and *Slit1*$^{+/-}$/*Slit2*$^{+/-}$ littermates (n=3), and immunostained them for FOXP2. Counts of FOXP2^{+} cells in the striatum of both groups of animals showed no significant difference between them (Fig. 3.15A-C), indicating that Slit signalling may not be required for the correct generation and/or positioning of striatal projection neurons. The fact that *Slit1*$^{-/-}$/*Slit2*$^{-/-}$ animals die few hours after birth prevented me from further exploring any putative effect of Slit1 and Slit2 signalling on mature striatal projection- or inter- neurons.

To explore whether the increased number of CB^{+} cells observed in *Robo1*$^{-/-}$ animals was similar in the absence of Slit1 and Slit2, I prepared coronal sections from E15.5 *Slit1*$^{-/-}$/*Slit2*$^{-/-}$ embryos (n=2) and *Slit1*$^{+/-}$/*Slit2*$^{+/-}$ littermates (n=3), and immunostained them for CB. Examination of labelled cells in the striatum showed no differences between the two groups of mice (Fig. 3.15D-F).Thus, previous (Marin et al., 2003) and current observations suggest that, unlike the Robo1 receptor, Slit1 and Slit2 signalling does not participate in the migration and/or positioning of GABAergic cells either within the neocortex or striatum.

3.3 Conclusions

Previous studies suggested that deletion of Robo1 receptor resulted in a presumptive increase in cortical interneurons (Andrews et al., 2006). In the present chapter, I have demonstrated that there exists an actual increase of GABAergic cells in the neocortex of animals that lack Robo1 receptors (Fig. 3.1A-D). Interestingly, deletion of Robo1 receptors leads to an increased number of GABAergic cells in the developing striatum (Fig. 3.1E,F). My efforts to identify the nature of those cells illustrated that deletion of Robo1 receptor does not alter the total number of developing striatal neurons (projection- or inter- neurons; Fig. 3.6, 3.7). Similarly, the absence of Robo1 receptors does not affect the number of mature striatal neurons (Fig. 3.8, 3.9). The fact that no differences were found in the number of developing or mature striatal neurons suggested that other population of cells might invade the developing striatum in the absence of Robo1 receptors. Support to this idea came from the observation that the increased number of GAD67-GFP^{+} cells does not co-localise with FOXP2^{+} cells in *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice (Fig. 3.11).

Using CB as a marker of migrating cortical interneurons (Anderson et al., 1997a; Andrews et al., 2006), I found that there is an actual increase of CB^{+} cells in the developing striatum of *Robo1*$^{-/-}$ mice when compared to control littermates (Fig. 3.10), illustrating that aberrant migration of cortical interneurons occurs in the striatum of these mice. Additional support to this idea comes from the fact that cortical interneurons preferentially express GAD67 proteins, whilst striatal cells tend to express GAD65 proteins (Greif et al., 1992; Mercugliano et al., 1992; Feldblum et al., 1993; Hendrickson et al., 1994).

Earlier observations illustrated that, unlike Robo1, Robo2 receptor is not required for the migration of cortical interneurons (Andrews et al., 2008). However, this receptor is expressed in the developing striatum (Fig. 3.3), indicating a putative role in the development of this area. Examination of the embryonic and adult striatum of *Robo2*$^{-/-}$ animals, shows no difference in the number of striatal neurons (projection- or inter-neurons) compared to control animals at all ages examined (3.12-3.14). Therefore, previous and present observations clearly show that Robo2 receptor is not important for the migration of either cortical interneurons or striatal neurons.

The observation that Robo1 and Robo2 receptors are present in cells that populate the developing striatum indicates a putative role of Slit signalling in the development of that area. However, my observations on E15.5 *Slit1*$^{-/-}$*/Slit2*$^{-/-}$ mice showed no differences

in the number of developing striatal cells compared to controls (Fig. 3.15), suggesting that, unlike Robo1, Slit signalling is not necessary for the normal migration, generation and/or positioning of either cortical or striatal GABAergic cells.

Taken together, all data generated in the present chapter suggest that Robo1, but not Robo2 or Slit1 and Slit2, signalling is pivotal for the correct cell migration of cortical interneurons around the striatum. The fact that *Robo1*$^{-/-}$ cortical interneurons invade the striatum might indicate that these cells do not detect the chemorepulsive action of Sema3A and/or Sema3F expressed in that area. Therefore, I decided to concentrate my efforts in testing if *Robo1*$^{-/-}$ cortical interneurons lose responsiveness to Sema3A and/or Sema3F and thus, become refractory to the chemorepulsive action of these molecules.

Chapter 4: *Robo1*$^{-/-}$ cortical interneurons are unresponsive to the chemorepulsive actions of Sema3A and Sema3F

4.1 Background

En route to the cerebral cortex, migrating cortical interneurons from the MGE encounter the developing striatum and avoid entering within it. It is thought that chemorepulsive cues expressed in the striatum maintain cortical interneurons away from that area and thus, those molecules participate in channelling these cells into their appropriate adjacent migratory paths (Marin et al., 2001; Marin and Rubenstein, 2003; Metin et al., 2006). Members of the chemorepulsive family of class 3 semaphorins, Sema3A and Sema3F, have been proposed to play a crucial role in this process (Marin et al., 2001; Tamamaki et al., 2003a). Indeed, Sema3A and Sema3F expression has been identified in the developing striatum (Marin et al., 2001). Moreover, migrating cortical interneurons contain Nrp1 and Nrp2 receptors and respond to Sema3A and Sema3F chemorepulsion *in vitro* (Marin et al., 2001; Tamamaki et al., 2003a). In addition, recent evidence suggests that members of the extracellular matrix family of proteoglycans might play a substantial role in maintaining cortical interneurons away from the developing striatum by stabilising the diffusion of class 3 semaphorins within this area (Zimmer et al., 2010). Thus, the presence of Sema3A and Sema3F in the developing striatm creates an exclusion zone for migrating cortical interneurons.

Interestingly, the analysis that I carried out on the developing striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ animals (Fig. 3.1), along with the quantification of CB^{+} cells in the striatum of *Robo1*$^{-/-}$ embryos, indicates that deletion of Robo1 receptor leads to aberrant migration of cortical interneurons through the developing striatum. This notion is supported by the observations that there exists no co-localisation between $FOXP2^{+}$ cells and the increased number of GAD67-GFP^{+} in *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ animals (Fig. 3.11). Thus, taking together all these pieces of evidence, it seems that migrating cortical interneurons, in absence of Robo1 signalling, fail to respond to Sema3A and/or Sema3F induced chemorepulsion and invade the developing striatum.

The aim of the present chapter is to evaluate whether cortical interneurons in the absence of Robo1 receptors lose responsiveness to Sema3A and/or Sema3F chemorepulsion.

4.2 Results

4.2.1 MGE-derived *Robo1*$^{-/-}$ cells are less sensitive to Sema3A and Sema3F induced chemorepulsion *in vitro*

Migrating cortical interneurons avoid the developing striatum during their migratory journey because of the chemorepulsive activity of the Sema3A and Sema3F cues emanating in this area (Marín et al., 2001; Tamamaki et al., 2003a). However, the observation reported in the previous chapter indicate that deletion of Robo1 receptor leads to aberrant migration of cortical interneurons through the developing striatum, suggesting that these cells might fail to respond to Sema3A and/orSema3F induced chemorepulsion. To evaluate this idea, I performed chemomigration studies using explants or dissociated cells taken from the MGE of *Robo1*$^{+/-}$ and *Robo1*$^{-/-}$ mice.

Given that GE-derived cells exhibit an astonishing intrinsic migratory capacity *in vitro* (Wichterle et al., 1999; Nery et al., 2002), I first wanted to assess the migratory potential of MGE *Robo1*$^{-/-}$ (n=260) and *Robo1*$^{+/-}$ (n=209) explants in the presence of control-CM (CM-myc or CM-FLAG). MGE explants prepared from E13.5 *Robo1*$^{-/-}$ and *Robo1*$^{+/-}$ mice were treated with CM-myc or CM-FLAG for 2 DIV. Semi-quantification of the migratory potential of the two groups of explants by the Sholl method (see Materials and Methods in Chapter 2) showed no significant differences after 2DIV [CM-myc (*Robo1*$^{+/-}$ 6.36±0.71 intersections; *Robo1*$^{-/-}$ 7.08±0.42 intersections; $p>0.05$) or CM-FLAG (*Robo1*$^{+/-}$ 7.55±0.32 intersections; *Robo1*$^{-/-}$ 6.74±0.29 intersections; $p>0.05$)], suggesting that there is no impairment in the motility of MGE-derived cells taken from *Robo1*$^{-/-}$ animals (Fig. 4.1A,B,E; 4.2A,B,E; 4.5A,B,E).

I then wanted to examine the response of MGE explants taken from *Robo1*$^{+/-}$ mice to CM-Sema3A (n=63) and CM-Sema3F (n=110) treatment. Semi-quantification of the migratory potential of *Robo1*$^{+/-}$ explants after treatment with CM-Sema3A or CM-Sema3F showed a significant reduction in their cell migration when compared to those treated with CM-myc (n=42) or CM-FLAG (n=130), respectively [(CM-myc, 6.36±0.71 intersections; CM-Sema3A, 1±0.071intersections; $p<0.001$); (CM-FLAG 7.55±0.32

intersections; CM-Sema3F 4.52±0.52 intersections; $p<0.01$); (Fig. 4.1A,C,E; 4.2A,C,E)]. These results confirmed the previously documented chemorepulsive action of Sema3A and Sema3F on MGE-derived cells (Marín et al., 2001; Tamamaki et al., 2003a).

Interestingly, the evaluation of *Robo1*$^{-/-}$ explants treated with CM-Sema3A (n=54) or CM-Sema3F (n=129) showed no differences in their migratory potential compared to similar explants treated with CM-myc (n=42) or CM-FLAG (n=111), respectively [(CM-myc, 7.08±0.42 intersections; CM-Sema3A, 7.48±0.08 intersections); (CM-FLAG, 6.34±0.3 intersections; CM-Sema3F 6.48±0.32 intersections; $p>0.05$); (Fig. 4.1B,D,E; 4.2B,D,E)], suggesting that MGE-derived cells from *Robo1*$^{-/-}$ mice do not respond to Sema3A or Sema3F induced chemorepulsion.

To confirm the above observations in a quantitative way, I assessed the ability of dissociated cells taken from the MGE of E13.5 *Robo1*$^{-/-}$ and *Robo1*$^{+/-}$ littermates to respond to control-CM, CM-Sema3A or CM-Sema3F using the Boyden's chamber. This assessment revealed that migration of cells taken from *Robo1*$^{-/-}$ and *Robo1*$^{+/-}$ mice was similar into compartments containing control-CM [CM-myc (*Robo1*$^{+/-}$, 548.7±76.3 cells/mm^2; *Robo1*$^{-/-}$, 451.8±55.99 cells/mm^2); CM-FLAG (*Robo1*$^{+/-}$, 510±25.4 cells/mm^2; *Robo1*$^{-/-}$, 500.6±16.8 cells/mm^2); $p>0.05$; Fig. 4.3A,B,E; 4.4A,B,E), supporting the notion that deletion of the Robo1 gene does not impair the normal motility of MGE-derived cells, and confirming my previous observations using explants (Fig. 4.1A,B,E; 4.2A,B,E).

I observed, as expected, a significant reduction in the number of MGE-derived cells taken from *Robo1*$^{+/-}$ mice that migrated towards compartments containing either CM-Sema3A or CM-Sema3F compared to those compartments containing control-CM [(CM-myc, 548.7±76.3 cells/mm^2; CM-Sema3A 350.3±19.7 cells/mm^2; $p<0.01$); (CM-FLAG, 510.3±25.4 cells/mm^2; CM-Sema3F 357.3±18.7 cells/mm^2; $p<0.01$); (Fig. 4.3A,C,E; 4.4A,C,E)].

Interestingly, MGE dissociated cells taken from *Robo1*$^{-/-}$ mice were less responsive to either CM-Sema3A or CM-Sema3F and migrate similarly to *Robo1*$^{-/-}$ cells that moved into compartments containing control-CM [(CM-myc, 451.8±55.99 cells/mm^2; CM-Sema3A 432.34±20.21 cells/mm^2; $p>0.05$); (CM-FLAG, 500.6±16.8 cells/mm^2; CM-Sema3F, 515.7±21.1 cells/mm^2; $p>0.05$); Fig. 4.3B,D,E; 4.4B,D,E)].

Taken together, this data suggest that cells derived from the MGE of *Robo1*$^{-/-}$ mice are unresponsive to Sema3A and Sema3F induced chemorepulsion compared to those taken from *Robo1*$^{+/-}$ animals.

4.2.2 MGE-derived *Robo1*$^{-/-}$ cells are less sensitive to Slit1 induced chemorepulsion *in vitro*

It has been documented that migrating cortical interneurons express Robo1, Robo2 and Robo3 proteins, the cognate receptors for Slit ligands (Andrews et al., 2008; Barber et al., 2009). In addition, there is evidence suggesting that Slit proteins can repel postmitotic cells away from the ventral proliferative zones of the GEs, particularly the LGE (Hu, 1999; Zhu et al., 1999). However, analysis of *Slit1*$^{-/-}$/*Slit2*$^{-/-}$ mice has illustrated that there is not alteration in the number of GABAergic cells that populate the developing neocortex (Marin et al., 2003) or striatum (present data, Fig. 3.15) in those animals, suggesting that Slit1 and Slit2 signalling might not be required for the migration of cortical or striatal GABAergic cells.

To clarify whether migrating MGE-derived cells respond or not to Slit induced chemorepulsion, I carried out *in vitro* chemomigration assays similar to the ones described above, using Slit1 treatment of MGE-derived cells taken from E13.5 *Robo1*$^{+/-}$ embryos. Explant assays illustrated that MGE-derived cells respond to Slit1 induced chemorepulsion when compared to those explants treated with control-CM [(CM-myc, 6.75±0.18 intersections; CM-Slit1, 1.24±0.43 intersections; $p<0.001$); Fig. 4.5A,C,E)]. This result was confirmed using MGE dissociated cells from these animals in Boyden's chamber [(CM-myc, 510±6.66 cells/mm^2; CM-Slit1, 362.3±17.95 cells/mm^2; $p<0.01$), Fig. 4.6A,C,E), indicating that GE-derived cells are indeed responsive to Slit signalling as reported elsewhere (Hu, 1999; Zhu et al., 1999).

To explore whether MGE-derived cells of *Robo1*$^{-/-}$ also lose responsiveness to its natural ligand, I performed similar studies using Slit1 treatment of MGE-derived cells taken from E13.5 *Robo1*$^{-/-}$ embryos. I found that *Robo1*$^{-/-}$ explants do not respond to Slit1 induced chemorepulsion compared to control treatment [(CM-myc, 8.04±0.31 intersection; CM-Slit1, 6.51±0.21 intersections; $p>0.05$); Fig. 4.5B,D,E]. I further confirmed this result using MGE dissociated cells taken from these mice [(CM-myc, 486±22.2 cells/mm^2; CM-SLIT1, 488.17±34.38 cells/mm^2; $p>0.05$) in Boyden's chamber (Fig. 4.B,D,E).

Thus, this *in vitro* data show that MGE-derived cells are actually responsive to Slit signalling. In addition, it seems that loss of Robo1 receptors also perturbs the chemorepulsive effect of Slit signalling in the migration of MGE-derived cells.

4.2.3 MGE-derived cells of *Robo1*$^{-/-}$ mice do not respond to the collapsing effect of Sema3A and Sema3F

Class 3 semaphorins are known to induce cytoskeleton reorganisation in neurons that leads to growth cone collapse and the retraction of cell processes (Winberg et al., 1998; Tran et al., 2007). Interestingly, examinations of *Robo1*$^{-/-}$ cortical interneurons have shown that a number of morphological defects occur in those cells, as they exhibit longer and more elaborated processes when compared to control littermates (Andrews et al., 2008), suggesting that *Robo1*$^{-/-}$ cortical interneurons might be unable to respond to chemotactic cues such as class 3 semaphorins that shape their morphology.

In order to evaluate whether deletion of Robo1 receptor can prevent the response of MGE-derived cells to the collapsing effect of class 3 semaphorins on their neurites, I prepared dissociated cell cultures from the MGE of *Robo1*$^{+/-}$ and *Robo1*$^{-/-}$ mice at E13.5. After 2DIV, I treated dissociated cells briefly (1 hour) with CM-myc, CM-Sema3A and CM-Sema3F. After incubation in the selected treatments, I fixed the dissociated cell cultures and inmunostained them for CB. Subsequently, I investigated qualitatively the effect of each treatment on the morphology of both groups of animals.

Qualitative analysis of *Robo1*$^{+/-}$ and *Robo1*$^{-/-}$ cells in the presence of CM-myc revealed that both groups of cells exhibit growth cones on their neurites (arrows in Fig. 4.7A-F), which indicates that they actively elaborate and elongate neurites. In contrast, *Robo1*$^{+/-}$ cells treated with CM-Sema3A (Fig. 4.7G-I) or CM-Sema3F (Fig. 4.7M-O) exhibited commonly absence of neurites, collapse of growth cones and severe retraction of their neurites (solid triangles in Fig. 4.7G-I,M-O) compared to cells treated with control medium (Fig. 4.7A-C). Nevertheless, the morphology of *Robo1*$^{-/-}$ cells seemed to be unaffected after treatment with either CM-Sema3A or CM-Sema3F, and their morphology resemble that of *Robo1*$^{-/-}$ cells treated with CM-myc (Fig. 4.7D-F,J-L,P-R).

Thus, this evidence shows that MGE-derived cells of *Robo1*$^{-/-}$ embryos do not respond to the collapsing effect triggered by Sema3A and Sema3F. Additionally, these results could also indicate that *Robo1*$^{-/-}$ cortical interneurons exhibit a more elaborated morphology (as reported in Andrews et al., 2008), because they do fail to respond to collapsing molecules such as class 3 semaphorins that shape their morphology during development.

4.2.4 GN11 cells as an *in vitro* model to study the putative interaction between Robo1 receptors and class 3 semaphorin signalling system

I have shown in previous sections of this chapter that MGE-derived cells, are less responsive to class 3 semaphorins in the absence of Robo1 receptors, suggesting that there might exist an interaction between Robo1 and the class 3 semaphorin signalling system. Since the MGE gives rise to distinct and heterogeneous subpopulations of cortical and subcortical GABAergic cells (see discussion), I wanted to develop a simpler and easier *in vitro* system that could allow me to examine further the putative interactions between Robo1 receptor and the class 3 semaphorin signalling system.

Thus, I wanted to use a cell line that could endogenously possess receptors for class 3 semaphorins and Slits (Nrp and Robo receptors, respectively) and also exhibit an intrinsic migratory activity *in vitro*. I found that an immortalised cell line derived from gonadotropin-realising hormone secreting neurons, GN11 cell line, possesses the receptors Nrp1 and Nrp2, responds to Sema3A and Sema3F induced chemorepulsion and also exhibits intrinsic migratory activity *in vitro* (Cariboni et al., 2007). Therefore, I investigated whether GN11 cells could also possess any of the three Robo receptors (Robo1, Robo2 and Robo3) and whether they could also respond to the chemorepulsive action of Slit1 and Slit2 *in vitro*.

Firstly, I evaluated whether GN11 cells, like cortical interneurons, expressed mRNA for Robo receptors. I used two sets of primers for each Robo receptor (namely Robo1A, Robo1B, Robo2A, Robo2B, Robo3A and Robo3B, respectively) and performed RT-PCR experiments. My evaluation showed that, similar to wildtype mice brains, GN11 cells actually express the three Robo receptors, as detected with the two sets of primers used for each receptor (Fig. 4.8A; Robo3B primer was the only exception and did not amplify any product for brain or GN11 samples). Specifically, GN11 cells appear to express strongly Robo1 and Robo3, but only weakly Robo2 receptor (Fig 4.8A). Secondly, I confirmed by immunofluorescence that GN11 cells do contain Robo1, Robo2 and Robo3 receptors on their membranes (Fig. 4.8B-D).

Given that GN11 cells have Robo receptors, I wanted to evaluate whether they are able to respond to the chemorepulsive action of Slit1 and Slit2 in Boyden's chamber. However, I first evaluated if GN11 cells were indeed responsive to Sema3A and Sema3F induced chemorepulsion. As reported previously (Cariboni et al., 2007), these cells showed reduced migration into compartments containing CM-Sema3A or CM-Sema3F when compared to cell migration into compartments containing CM-myc [(CM-myc,

648±42 cells/mm^2; CM-Sema3A, 301±51 cells/mm^2; CM-Sema3F, 244±36 cells/mm^2; $p<0.01$); Fig. 4.8E]. Similarly, I observed reduced migration of GN11 cells into compartments containing CM-Slit1 or CM-Slit2 when compared to those cells migrating into compartments containing control-CM [(CM-myc, 648±42 cells/mm^2; CM-Slit1 308±19 cells/mm^2; Slit2 232±44 cells/mm^2; $p<0.01$); Fig. 4.8]. Together, this data demonstrate that migration of GN11 cells is severely reduced in presence of both Slit and class 3 semaphorins. To confirm further the chemorepulsive effect of Slit and class 3 semaphorin cues on GN11 cells, I evaluated the morphology (collapse response) of these cells after treatment with CM-myc, CM-Sema3A, CM-Sema3F, CM-Slit1 and CM-Slit2. In the presence of CM-myc, GN11 cells presented a flat and extended cytoplasm (thin arrows in Fig. 4.9A,B) and very few neurite-like processes (arrow head in Fig. 4.9A). After treatment with CM-Sema3A and CM-Sema3F, these cells elaborated numerous neurite-like processes (arrow heads in Fig. 4.9C,D) and exhibited some remnants of their extended cytoplasm (asterisks in Fig. 4.9C,D). Interestingly, GN11 cells in the presence of CM-Slit1 or CM-Slit2 showed also some neurite-like processes (arrow heads in Fig. 4.9E,F), but mainly the retraction of numerous cell processes (thick arrows in Fig, 4.9E,F).

The fact that GN11 cells express Nrp and Robo receptors and that they respond to class 3 semaphorins and Slits, prompted me to speculate whether the loss/lack of Robo receptors in GN11 cells could mimic the loss of responsiveness to class 3 semaphorins that MGE-derived cells showed in the absence of Robo1 receptors (Fig. 4.1-4.7). Thus, I recreated the conditions of *Robo1*$^{-/-}$ cells in GN11 cells by transfecting these cells with Robo1 dominant negative plasmids (Robo1-DN), which have been shown to perturb the projection of chick cranial motor neurons (Hammond et al., 2005). I also transfected GN11 cells with GFP plasmid to use them as control cells.

Following transfection, I evaluated the ability of GFP-transfected and non-transfected GN11 cells to migrate in Boyden's chamber. Firstly, I estimated the migration of these cells in the presence on control-CM (non-transfected cells, 648±42 cells/mm^2; GFP-transfected cells, 693±37 cells/mm^2; $p>0.05$), my results illustrated that transfection of plasmids does not alter the migratory potential of GN11 cells. As expected, GFP-transfected GN11 cells showed reduced migration into compartments containing CM-Sema3A, CM-Sema3F, CM-Slit1 or CM-Slit2 compared to GFP-transfected cells migration into compartments containing control-CM [Sema3A (CM-myc, 693±37 cells/mm^2; CM-Sema3A, 287±36 cells/mm^2; $p<0.01$); Sema3F (CM-FLAG 693±37 cells/mm^2; Sema3F 242±28 cells/mm^2; $p<0.01$); Slit1 (CM-myc, 693±37 cells/mm^2; CM-

Slit1, 392±43 cells/mm^2; p<0.01); Slit2 (CM-myc, 693±37 cells/mm^2; CM-Slit2, 397±32 cells/mm^2; p<0.01); Fig. 4.10].

Interestingly, Robo1-DN-transfected GN11 cells were drastically less sensitive to the chemorepulsion of CM-Sema3A and CM-Sema3F compared to GFP-transfected cells [CM-Sema3A (GFP-transfected cells, 287±36 cells/mm^2; Robo1-DN-transfected cells, 497±28 cells/mm^2; p<0.01; Fig. 4.10A); CM-Sema3F (GFP- transfected cells, 242±28 cells/mm^2; Robo1-DN-transfected cells, 524±41 cells/mm^2, p<0.01; Fig. 4.10B)]. Thus, it seems that Robo1-DN-transfected GN11 cells can emulate the refractory behaviour of MGE-derived *Robo1*$^{-/-}$ cells to class 3 semaphorins.

Surprisingly, Robo1-DN-transfected GN11 cells were significantly more sensitive to the chemorepulsion of CM-Slit1 and CM-Slit2 compared to GFP-transfected cells [CM-Slit1 (GFP-transfected cells, 392±43 cells/mm^2; Robo1-DN-transfected cells, 248±37 cells/mm^2; p<0.01; Fig. 4.10C); CM-Slit2 (GFP-tranfected cells, 397±32 cells/mm^2; Robo1-DN-transfected cells, 287±29 cells/mm^2; p<0.01; Fig. 4.10D)], suggesting that in the absence of Robo1, Robo2 or Robo3 receptors might potentiate the effect of Slit1 and Slit2 ligands in GN11 cells.

Thus, taken together this *in vitro* data suggest that GN11 cells are ideal for further explorations on the putative interaction between Robo1 receptor and class 3 semaphorin signalling system. In addition, these results suggest that migrating gonadotropin-realising hormone secreting neurons might utilise Slit-Robo signalling to be directed to their right positions within the developing brain.

4.3 Conclusions

Evidence presented in the previous chapter shows that *Robo1*$^{-/-}$ cortical interneurons migrate through the developing striatum (Fig. 3.1, 3.10, 3.11), suggesting that these cells fail to respond to Sema3A and Sema3F chemorepulsive cues, which emanate from the striatal area. In the present chapter, I have shown by explants assays (Fig. 4.1, 4.2) and Boyden's chamber (Fig. 4.3, 4.4) that MGE-derived *Robo1*$^{-/-}$ cells are actually unresponsive to Sema3A and Sema3F induced chemorepulsion. Therefore, it seems that *Robo1*$^{-/-}$ cortical interneurons do migrate through the developing striatum because they do not respond to these chemorepulsive cues. Previous studies have illustrated that disruption of Nrp receptors signalling results in aberrant migration of cortical interneurons to the developing striatum (Marin et al., 2001; Zimmer et al., 2010). Thus, I will investigate in the following chapter whether the absence of Robo1 receptors results in a disruption of Nrp signalling in cortical interneurons.

In addition to their well-known function in directing the migration of interneurons, Sema3A and Sema3F have been documented to play a pivotal role in shaping the morphology of neurons (Winberg et al., 1998; Tran et al., 2007). Interestingly, migrating *Robo1*$^{-/-}$ cortical interneurons exhibit severe morphological alterations when compared to control counterparts (Andrews et al., 2008). The fact that migrating MGE-derived *Robo1*$^{-/-}$ cells do not respond to Sema3A and Sema3F chemorepulsion prompted me to speculate if these cells also fail to respond to the collapsing effect of class 3 semaphorins. Morphological examination of dissociated cells taken from the MGE of *Robo1*$^{-/-}$ mice and treated with Sema3A or Sema3F cues revealed that the morphology of these cells is indeed not affected by these cues (Fig. 4.7), illustrating once again that *Robo1*$^{-/-}$ cortical interneurons are unresponsive to class 3 semaphorins. This data also suggests that the altered morphology of *Robo1*$^{-/-}$ cortical interneurons (reported by Andrews et al., 2008) could emerge from the loss of responsiveness that the cells exhibit to some chemorepulsive cues that shape up their morphology.

In spite of expressing Robo receptors, it was unclear whether MGE-derived cortical interneurons respond to Slit signalling. In the present chapter, I provide evidence that MGE-derive cells do respond to Slit1 induced chemorepulsion, as assessed by explants assays and Boyden's chamber (Fig. 4.5A,C,E; 4.6A,C,E). Moreover, analysis of MGE-derived cells taken from *Robo1*$^{-/-}$ mice revealed that loss of Robo1 receptors also

perturbs the effect of Slits on the migration of MGE-derived cells (Fig. 4.5B,D,E; 4.6B,D,E).

An attempt to find a simpler and easier *in vitro* model to further explore the presumptive interactions between Robo1 receptor and the class 3 semaphorin signalling system led me to identify the cell line GN11 as a potential candidate for this enterprise. Indeed, GN11 cells expressed Nrp and Robo receptors and respond to class 3 semaphorins and Slits (Fig. 4.8;4.10). Additionally, disruption of Robo1 receptor in GN11 cells results in loss of responsiveness to Sema3A and Sema3F (Fig. 4.10A,B), mimicking the phenotype observed in MGE-derived *Robo1*$^{-/-}$ cells. Interestingly, these results also suggest that migrating gonadotropin-realising hormone neurons might be directed by Slit signalling during their migration from their birthplaces to their final destination. This possibility will not be explored in the present study.

The fact that Robo1-DN-transfected GN11 cell become more sensitive to Slit1 or Slit2 chemorepulsion suggests that Robo2 or Robo3 receptors might potentiate the effect of Slit1 and Slit2 ligands in the absence of Robo1 signalling. This result, although very interesting, will not be investigated any further in the present work. Nevertheless, I will discuss it in chapter 6. Instead, I will focus my efforts to investigate why *Robo1*$^{-/-}$ cortical interneurons do not respond to class 3 semaphorins.

Chapter 5: Deletion of Robo1 receptors results in down regulation of distinct components of the class 3 semaphorin signalling system in cortical interneurons

5.1 Background

I have shown in chapter 3 that deficiency of Robo1 receptors results in an increased number of GABAergic neurons in the developing neocortex and striatum (Fig. 3.1). My detailed examination on the developing striatum of *Robo1*$^{-/-}$ mice illustrates that the additional number of GABAergic cells in this structure is not due to an increase in its neural populations (projection- or inter- neurons) (Fig. 3.6-3.9). Counts of CB^{+} cells in the striatum of *Robo1*$^{-/-}$ mice (Fig. 3.10), along with the qualitative observations of greater numbers of GAD67-GFP^{+} cells in *Robo1*$^{-/-}$*;GAD-67-GFP*$^{neo/-}$ animals (Fig. 3.1), suggests that an influx of cortical interneurons occurs in the absence of Robo1 receptors. This notion is further supported by the fact that there exists virtually no co-localisation between the increased number of GAD7-GFP^{+} cells and those cells positive for FOXP2 in *Robo1*$^{-/-}$ mice (Fig. 3.11). Interestingly, the increased number of CB^{+} cells (cortical interneurons) reaches a peak at about E18.5 that diminishes towards birth (Fig. 3.10), indicating that these cells pass through the developing striatum in order to populate the neocortex. This idea is in agreement with the reported increase of cortical interneurons in the adult neocortex of *Robo1*$^{-/-}$ mice (Andrews et al., 2006, 2008). Since the developing striatum is an exclusion zone for migrating cortical interneurons, it seems that deletion of Robo1 receptors alters the response of these cells to the chemorepulsive cues expressed in that area.

The chemorepulsive molecules Sema3A and Sema3F have been documented to be expressed within the developing striatum and to repel cortical interneurons (Marin et al., 2001; Tamamaki et al., 2003a). Therefore, it seems that migrating *Robo1*$^{-/-}$ cortical interneurons are unresponsive to the chemorepulsive action of Sema3A and/or Sema3F. Indeed, I demonstrated in chapter 4, that MGE-derived cells taken from *Robo1*$^{-/-}$ mice are actually unresponsive to Sema3A and Sema3F *in vitro* (Fig. 4.1-4.4). In addition, I also documented that the morphology of MGE-derived *Robo1*$^{-/-}$ cells is unaltered in presence of Sema3A or Sema3F compared to MGE-derived *Robo1*$^{+/-}$ cells (Fig. 4.7).

Class 3 semaphorins signal through Nrp-PlexinA receptor complexes to induce a number of events in the developing brain (Winberg et al., 1998; Zhou et al., 2008; Gelfand et al., 2009). Experimental reports indicate that loss of Nrp function in cortical interneurons results in abnormal migration of them through the striatum (Marín et al., 2001; Zimmer et al., 2010). Given that my *in vivo* and *in vitro* data showed in the previous chapters indicate that cortical interneurons are less responsive to class 3 semaphorins and that they invade the striatum, I hypothesised that alterations in the expression and/or synthesis of Nrp and/or PlexinA receptors might result from the deletion of Robo1 receptors in cortical interneurons.

The aim of the present chapter is to evaluate the levels of class 3 semaphorin receptors in MGE-derived *Robo1*$^{-/-}$ cells. Additionally, I wish to assess whether there is an imbalance in the expression of intracellular effectors triggered by class 3 semaphorins these cells.

5.2 Results

5.2.1 Down regulation of *Nrp1, Nrp2, PlexinA1* and *PlexinA2* receptors in MGE-derived cells of *Robo1*$^{-/-}$ mice

To elucidate whether deletion of Robo1 receptor alters the expression of class 3 semaphorin receptors (Nrp1-2 and PlexinA1-A4 receptors) in MGE-derived cells, I carried out a QPCR analysis on FACS-sorted MGE-derived cells taken from E15.5 *Robo1*$^{-/-}$;GAD67-GFP$^{neo/-}$ (n=3) and *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ (n=3) littermates. This analysis revealed a significant reduction in the expression of *Nrp1* (-3.6 Fold; $p<0.01$), *PlexinA1* (-11.2 fold; $p<0.001$) and *PlexinA2* (-2.7 fold; $p<0.01$), but not *Nrp2* (-0.8 fold; $p>0.05$), expression in *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ cells when compared to *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ counterparts. I also found a modest increase of *PlexinA4* (+1.5 fold; $p<0.05$), and no significant change in *PlexinA3* (+0.3 fold) expression (Fig. 5.1A). Therefore, this data illustrates that loss of Robo1 receptors in MGE-derived cells leads to down regulation of *Nrp1*, *Nrp2*, *PlexinA1* and *PlexinA2* in cortical interneurons. In addition, these results might explain the reduced responsiveness of MGE-derived *Robo1*$^{-/-}$ cells to Sema3A and Sema3F chemorepulsion (Fig. 4.1-4.4), and the aberrant migration of *Robo1*$^{-/-}$ cortical interneurons through the developing striatum of *Robo1*$^{-/-}$ mice (Fig. 3.1, 3.10).

Numerous studies illustrate that Nrp receptors, in addition to forming heterodimers with PlexinA receptors and mediating the signalling of class 3 semaphorins, can also function as co-receptors with Kdr, Flt1 and VEGFR3 for vascular endothelial growth factor (VEGF) in endothelial cells (Soker et al., 1998), and they participate in the mediation of the VEGF response (Giraudo et al., 1998; Fuh et al., 2000; Gluzman-Poltorak et al., 2001; Catalano et al., 2004). Therefore, I wanted to determine whether reduction in the expression of Nrp receptors in MGE-derived *Robo1*$^{-/-}$ cells also affects the expression of the VEGF receptors. I performed a QPCR analysis for *Kdr*, *Flt1* and *VEGFR3* on FACS-sorted cells taken from the MGE of E15.5 *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ (n=3) and *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ (n=3) littermates. This analysis showed no expression of any of the three VEGF receptors in MGE-derived cells taken from both groups of animals, suggesting that cortical interneurons do not interact with VEGF signalling, at least during their initial migration (Fig. 5.1A).

In order to determine whether the reduced expression of class 3 semaphorin receptors in MGE-derived cells of *Robo1*$^{-/-}$ animals translate into corresponding changes in their protein levels, I performed WB analysis on dissociated cells taken from the MGE of E15.5 *Robo1*$^{-/-}$ and *Robo1*$^{+/-}$ animals for Nrp1 and PlexinA1 (the receptors that showed the greatest reduction in the QPCR examination). This analysis showed, in agreement with the QPCR data, a reduction in the levels of Nrp1 and PlexinA1 proteins in *Robo1*$^{-/-}$ cells when compared to control counterparts (Fig. 5.1B). Therefore, this data further supports the notion that Nrp and PlexinA receptors are down regulated in MGE-derived *Robo1*$^{-/-}$ cells.

It has been extensively shown that the class 3 semaphorin signalling system has diverse roles during brain development (Winberg et al., 1998; Gelfand et al., 2009). To clarify whether loss of Robo1 receptor leads to a general down regulation of *Nrp1-2* and/or *PlexinA1-A2* receptors within the developing forebrain of *Robo1*$^{-/-}$ mice, I assessed by *in situ* hybridisation the expression of *Nrp1-2* and *PlexinA1-2* receptors in coronal sections taken from *Robo1*$^{-/-}$ embryonic forebrains and compared to *Robo1*$^{+/-}$ littermates at E13.5, E15.5 and E18.5. This assessment showed that the expression of *Nrp1-2* and *PlexinA1-A2* is not perceptibly altered in the forebrain of *Robo1*$^{-/-}$ mice when compared to *Robo1*$^{+/-}$ counterparts at any of the three embryonic ages examined (Fig. 5.2, 5.3), suggesting that the reduction of class 3 semaphorin receptors in MGE-derived *Robo1*$^{-/-}$ cells might be specific for these cells and not a common phenomenon in the developing brain of these mice.

To confirm the reduction of Nrp levels in migrating cortical interneuron, I took coronal sections from E15.5 *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ (n=2) and *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ (n=2) brains and immunostained them for Nrp1 and Nrp2. The immunoreactivity for Nrp1 and Nrp2 was extremely low in both groups of animals and it was identified mainly in axonal tracks in cortical and subcortical structures, but not in migrating cortical interneurons (data not shown). Surprisingly, the striatum of *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ presented relatively strong immunoreactivity for both Nrp1 (Fig. 5.4B) and Nrp2 (Fig. 5.4H) receptors. However, Nrp1^{+} or Nrp2^{+} cells in the striatum of *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ do not co-localise with GAD67-GFP^{+} cells (Fig. 5.4A-C,G-I, and data not shown). Examination of Nrp1 and Nrp2 staining in the striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ showed reduced immunoreactivity for these two receptors (Fig. 5.4D-F, J-L). In addition to observing Nrp staining in striatal cells of *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$, Nrp1 and Nrp2 immunoreactivity was also detected in blood vessels irrigating the striatum (arrows in Fig. 5.4B,H). Interestingly, blood vessel in the striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ were also immunoreactive for both Nrp receptors (arrows in Fig. 5.4E,K). Since Robo1 receptor is expressed in striatal cells but was not observed in blood vessels (Fig. 3.2), it is very likely that absence of Robo1 receptors in striatal cells leads also to down regulation of their Nrp receptors, as it does in MGE-derived *Robo1*$^{-/-}$ cells (Fig. 5.1). The fact that Nrp1 and Nrp2 receptors were not detected in migrating cortical interneurons suggests that these cells produce very low levels of these proteins that might not be detected by immunohistochemistry.

5.2.2 Robo1 signalling is necessary, but not sufficient, to maintain the expression of Nrp and Plexin receptors

In the previous section, I showed that MGE-derived cells from *Robo1*$^{-/-}$ embryos contain reduced levels of Nrp and PlexinA receptors compared to control animals. Nevertheless, these results raised the following question: Is Robo1 expression or signalling necessary to induce and/or maintain the expression of Nrp and PlexinA receptors?

Since Robo1 gene is completely absent in *Robo1*$^{-/-}$ mice, and no expression of Robo1 receptor occurs at all, these animals were not suitable to address this issue properly. To solve this limitation, I transfected GN11 cells with Robo1-DN (Robo1-DN blocks the signalling but not the expression of Robo1 receptor; Hammond et al., 2005), treated them for 2 hours with CM-FLAG or CM-Sema3F after 2DIV and estimated by QPCR the levels of Nrp and PlexinA receptor in these cells.

As shown in chapter 4, GN11 cells contain both Robo and Nrp Receptors (Fig. 4.8) and respond to class 3 semaphorin and Slit ligands (Fig. 4.8-4.9). Additionally, Robo1-DN-transfected GN11 cells behave similar to MGE-derived *Robo1*$^{-/-}$ cells in Boyden's chamber assays (Fig. 4.10). Therefore, Robo1-DN-transfected GN11 cells seem to be a suitable system to explore the above question further.

Firstly, I assessed the expression of Nrp and PlexinA receptors in GFP-transfected GN11 cells treated with CM-FLAG. This analysis showed that *Nrp1* and *Nrp2* are expressed in GFP-transfected GN11 cells, confirming previous studies (Cariboni et al., 2007), and also illustrated that these cells express the four genes for *PlexinA (A1-A4)* (Fig. 5.5).

Secondly, I evaluated the expression of these receptors in GFP-transfected GN11 cells treated with CM-Sema3F. This examination revealed that *PlexinA4* expression is not altered by CM-Sema3F treatment, whereas *PlexinA1* expression is severely reduced (fold -51.8; $p<0.001$) and *Nrp1*, *Nrp2*, *PlexinA2* and *PlexinA3* expression is not detected at all in GFP-transfected GN11 cells, as compared to similar cells treated with CM-FLAG (Fig. 5.5). Thus, it seems that the excess of class 3 semaphorins results not only in the internalization of class 3 semaphorin receptors (Castellani et al., 2004; Narazaki and Tosato, 2006; Cariboni et al., 2007), but also in down regulation of them.

Thirdly, I estimated the expression of class 3 semaphorin receptors in Robo1-DN-transfected GN11 cells treated with CM-FLAG. This assessment showed that *PlexinA4* and *Nrp2* expression is not significantly altered by the blocking of Robo1 signalling, whilst *Nrp1* and *PlexinA1-3* expression is not detected in these cells when compared to GFP-transfected GN11 treated with the control-CM (Fig 5.5), suggesting that Robo1 signalling is necessary for the expression of Nrp and PlexinA receptors.

Finally, I wanted to examine the expression of Nrp and PlexinA receptors in Robo1-DN-transfected GN11 cells treated with CM-Sema3F. Surprisingly, this evaluation showed up regulation of *Nrp1* (fold +4.93; $p<0.5$) and *Nrp2* (fold +11.7; $p<0.01$) in Robo1-DN-transfected GN11 cells after treatment with CM-Sema3F when compared to GFP-transfected GN11 treated with CM-FLAG (Fig. 5.5). Additionally, this assessment showed low levels of *PlexinA2* (fold -2.75; $p<0.05$) and *PlexinA3* (fold -2.24; $p<0.05$), but no significant changes of *PlexinA1* (fold -0.84; $p>0.5$) or *PlexinA4* (fold +0.36; $p>0.5$) in Robo1-DN-transfected GN11 cells after treatment with CM-Sema3F when compared to GFP-transfected GN11 treated with CM-FLAG (Fig. 5.5), illustrating that Sema3F treatment can rescue, to some extend, the expression of class 3 receptors in Robo1-DN-transfected GN11 cells.

Taken together, these results indicate that the expression of class 3 semaphorin receptors depends on the presence of their natural ligands. Moreover, Robo1 signalling seems to be necessary to maintain, but not to induce, the expression of Nrp and PlexinA receptors. This conclusion is based on the fact that blocking of Robo1 signalling leads to drastic down regulation of class 3 semaphorin receptors, but the expression of these receptors can be partially rescued by the presence of their natural ligands.

5.2.3 Levels of transcription factors in MGE-derived *Robo1*$^{-/-}$ cells

Recent work suggests that Nkx2.1, Dlx1 and Dlx2 transcription factors regulate the expression of Nrp receptors and thus, the response of MGE-derived cells to the class 3 semaphorin signalling (Le et al., 2007; Nobrega-Pereira et al., 2008). Moreover, experimental evidence suggests that Nkx2.1 in proliferating and early postmitotic MGE cells can also activate the expression of Lhx6 (Du et al., 2008). Absence of Lhx6 results in delayed migration of interneurons and also affects their targeting within the cortex (Alifragis et al., 2004; Liodis et al., 2007; Zhao et al., 2008).

The fact that reduced levels of Nrp and PlexinA receptors are observed in MGE-derived *Robo1*$^{-/-}$ cells and aberrant migration of cortical interneurons occurs through the striatum of *Robo1*$^{-/-}$ mice, prompted me to ask whether absence of Robo1 receptor disrupts the expression of these transcription factors in *Robo1*$^{-/-}$ cortical interneurons, and thus, their response to class 3 semaphorin induced chemorepulsion. To clarify this idea, I carried out *in situ* hybridisation experiments on *Robo1*$^{+/-}$ and *Robo1*$^{-/-}$ mice at E13.5 and E15.5 (n=3 per age and condition), and QPCR on E15.5 FACS-sorted cells prepared from the MGE of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ (n=3) and *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ (n=3) littermates for *Dlx1, Dlx2, Nkx2.1*, *Lhx6* and *Lhx8* mRNA. The expression of Dlx and Lhx8 transcription factors showed no differences between *Robo1*$^{+/-}$ and *Robo1*$^{-/-}$ embryos at any of the ages analysed by in situ hybridisation (Fig. 5.6) or by QPCR [*Dlx1* (1.03 fold; $p>0.05$); *Dlx2* (1.38 fold; $p>0.05$); Lhx8 (1.01 fold; $p>0.05$)]. However, expanded expression of *Nkx2.1* (at E13.5 but not E15.5) and *Lhx6* was detected by *in situ* hybridisation in the MGE of *Robo1*$^{-/-}$ animals compared to *Robo1*$^{-/-}$ littermates (Fig. 5.7). Surprisingly, no significant changes in the levels of these transcription factors were detected by QPCR [*Nkx2.1* (+1.32 fold, $p>0.05$); *Lhx6* (+1.68 fold, $p>0.05$)] between these animals, suggesting that the increased expression of *Nkx2.1* and *Lhx6* observed in the MGE of *Robo1*$^{-/-}$ mice may be due to the reported increase of proliferating cells

reported in the MGE of those animals (Andrews et al., 2008), rather than increase in the expression of these transcription factors.

5.2.4 Deletion of Robo1 results in down regulation of endogenous *Sema3F*, but not *Sema3A*, as well as some of its intracellular effectors activated by class 3 semaphorins

One of the main functions of class 3 semaphorins is to induce cytoskeletal reorganization, which leads to growth cone collapse and retraction of cell processes in neurons (Winberg et al., 1998; Tran et al., 2007). Analysis of *Robo1*$^{-/-}$ mice illustrated that loss of Robo1 receptor results in a number of morphological alterations in migrating cortical interneurons (Andrews et al., 2008). Particularly, it was reported that migrating *Robo1*$^{-/-}$ cortical interneurons exhibit longer and more elaborated cell processes compared to control littermates (Andrews et al., 2008).

Since my *in vitro* studies, showed in chapter 4, illustrate that MGE-derive cells taken from *Robo1*$^{-/-}$ fail to respond to class 3 semaphorins in chemotaxis experiments (Fig. 4.1-4.4) as well as in collapse assays (Fig. 4.7), it is very likely that deletion of Robo1 receptor might also affect the expression of intracellular effectors triggered by class 3 semaphorins and related to cytoskeleton dynamics.

To elucidate whether loss of Robo1 receptors affects the expression of intracellular effectors triggered by class 3 semaphorin signaling, I examined by QPCR analysis the expression of *Akt*, *Cdc42*, *Crimp2*, *Farp2*, *Rac1* and *Rnd1* (well-known class 3 semaphorin intracellular effectors; Puschel, 2007; Zhou et al., 2008), as well as the endogenous levels of *Sema3A* and *Sema3F* in MGE-derived cells prepared from *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ (n=2) and *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ (n=2) littermates. This analysis showed significant reduction in the level of *Farp2* (-7.46 fold; $p<0.01$), *Rnd1* (-3.08 fold; $p<0.01$), *Sema3F* (-3.2 fold; $p<0.01$) *and Cdc42* (-2.07 fold; $p<0.05$), but an increase of *Sema3A* (+3.2 fold; $p<0.01$) and *Akt* (+2.03 fold; $p<0.05$) in *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ cells when compared to control littermates (Fig. 5.8). Taken together, this data suggests that absence of Robo1 receptors does not only alter the expression of class 3 semaphorin receptors (Fig. 5.1), but also disrupts the expression of distinct components of the class 3 semaphorin signalling system. Additionally, changes in the expression of intracellular effectors observed in *Robo1*$^{-/-}$ mice might also explain, at least in part, the abnormal migration and morphology of cortical interneurons in these animals.

5.2.5 Physical interaction between Robo1 and Nrp1, Nrp2 and PlexinA1

In addition to forming dimers with Plexin and VEGF receptors, Nrp proteins have also been shown to bind the cell adhesion molecule, L1 (Castellani et al., 2002, 2004). L1 is a member of the IgCAM superfamily and plays a critical role in the formation of neuronal networks (Castellani et al., 2004). Given that Robo1 receptor is also a well-known member of the IgCAM family (Liu et al., 2004), I asked whether there exists an actual physical interaction between Robo1 and Nrp and/or PlexinA receptors.

To answer this question I prepared cell lysates from E13.5 wildtype dissociated cell cultures and separately immunoprecipitated them with Nrp1, Nrp2, PlexinA1 or Robo1 antibodies. After gel electrophoresis and membrane transference, adhered proteins on membranes were independently detected using Nrp1, Nrp2, PlexinA1 or Robo1 antibodies. These experiments showed that Nrp1-, Nrp2- or PlexinA1 imunoprecipitated lysates can be detected with Robo1 antibodies (red box in Fig 5.9A). Moreover, Robo1 immunoprecipitated lysates can be detected with Nrp1, Nrp2 or PlexinA1 antibodies (black box in Fig. 5.9A). Specificity of antibodies was confirmed by the strong detection between antibodies used to precipitate the lysates and those used for immunodetection (blue boxes in Fig. 5.9A). Thus, this data demonstrates that there is a physical interaction between Robo1 and class 3 semaphorin receptors in MGE-derived cells.

Since GN11 cells exhibit similar characteristics to migrating MGE-derived cells, I wanted to test whether the binding between Robo1 and class 3 semaphorin receptors was specific to MGE-derived cells or a common phenomenon in migrating neurons. Thus, I prepared cell lysates from full length Robo1-myc, Nrp1-myc or Nrp2-myc transfected GN11 cells, immunoprecipitated them with myc antibodies, and separately detected them with Nrp1, Nrp2 or PlexinA1 antibodies. This examination also showed that Robo1 receptor can be detected with Nrp1, Nrp2 or PlexinA1 antibodies (black box in Fig. 5.9B), indicating that binding between Robo1 and Nrp and PlexinA receptors might be a common mechanism in migrating neurons.

5.3 Conclusions

In chapter 3, I presented evidence suggesting that deficiency of Robo1 receptor results in aberrant migration of cortical interneurons through the developing striatum (Fig. 3.1, 3.10). I observed, in chapter 4, that MGE-derived cells taken from *Robo1*$^{-/-}$ are unresponsive to the chemorepulsive action of Sema3A and Sema3F (Fig. 4.1-4.4, 4.7), suggesting that deletion of Robo1 receptors in cortical interneurons affects their response to class 3 semaphorin signalling. Previous studies illustrate that disruption of Nrp signalling affects the targeting of cortical interneurons as well their response to Sema3A and Sema3F induced chemorepulsion (Marin et al., 2001; Zimmer et al., 2010). Therefore, I hypothesised in the present chapter that aberrant migration of cortical interneurons through the developing striatum of *Robo1*$^{-/-}$ mice and loss of responsiveness to Sema3A and Sema3F observed in MGE-derived *Robo1*$^{-/-}$ cells might result from a reduction in the expression and/or synthesis of class 3 semaphorin receptors in *Robo1*$^{-/-}$ animals.

Analysis of MGE-derived cells prepared from *Robo1*$^{-/-}$*;GAD-67-GFP*$^{neo/-}$ animals by QPCR demonstrated that these cells express reduced levels of *Nrp1*, *Nrp2*, *PlexinA1* and *PlexinA2* compared to control cells (Fig. 5.1A). The reduced expression of *Nrp* and *PlexinA* transcripts in MGE-derived cells from *Robo1*$^{-/-}$ embryos is actually translated into their corresponding proteins levels (Fig. 5.1B). Interestingly, the reduction of Nrp and PlexinA expression seems to be restricted to *Robo1*$^{-/-}$ GABAergic neurons since the general pattern of expression of these receptors is not obviously altered in the forebrain of *Robo1*$^{-/-}$ embryos compared to control littermates (Fig. 5.2,5.3).

Nrp1 and Nrp2 immunoreactivity is extremely low in both *Robo1*$^{-/-}$*;GAD-67-GFP*$^{neo/-}$ and *Robo1*$^{+/-}$*;GAD-67-GFP*$^{neo/-}$ forebrain, and no co-localisation between Nrp1 or Nrp2 immunoreactivity and GAD67-GFP^{+} cells could be found in either the pallium or subpallium of both groups of animal (Fig. 5.4 and data not shown), suggesting that GABAergic cells contain very low levels of these proteins that cannot be detected by immunohistochemestry. Strikingly, there exists an enormous amount of cells immunoreactive for Nrp1 and Nrp2 receptors within the striatum of Robo1$^{+/-}$*;GAD-67-GFP*$^{neo/-}$, but not in *Robo1*$^{-/-}$*;GAD-67-GFP*$^{neo/-}$ mice (Fig. 5.4). Since striatal cells express Robo1 receptors (Fig. 3.2), it is likely that absence of this receptor also results in down regulation of class 3 semaphorin receptors in striatal cells. Interestingly, blood vessels in both *Robo1*$^{+/-}$*;GAD-67-GFP*$^{neo/-}$ and *Robo1*$^{-/-}$*;GAD-67-GFP*$^{neo/-}$ mice are strongly immunoreactive for both Nrp1 and Nrp2 (Fig. 5.4B,E,H,K), suggesting again that deletion

of Robo1 receptor affects the expression of class 3 semaphorins in GABAergic cells but not other systems.

Similar to MGE-derived cells of *Robo1*$^{-/-}$ embryos, Robo1-transfected GN11 cells exhibit down regulation of class 3 receptor, this result clearly indicates that Robo1 signalling is necessary for the expression of these receptors. Interestingly, treatment of Robo1-transfected GN11 cells with Sema3F partially rescued the normal levels of class 3 semaphorin receptors, suggesting that class 3 semaphorins are essential for the correct expression of their receptors, whereas Robo1 signalling contributes to maintain the expression of those receptors

No differences in the expression of *Dlx1*, *Dlx2*, *Nkx2.1*, *Lhx6* or *Lhx8* transcription factors are detected between MGE-derived cells of *Robo1*$^{-/-}$ embryos and control counterparts by QPCR. Interestingly, I observed expanded expression of *Nkx2.*1 and *Lhx6* in the MGE of *Robo1*$^{-/-}$ mice compared to *Robo1*$^{+/-}$ littermates by *in situ* hybridisation (Fig. 5.7). I reasoned that the expanded expression of these transcription factors in the MGE of *Robo1*$^{-/-}$ embryos might reflect the reported increase of MGE progenitors in these animals (Andrews et al., 2008) rather than an increase of their expression.

In addition to down regulation of class 3 semaphorin receptors in MGE-derived cells of Robo1$^{-/-}$ embryos, I observed that the absence of Robo1 receptor in these cells also produces significant down regulation of a number of class 3 semaphorin intracellular effectors (Fig. 5.8), which might explain, at least in part, the abnormal migration and morphology of *Robo1*$^{-/-}$ cortical interneurons (Fig. 3.10,4.7).

The fact that Robo1, but not Slit1 or Slit2, signalling is crucial for the correct migration of cortical interneurons around the striatum indicates that other mechanisms independent of Slit might activate its signalling. Here, I found that there exists a physical interaction between Robo1 and class 3 semaphorin receptors (Fig. 5.9), suggesting that the cell sorting of cortical interneurons around the striatum might result from a collaborative effort between Robo1 and the class 3 semaphorin signalling system.

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Chapter 6: Discussion

6.1 Cortical interneuron migration

Interneurons that populate the adult neocortex emerge from embryonic transient structures situated in the ventral telencephalon (namely the GEs). After becoming postmitotic, they migrate away from their proliferative zones in the GEs and move along tangential corridors or paths towards the developing neocortex. (Anderson et al., 1997a; Tamamaki et al., 1997; Lavdas et al., 1999; Wichterle et al., 1999, 2001).

Interest in understanding the mechanisms that control the migration of cortical interneuron has increased in recent years, as numerous mental disorders have been related to deficiencies in the migration of these cells. (Mallet et al., 2006; Di Cristo, 2007; Birchmeier, 2009). Since cortical interneurons appear to migrate independently of a glial scaffold, it is thought that a combination of chemoattractive and chemorepulsive cues expressed within the neocortex and ventral telencephalon, respectively, imparts directionality to them during their journey from the GEs to their final destination in the neocortex (Polleux et al., 2002; Marin et al., 2003; Wichterle et al., 2003). Attempts to uncover the extrinsic cellular mechanisms that direct the migration of cortical interneurons have identified some soluble chemotactic cues/and their corresponding receptors such as: SDF1/CXCR4 (Tiveron et al., 2006; Stumm et al., 2007) and NGR1/Erb4 (Flames et al., 2004) in the neocortex, and the membrane-bound EphrinA5/EphA4 (Zimmer et al., 2008) in the GEs. Given that EphrinA5 is a membrane-bound protein exclusively expressed in the proliferative zones of the GEs (Zimmer et al., 2008), it is likely that this protein exerts its chemorepulsive action locally on cortical interneurons and that other soluble factors repel cortical interneurons away from ventral telencephalon.

6.2 Multiple roles of Slit-Robo signalling in the development of cortical interneurons

Several lines of research suggest that Slit proteins, a soluble family of chemorepulsive ligands, might be involved in directing cortical interneurons away from the ventral telencephalon. First, *Slit1* is strongly expressed in the VZ of the GEs (Bagri et al., 2002; Marillat et al., 2002). Second, cortical interneurons contain Robo1, Robo2 and Robo3

receptors, the cognate receptors for Slit ligands (Andrews et al., 2006, 2008; Barber et al., 2009). Third, *in vitro* experiments show that LGE-derived cells are repelled by aggregates of cells expressing Slit ligands (Hu, 1999; Zhu et al., 1999). In agreement with this evidence, my *in vitro* data shows that Slit1 has a strong effect on the migration of MGE-derived cells. Indeed, my explants and chemotactic assays illustrate that MGE-derived cells exhibit reduced migration after treatment with Slit1. It is important to note that the data presented here does not reveal whether Slit1 inhibits or repels the migration of these cells. However, I consider the effect of Slit1 on MGE-derived cells as repulsive rather than inhibitory (as reported by Zhu et al., 1999 using LGE-derived cells); as cortical interneurons migrate actively from the subpallium to the neocortex, and if Slit1 had an inhibitory activity on these cells, it would delay their migration or stop it in the ventral telencephalon. Nevertheless, future work (possibly co-explanting MGE tissues and aggregates of cells expressing Slit1) is still needed to directly clarify the effect of Slit1 on these cells. Qualitative observations in the subpallium of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice seem to support the idea that Slit1 has a repulsive effect on cortical interneurons, as a number of those cells can be found misplaced in the VZ of the GEs when compared to control littermates (data not shown), which might indicate that some of these cells are actually not repelled by Slit1. Interestingly, the chemorepulsive action of Slit1 on cortical interneurons seems to be mediated by Robo1, but not Robo2 or Robo3, receptors as explants or dissociated cells taken from the MGE of *Robo1*$^{-/-}$ mice are not responsive to this chemotactic cue, even though they contain Robo2 and Robo3 (see below).

In contrast to my current data and previous reports (Hu, 1999; Zhu et al., 1999), the analysis of *Slit1*$^{-/-}$*/Slit2*$^{-/-}$ mice has illustrated no alterations in the number and position of GABAergic cells that populate the developing neocortex (Marin et al., 2003). Thus, it seems that Slit1 and Slit2 signalling is not required for the correct migration and/or positioning of GABAergic cells in the developing neocortex. As pointed out by Marin et al. (2003), a possible explanation for this observation is that Slit proteins do not direct the migration of cortical interneurons, but the migration of their striatal counterparts; particularly, these authors found that the migration of striatal NPY^{+} and ChAT^{+} interneurons is mediated by Slit signalling. In the present work, I found that most cells that make up the developing striatum are highly immunoreactive for Robo1 and Robo2 proteins, receptors for Slit ligands, suggesting that Slit proteins do not only direct the migration of striatal interneurons, but also the migration of striatal projection cells. Support to this idea comes from the facts that LGE is the source of striatal projection neurons (Deacon et al., 1994; Olsson et al., 1995, 1998) and Slit proteins repel LGE-

derived cells *in vitro* (Hu, 1999; Zhu et al., 1999). However, my analysis of developing and mature striatal projection neurons does not revealed any significant difference between *Robo1*$^{-/-}$, *Robo2*$^{-/-}$and *Slit1*$^{-/-}$/ *Slit2*$^{-/-}$ mice when compared to their corresponding control littermates, indicating that Slit-Robo signalling does not affect the migration and/or positioning of these cells.

How then can we reconcile the inconsistencies between the *in vitro* (Slit proteins have a repulsive effect on the migration of LGE- and MGE- derived cells; Hu, 1999; Zhu et a., 1999; and present work) and the *in vivo* (no differences in the number and/or positioning of striatal projection neurons and cortical interneurons; present work; Marin et al., 2003) data regarding the role of Slit signalling on interneuron migration? Since Slit1 and Slit3 are also expressed in the developing neocortex, Marin and others (2003) have suggested that cortical interneurons might, indeed, respond to Slit chemorepulsion, but only upon arrival in the neocortex. Particularly, expression of Slit1 and Slit3 has been observed in the developing CP (Bagri et al., 2002; Marillat et al., 2002), which may prevent cortical interneurons from invading prematurely this layer. In this regard, work in the Parnavelas laboratory has documented that mice deficient of Robo1, but not Robo2 or Robo3; receptors contain significantly more CB^{+} cells (presumptive cortical interneurons) in their neocortices than wildtype littermates (Andrews et al., 2006, 2008; Barber et al., 2009; present observations). This findings support the notion that Slit ligands through activating Robo1 receptor might prevent the premature entry of interneurons to the CP. Thus, the response of MGE-derived cells to Slit1 induced chemorepulsion observed *in vitro* would reflect how this molecule affects cortical interneurons once they reach the cortical wall. It is then very intriguing that Marin et al., (2003) did not observe any difference in the position of these cells within the neocortex of *Slit1*$^{-/-}$/ *Slit2*$^{-/-}$ mice. Nevertheless, it is likely that Slit3 compensates the absence of Slit1 ligands. In support of this view, it has been shown that Robo receptors can bind similarly to Slit1, Slit2 and Slit3 ligands (Liu et al., 2004; Mambetisaeva et al., 2005; Cammuri et al., 2005) and therefore, it seems that cortical interneurons containing Robo1 receptor could still respond to Slit3 in *Slit1*$^{-/-}$/*Slit2*$^{-/-}$ mice.

Given that no more cortical interneurons are found in the neocortex of *Robo2*$^{-/-}$ and *Robo3*$^{-/-}$ mice, it is possible that these receptors do not participate in cortical interneuron migration. This idea is also supported by the observations that MGE-derived cells taken from *Robo1*$^{-/-}$ are not responsive to the chemorepulsive action of Slit1, even though they contain Robo2 and Robo3 receptors.

The observations described above indicate an active role of Slit1 and Slit3, through the activation of Robo1, signalling in the migration of interneurons once they arrive to the neocortex. However, they do not fully explain the expression of Slit1 and Robo1 in the VZ of the GE (Marillat et al., 2002), as *Robo1*$^{-/-}$ and *Slit*$^{-/-}$*/Slit2*$^{-/-}$ cortical interneurons still migrate from the ventral telencephalon to the neocortex. Thus, it is likely that Slit-Robo signalling might not affect the migration of these cells within the ventral brain but, instead, have other functions such as the control of cell proliferation. In this regard, Slit signalling has been reported to direct the proliferation of distinct neuronal precursors in *Drodophila* (Mehta and Bhat, 2001). Therefore, Slit-Robo1 signalling in the VZ of the GEs could be involved in the proliferation of cortical interneurons, but not in the proliferation of striatal cells (projection or interneurons), as I observed no differences in the number of striatal cells in *Robo1*$^{-/-}$, *Robo2*$^{-/-}$ or *Slit1*$^{-/-}$*/ Slit2*$^{-/-}$ mice. Work by Andrews and colleagues (2008) actually demonstrated that Slit-Robo1 signalling regulates the proliferation of GE progenitors. Specifically, these authors documented increased numbers of mitotic cells in the GE of *Robo1*$^{-/-}$ mice compared to their control littermates. Moreover, these authors also observed reduced proliferation in dissociated GE-derived cells after treatment with Slit proteins. Thus, this increased proliferation in the GEs of *Robo1*$^{-/-}$ mice might explain why those animals contain more cortical interneurons.

Another putative function of Slit-Robo signalling in the ventral telencephalon could be the regulation of cortical interneuron morphology, possibly to facilitate their migration and/or response to other chemotactic cues. *In vitro* experiments have shown that Slit1 promotes neurite branching in MGE-derived cells (Sang et al., 2002; Sang and Tan, 2003). Interestingly, the action of Slit1 on MGE-derived cells seems to be time dependent as it promotes neurite branching in those cells after several days in culture (Sang et al., 2002). Evidence from the analysis of *Robo1*$^{-/-}$, *Robo2*$^{-/-}$, *Robo3*$^{-/-}$ or *Slit1*$^{-/-}$*/ Slit2*$^{-/-}$ mice shows severe alterations in the morphology of migrating cortical interneurons in those animals, as they exhibit longer and more elaborated neurites compared to corresponding controls (Andrews et al., 2008; Barber et al., 2009), which strongly emphasises the role of Slit-Robo signalling in shaping up their morphology. Interestingly, it appears that Slit-Robo signalling promotes neurite branching exclusively in cortical interneurons, as no alterations are found in the morphology of cortical projection neurons in mixed cultures (Sang et al., 2002). At present, it is unclear whether Slit signalling also regulates the morphology of striatal projection neurons and future experiments are needed to clarify this point.

Taking together the evidence described above, it seems that Slit-Robo signalling has multiple functions in the development of cortical interneurons. First, Slit1 through the activation of Robo1 receptor controls the proliferation of GE progenitors in the subpallium. Second, Slit1 by activating Robo-1, -2, -3 receptors contributes to shape up and possibly constrain the morphology of migrating cortical interneurons. Third, Slit1 and Slit3 through Robo1 receptor might prevent cortical interneurons from prematurely invading the CP. The fact that there are no major alterations in the migration of cortical interneurons or striatal cells from the ventral telencephalon to their final destination in *Slit1*$^{-/-}$*/ Slit2*$^{-/-}$ mice, suggests that other yet unknown soluble factors direct the migration of those cells away from their proliferative zones and towards the neocortex and striatum, respectively. Nevertheless, this idea does not rule out the possibility that Slit signalling (or an unknown member of the Slit family) might also have a mild effect on migrating GABAergic cells, possibly giving them the first "push" to leave their proliferative zones, but it would not be sufficient for controlling cell migration from the LGE and MGE.

6.3 Robo1 receptor cooperates with class 3 semaphorins to steer cortical interneurons around the striatum

En route to the neocortex, cortical interneurons migrate around the developing striatum and steer clear of that structure (Marin and Rubenstein, 2003; Metin et al., 2006). It has been proposed that these cells avoid entering to the developing striatum because they are repelled by chemorepulsive cues emanating from that area (Marin and Rubenstein, 2003; Metin et al., 2006). In an early study, Andrews and colleagues (2006) qualitatively observed increased CB^{+} staining in the developing striatal area of Robo1 deficient (exon 5 deleted) mice when compared to control littermates. Given that CB is a marker of migrating cortical interneurons, Andrews et al. (2006, 2008) speculated that cortical interneurons lacking Robo1 receptor aberrantly migrate into the developing striatum, possibly taking a "short-cut" towards the neocortex. However, no quantification of the presumptive increased number of CB^{+} cell in the developing striatum of Robo1 deficient mice was carried out by these authors, casting some doubts as to whether there exists an actual increase of these GABAergic cells in those animals.

My analysis of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ undoubtedly demonstrated that deletion of Robo1 receptor results in an increased number of GABAergic cells in the developing striatum. Moreover, these observations strongly suggest that aberrant migration of cortical interneurons does occur thought the developing striatum of *Robo1*$^{-/-}$ animals, as previous

studies have shown that striatal neurons preferentially express GAD65, whereas cortical interneurons express higher levels of GAD67 (Greif et al., 1992; Mercugliano et al., 1992; Feldblum et al., 1993; Hendrickson et al., 1994). The lack of specific markers for cortical interneurons prevented me from exploring in detail the exact nature of those misplaced cells in the striatum of *Robo1*$^{-/-}$ mice. However, in an attempt to confirm and quantify the size of this aberrant migration in *Robo1*$^{-/-}$ mice, I quantified CB^{+} cells in the developing striatum of these and control animals at different embryonic ages. It is important to note here that CB is also expressed in the vast majority of mature striatal projection neurons, particularly in those that make up the striatal matrix compartment (Ouimet et al., 1988; Liu and Gaybriel, 1992), but since I found no differences in developing or mature striatal cells (projection or interneurons) in *Robo1*$^{-/-}$ mice and control littermates, I considered CB as an acceptable marker for migrating cortical interneurons (as reported in Anderson et al., 1997; Andrews et al., 2006). The quantification of CB^{+} showed an increase of these cells in the developing striatum of *Robo1*$^{-/-}$ mice compared to control littermates at all ages examined, confirming previous qualitative observations of greater CB staining in the striatum of Robo1 deficient embryos (Andrews et al., 2006) and also indicating that cortical interneurons move as a wave within the developing striatum of *Robo1*$^{-/-}$ embryos, which reaches a peak at E18.5, and subsequently diminishes as development proceeds (i.e. E15.5= ~23%; E18.5= ~39%; P0= ~16% more cells than control littermates). This movement mirrors the migration of newly generated cortical interneurons through the subpallium *en route* to the cortex (Parnavelas et al., 2000; Marin and Rubenstein, 2003; Metin et al., 2006). Interestingly, the approximate 30% increase of CB^{+} in the developing striatum of *Robo1*$^{-/-}$ embryos matches the increased number of interneurons (approximately 30%) reported in the mature neocortex of these animals (Andrews et al., 2008), suggesting that those cortical interneurons migrating through the developing striatum might be the ones that are more numerous in the mature neocortex of *Robo1*$^{-/-}$ mice.

Expression of Sema3A and Sema3F in the developing striatum creates an exclusion zone for migrating cortical interneurons. Here, it seems that *Robo1*$^{-/-}$ cortical interneurons migrate through the striatal area because they do not respond to the chemorepulsive action of class 3 semaphorins. My *in vitro* data showed that MGE-derived cells from *Robo1*$^{-/-}$ mice are indeed unresponsive to both chemorepulsive cues. Interestingly, approximately 30% of the MGE-derived cells taken from *Robo1*$^{-/-}$ mice are not responsive to the chemorepulsive effect of Sema3A or Sem3F, which is reflected in the observed increase of CB^{+} cells in the striatum of these mice. Thus, failure of *Robo1*$^{-/-}$

cortical interneurons to respond to the chemorepulsive action of Sema3A and Sema3F might be the key factor for their aberrant presence in the developing striatum.

Class 3 semaphorins are known to induce cytoskeletal reorganisation leading to growth cone collapse and retraction of cell processes (Winberg et al., 1998; Tran et al., 2007). Andrews and others recently reported that migrating *Robo1*$^{-/-}$ cortical interneurons exhibit longer and more elaborated processes when compared to control littermates (Andrews et al., 2008). While this abnormal morphology may be caused by the disruption of Slit-Robo1 signalling, it might also reflect the inability of these cells to respond to factors that shape up their morphology, such as class 3 semaphorins. Here, I qualitatively observed that MGE-derived cells taken from *Robo1*$^{-/-}$ mice are unresponsive to the collapsing effect of class 3 semaphorins compared to control counterparts. Thus, these observations seem to explain the abnormal morphology that *Robo1*$^{-/-}$ cortical interneurons exhibit *in vivo*, as they are refractory to the collapsing effect of class 3 semaphorins. The alterations in the expression of class 3 semaphorin intracellular effectors observed in *Robo1*$^{-/-}$ cortical interneurons might also explain why these cells are less responsive to Sema3A and Sema3F, as they seem to have affected the cytoskeletal dynamics that shape up their morphology and migration. Interestingly, the analysis of *Robo2*$^{-/-}$ or *Robo3*$^{-/-}$ animals revealed similar alterations in the morphology of cortical interneurons than *Robo1*$^{-/-}$ mice, which supports the view that a plausible function of Slit1-Robo -1,-2,-3 signalling in the ventral telencephalon is to regulate the morphology of those cells during their migratory journey

Early studies by Marin and colleagues (2001) suggested that loss or disruption of Nrp function results in perturbations in cortical interneuron migration (Marín et al., 2001). Specifically, loss of function studies, using focal electroporation of *Nrp1* dominant negative plasmids in the MGE of wildtype embryos or similar delivery of *GFP* plasmids in *Nrp2*$^{-/-}$ mice, revealed an influx of cortical interneurons to the developing striatum (Marin et al., 2001). Since my *in vivo* and *in vitro* findings suggested that at least a subpopulation of cortical interneurons do not respond or are less sensitive to Sema3A and Sema3F induced chemorepulsion, I speculated that down regulation of Nrp and/or PlexinA (receptors for class 3 semaphorins) proteins might occur in the absence of Robo1 receptor. My results clearly illustrate a significant down regulation of *Nrp1*, *Nrp2*, *PlexinA1* and *PlexinA2* transcripts in *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ cells when compared to controls, a finding that I confirmed at the protein level from MGE dissociated cells of *Robo1*$^{-/-}$ embryos. Thus, my data suggests that loss of Robo1 receptor leads to specific down regulation of Nrp1, Nrp2, PlexinA1 and PlexinA2 receptors in cortical

interneurons, which results in reduced responsiveness to Sema3A and Sema3F induced chemorepulsion and their subsequent migration through the developing striatum. Furthermore, this data indicates that Robo1 signalling is required for the correct expression of multiple components of the class 3 semaphorin signalling system.

In addition to modulating class 3 semaphorins, Nrp receptors have also been shown to function as co-receptors with Kdr, Flt1 and *VEGFR3* for vascular endothelial growth factor (VEGF) in endothelial cells (Soker et al., 1998) and to participate in the mediation of the VEGF response (Giraudo et al., 1998; Fuh et al., 2000; Gluzman-Poltorak et al., 2001; Catalano et al., 2004). Therefore, I asked whether reduction in Nrp levels in *Robo1*$^{-/-}$ cortical interneurons also affects VEGF receptor levels. My analysis showed no expression for the three VEGF receptors in MGE-derived cells in these animals or in control litermates, indicating that cortical interneurons do not interact with VEGF signaling, at least during their initial migration.

Slit-Robo and class 3 semaphorin signalling system participate in numerous events during brain development (Winberg et al., 1998; Gelfand et al., 2009). Then, it was plausible to assume that reduction of Nrp and PlexinA receptor could also be observed in other developing brain systems of *Robo1*$^{-/-}$ mice. My examination by *in situ* hybridisation of *Nrp1, Nrp2, PlexinA1 and PlexinA2* expression in *Robo1*$^{+/-}$ and *Robo1*$^{-/-}$ embryos at different embryonic ages, illustrates that there exists no differences in the general expression pattern of these receptors between both groups of animals, which might indicate that the regulation of class 3 semaphorin receptors by Robo1 signaling is specific for cortical interneurons. To confirm this suggestion, I immunostained sections taken from *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice and control littermates for Nrp1 and Nrp2. Different protocols were applied to label cortical interneurons with Nrp1 and Nrp2 antibodies, but I was unable to detect immunoreactivity with any of them (i.e. no staining in GAD67-GFP^{+} cells present within the subpallium or neocortex). It is likely that cortical interneurons produce very low levels of both receptors that might not be detected by immunohistochemitry. Interestingly, I observed very reduced immunoreactivity for both Nrp receptors in the striatum of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice. As described above, striatal projection neurons contain Robo1 (and Robo2) proteins and therefore, the absence of Robo1 receptors in those cells might also lead to the down regulation of class 3 semaphorin receptors. Thus, these observations seem to indicate that Robo1 signalling controls the expression of class 3 semaphorin receptors in GABAergic cells. It is still intriguing that there are no differences in the number of striatal projection neurons in differentiating or mature *Robo1*$^{-/-}$ *or Robo2*$^{-/-}$ mice. One possible explanation for this

phenomenon is that Robo (Robo1 and Robo2) signalling regulates postnatal events such as dendritogenesis or synaptogenesis in the striatum, as it has been reported in other brain areas (Campbell et al., 2007; Furrer et al., 2007).

Several lines of research have documented that transcription factors, such as Nkx2.1, Lhx6, Lhx8, Dlx1 and Dlx2, among others, participate in multiple events during the migration of cortical interneurons (see Elias et al., 2008). In addition to specifying the identity of MGE cortical interneurons, the transcription factor Nkx2.1 modulates the correct targeting of MGE derived interneurons (Nobrega-Pereira et al., 2008). Different reports have also suggested that Nkx2.1 in proliferating and early postmitotic MGE cells activates the expression of Lhx6 (Alifragis et al., 2004; Liodis et al., 2007; Du et al., 2008), which participates actively in cortical interneuron migration. The absence of Lhx6 results in a delay of interneuron migration and affects the targeting of interneurons within the cortex (Alifragis et al., 2004; Liodis et al., 2007; Zhao et al., 2008). Interestingly, It has also been reported that Nkx2.1 and Dlx proteins regulate the response of MGE-derived cells to class 3 semaphorin chemorepulsion by negatively controlling the expression of Nrp receptors (Le et al., 2007; Nobrega-Pereira et al., 2008). The fact that I observed reduced levels of Nrp and Plexin receptors in *Robo1*$^{-/-}$ cortical interneurons and aberrant migration of these cells through the striatum of *Robo1*$^{-/-}$ mice, prompted me to ask whether the absence of Robo1 receptor disrupts the expression of these transcription factors in *Robo1*$^{-/-}$ cortical interneurons an thus, their response to class 3 semaphorin chemorepulsion. Here, I found by *in situ* hybridisation expanded expression of *Lhx6* and *Nkx2.1*, but not *Lhx8* or *Dlx1*, in the MGE of *Robo1*$^{-/-}$ mice. However, the estimation of the level of expression of *Nkx2.1*, *Lhx6*, *Lhx8*, *Dlx1* and *Dlx2* in FACS-sorted MGE cells taken from GAD67-GFP^{+} embryos by QPCR, revealed no significant differences between *Robo1*$^{-/-}$ mice and control animals. Thus, it seems that the expanded expression of *Nkx2.1* and *Lhx6* in the MGE of *Robo1*$^{-/-}$ mice may be caused by the observed increase in proliferation in the MGE of these animals (as reported in Andrews et al., 2008), rather than an increase in the expression of this transcription factor. The fact that I found no differences in the expression of *Nkx2.1*, *Lhx6*, *Lhx8*, *Dlx1* and *Dlx2* in FACS-sorted MGE cells taken from *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice seems to indicate that other transcription factors, different to the ones studied in the present thesis, are involved in the expression of Nrp and Plexin receptors. This idea, however, does not rule out the possibility that these transcription factors might specifically contribute to the expression of class 3 semaphorin receptors in cortical interneurons, possibly acting as additional controllers in the expression of Nrp and PlexinA receptors.

As described above, Robo1, but not Slit1 or Slit2, signalling appears to be pivotal for the correct migration of cortical interneurons around the developing striatum. Thus, it is likely that other mechanisms independent of Slit cues activate Robo1 receptors in these cells. It is important to note that the cytoplasmatic domains of Robo receptors do not posses autonomous catalytic activity and, therefore, they need to interact with other receptors to mediate their specific effects (Ypsilanti et al., 2010). It has been reported that Nrp proteins, in addition to forming dimers with PlexinA and VEGF receptors, can bind members of the IgCAM superfamily of cell adhesion proteins, such as L1, and together mediate axonal navigation during development (Castellani et al., 2002, 2004). Since Robo1 receptor is also a well-known member of the IgCAM family (Liu et al., 2004), I investigated whether there exists a physical interaction between Robo1 and Nrp and/or PlexinA receptors. My investigation showed that there exists indeed a physical interaction between Robo1 and class 3 semaphorin receptors (at least Nrp1, Nrp2 and PlexinA1). These data indicated that the cell sorting of cortical interneurons around the striatum might result from a collaborative effort between Robo1 and class 3 semaphorin signalling system.

6.4 GN11 cells as an *in vitro* model to study interactions between Robo1 receptor and the class 3 semaphorin signalling system

As mentioned in the introduction of the present work, MGE-derived cells are a very heterogeneous groups. Indeed, MGE gives rise to distinct classes of cortical interneurons that together correspond to more than 70% of the total number of those cells in the neocortex (Fogarty et al., 2007). In addition to generating most of cortical interneurons, the MGE has also been reported to provide cells to the striatum, hippocampus and most of the ventral subcortical nuclei including the dorsal pallidum, ventral pallidum, basal magnocellular complex, the nucleus basalis magnocellularis, diagonal band, and medial septum (Mesulam et al., 1983a,b; Semba et al.,1988; Brady et al., 1989; Phelps et al., 1989; Kita and Kitai, 1994; Kawaguchi et al., 1995; Olsson et al., 1995, 1998; Parent and Hazrati, 1995; Rubenstein et al., 1998; Marín et al., 2000; Pleasure et al., 2000; Cobos et al., 2001; Wichterle et al., 2001). In order to develop a simpler and easier *in vitro* system to further study the interactions between Robo1 receptor and the class 3 semaphorin signalling system, I sought a cell line that could exhibit a dynamic migratory activity *in vitro* and also possess intrinsically the receptors for class 3 semaphorins and Slit ligands. I found that an immortalised cell line derived from gonadotropin-realising hormone

secreting neurons (GN11 cell line) possesses the receptors Nrp1 and Nrp2 and responds to Sema3A and Sema3F *in vitro* (Cariboni et al., 2007). My chemotactic and expression studies on these cells also revealed that they contain Robo receptors (specifically these cells express strongly Robo1 and Robo3) and respond to Slit1 and Slit2 induced chemorepulsion. I also found that blocking the Robo1 receptor in these cells by using Robo1-DN plasmids, I could mimic those conditions observed in *Robo1*$^{-/-}$ cortical interneurons, as they became less responsive to Sema3A and Sema3F induced chemorepulsion. Thus, these cells are suitable to further explore those dynamics behind the interaction between Robo1 receptor and class 3 semaphorins. Indeed, using GN11 cells, it was possible to confirm the physical binding between Robo1 and class 3 semaphorin receptors, but most important this *in vitro* system revealed that Robo1 signalling is crucial for maintaining, although not sufficient, the expression of Nrp and PlexinA receptors. To date little is known about the molecular mechanisms that directly control the expression of class 3 semaphorin receptors. Evidence from the Marin laboratory suggests that Nkx2.1 regulates the response of MGE-derived cells to class 3 semaphorins by negatively controlling the expression of Nrp receptors (Nobrega-Pereira et al., 2008). Particularly, it was shown that Nkx2.1 by directly binding Nrp2 promoter seems to repress the expression of this receptor (Nobrega-Pereira et al., 2008). Given that GN11 cells do not express Nkx2.1, other transcription factors must regulate the expression of class 3 semaphorin receptors. Therefore, the *in vitro* system that I developed in the present work could facilitate future explorations in this field and unveil the precise molecular mechanisms that control the expression of these receptors. Here, I am tempted to imagine that those transcription factors might also direct the expression of class 3 semaphorin receptors in cortical interneurons, as my analysis of MGE-derived *Robo1*$^{-/-}$ cells did not reveal any significant change in the expression of *Nkx2.1* or *Dlx* genes. Moreover, class 3 semaphorin receptors are spread through distinct tissues in developing and mature animals, where Dlx or Nkx2.1 proteins are not expressed, which strongly suggests the existence of these hypothetical other transcription factors. This idea does not exclude that Nkx2.1 or Dlx genes participate in orchestring the expression of Nrp and Plexin receptors, but points out that are not sufficient for their expression.

As noted in chapter 5, Robo1-DN-transfected GN11 cells are more sensitive to Slit1 or Slit2 induced chemorepulsion. In spite of sharing numerous structural features and molecular partners, individual Robo receptors exert distinct fuctions. As recently pointed out by Ypsilanti et al. (2010), Robo3 differs from Robo1 and Robo2 receptors in several ways. Specifically, Robo3 receptor exhibit great structural heterogeneity and lacks

of some intracellular motifs (Yuan et al., 1999a; Chen et al., 2008). Moreover, it is exclusively expressed in restricted time windows within the nervous systems (Marillat et al., 2004; Camurri et al., 2005; Barber et al., 2009). The function of Robo3 receptor was uncovered after analysing the spinal cord of *Robo3*$^{-/-}$ mice, where virtually no commissural axon crosses the midline, as they are more sensitive to Slit chemorepulsion in the midline. In wild type animals Robo1, Robo2 and Robo3 receptors are expressed strongly in those axons, whilst Slit1 and Slit2 are expressed by midline cells (Marillat et al., 2004; Sabatier et al., 2004; Tamada et al., 2008). Interestingy, after crossing the midline commissural axons down regulate Robo3 receptors. It has been hypothesised that Robo3 receptor interferes with Slit chemorepulsion by binding and blocking the other Robo proteins (Robo1 and Robo2) and thus, it allows commissural axons to cross the midline. Once Robo3 is down regulated the other two Robo receptors become active and respond to Slit chemorepulsion an then, this axons do not re-cross the midline. Support to this model comes from the analysis of *Robo*$^{-/-}$*/Robo3*$^{-/-}$ or *Robo*$^{-/-}$*/Robo2*$^{-/-}$*/Robo3*$^{-/-}$ mice, where these animals have normal phenotype (Sabatier et al., 2004; Chen et al., 2008). Since GN11 cells express the three Robo receptors, it is likely that a similar interference exists among them. Blocking the Robo1 receptor might release the other Robo receptors to freely respond to Slit induced chemorepulsion and thus, they are more sensitive to these cues. Unlike Robo1-DN-transfected GN11 cells, *Robo1*$^{-/-}$ cortical interneurons are not more sensitive to Slit induced chemorepulsion. In fact these cells lose responsiveness to this chemorepulsive cue, which seems to reflect that the model described above does not apply to these cells.

6.5 Personal reflexion and future work

The present thesis provides several pieces of evidence that indicate a significant role for Slit-Robo signalling in the migration of cortical interneurons, as well as showing that the absence of Robo1 receptor in cortical interneurons results in down regulation of class 3 semaphorin receptors, leading to their aberrant migration through the striatum of *Robo1*$^{-/-}$ mice. However, a number of questions emerged through the writing of my data, and I consider that future work should address the following points to further complete my current observations.

First, it seems that Slit3 can compensate for the absence of Slit1 cues in the CP of *Slit1*$^{-/-}$*/Slit2*$^{-/-}$ mice and thus, prevents the premature invasion of cortical interneurons to this layer. To test this point it would be of interest to analyse the neocortex of Slit triple

knockout (*Slit1*$^{-/-}$*/Slit2*$^{-/-}$*/Slit3*$^{-/-}$; Long et al., 2004) mice and observe whether cortical interneurons invade the developing CP of these animals, as it happens in *Robo1*$^{-/-}$ animals. Using *Slit1*$^{-/-}$*/Slit2*$^{-/-}$*/Slit3* mice, it could also be tested whether Slit1 in the ventral telencephalon participates in the chemorepulsion of cortical interneurons away from their proliferative zones. One possibility would be to take the SVZ from the MGE of control animals and co-explant them with homotopic or heterotopic pieces of VZ from the MGE of control or *Slit1*$^{-/-}$*/Slit2*$^{-/-}$*/Slit3*$^{-/-}$ mice.

Second, my data shows that most striatal projection neurons contain Robo1 and/or Robo2 receptor. However, the quantification of these cells in *Robo1*$^{-/-}$, *Robo2*$^{-/-}$ or *Slit1*$^{-/-}$*/Slit2*$^{-/-}$ mice do not reveal any significant difference when compared to their corresponding littermates. Thus, it is currently unclear what the role of Slit-Robo signalling is in the development of striatal projection neurons. One possibility is that it participates in events of dendritogenesis or synaptogenesis during the postnatal development of the striatum. Thus, it would be of interest to study by Golgi impregnation the morphology of striatal projection neurons in adult *Robo1*$^{-/-}$, *Robo2*$^{-/-}$ mice. Additionally, it would be important to analyse the formation of synaptic contacts by electron microscopy or immunohistochemistry in the early postnatal striatum.

Third, the lack of co-localisation between FOXP2^{+} and GAD67-GFP^{+} cells *in* *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice suggested aberrant migration of cortical interneurons through the striatum. However, the developing striatum of control (*Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$) mice also contained numerous GAD67-GFP^{+} cells that do not express FOXP2, indicating that at least a subpopulation of cortical interneurons migrate through the striatum en route to the neocortex. To clarify this point, it would be necessary to electroporate focally GFP plasmids in the developing striatum of wildtype animals and see whether GFP^{+} cells can migrate to the neocortex. If this is the case, a new interpretation of my data would emerge and thus, my data would reflect an increase of those cortical interneurons that normally migrate through this area, but not aberrant migration of cortical interneurons through the developing striatal area. This additional number of cortical interneurons in the striatum of *Robo1*$^{-/-}$ mice could also be the result of increased proliferation observed in the MGE of these animals (as reported by Andrews et al., 2008).

Fourth, my current data suggest that *Robo1*$^{-/-}$ cortical interneurons invade the striatum *en route* to the cortex. However, it is presently unknown whether class 3 semaphorins are expressed in the striatum of *Robo1*$^{-/-}$ mice; the absence of these molecules could also explain why these cells are unresponsive to these chemorepulsive

cues. *In situ* hybridisation studies using probes for Sema3A and Sema3F could clarify this point. Alternatively, Western blotting experiments on isolated bids of the striatum of *Robo1*$^{-/-}$ mice could also bring light to this issue.

Fifth, my *in vitro* experiments showed that MGE-derived cells from *Robo1*$^{-/-}$ mice are less responsive to the chemorepulsion of class 3 semaphorins. Nevertheless, the MGE contributes with cells to several cortical and subcortical structures in the developing brain, making unclear whether only cortical interneurons are affected by the deletion of Robo1 receptor. My qualitative observations on the developing brain of *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ mice suggested that not only the neocortex or striatum contains more GAD67-GFP^{+} cells, but also the hippocampus, globus pallidus, olfactory cortex, among others (data not shown). Therefore, it would be of interest to study those structures and confirm whether they contain more GABAergic cells. An increase of cells in those areas could also be explained by the fact that *Robo1*$^{-/-}$ mice contain more mitotic cells in their MGEs.

Sixth, my data seems to suggest that Robo1 signalling controls the expression of different components of the class 3 semaphorin signalling system (ligands, receptors and intracellular factors) in MGE-derived cells. However, I have no evidence of what intracellular mechanism links Robo1 receptor with the expression of this system Here, it would be interesting to carry out a microarray analysis on FACS-sorted MGE cells taken from *Robo1*$^{-/-}$*;GAD67-GFP*$^{neo/-}$ animals and *Robo1*$^{+/-}$*;GAD67-GFP*$^{neo/-}$ mice and assess the expression of transcription factor in *Robo1*$^{-/-}$ cells. Candidate genes could be further confirmed by *in situ* hybridisation and/or imunohistochemistry in *Robo1*$^{-/-}$ mice. Similar microarray analysis could be carried out on Robo1-DN-transfected-GN11 cells.

Finally, my data clearly shows that Robo1 can bind to Nrp and PlexinA receptors. Future experiment should address which domains of Robo1 proteins are required to form dimmers with class 3 semaphorin receptors. One possibility could be to use COS-7 cells transfected with constructs of Robo1 receptor containing different deletions in the extracellular or intracellular domains of Robo1 proteins and immunoprecipitate them with antibodies for Nrp and PlexinA receptors.

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