Semaphorin 5B:
An Inhibitory Transmembrane Guidance Cue Reveals Its Secretable Function

by

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Abstract

Corticofugal axons projecting to the thalamus, brainstem and spinal cord must travel the same initial trajectory through the subcortical lateral and medial ganglionic eminences, and are therefore likely subject to the same sets of guidance cues. These cues direct the course of corticofugal axons bringing them to each intermediate target, until they reach the diencephalic-telencephalic boundary, where axons targeting the thalamus turn dorsally and brainstem and spinal cord targeting axons turn ventrally. Many of these guidance cues have been elucidated, yet there are still gaps in our understanding of the formation of the corticofugal projection.

I found that Sema5B expression flanked the presumptive internal capsule during its formation, and was therefore ideally situated both spatially and temporally to act as an instructive cue for descending cortical axons. In Chapter 2, I show that Sema5B is not only capable of inhibiting cortical axons in vitro, but can cause misguidance of cortical axons in slice culture when placed ectopically over normally non-Sema5B expressing regions. In addition, I show that the loss of Sema5B from the neocortical VZ resulted in aberrant penetration of this normally avoided region. Therefore Sema5B is both necessary and sufficient to inhibit the corticofugal projection.

Semaphorins and their plexin receptors are frequently proteolyzed to modulate the elicited responses in navigating growth cones. In Chapter 3 I show that Sema5B cleavage results in an inhibitory fragment that in heterologous cells can produce inhibitory gradients for cortical

explants in collagen gel co-cultures, and collapse dissociated cortical neuronal growth cones, an effect that can be blocked with a function disrupting antibody to the cell adhesion molecule TAG-1.

This thesis shows that Sema5B, a guidance cue with a hitherto unknown function, is responsible for a very important aspect of cortical development. My work leads to a final proposal that Sema5B is in fact a two-in-one protein with separable inhibitory and alternate complex functions, the implications of which are discussed thoroughly in Chapter 4.

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List of Abbreviations

Anatomical/Developmental Terms

CFA..... corticofugal axons
CGE..... caudal ganglionic eminence
CNS ..... central nervous system
CP..... cortical plate
CSB ..... corticostriatal boundary
CR ..... Cajal-Retzius neuron
dctx ..... dorsal cortex
Dien ..... diencephalon
DTB..... dien-telencephalic boundary
dTh ..... dorsal thalamus
E..... embryonic day (gestational)
HEK293 ..... human embryonic kidney cells
Hind ..... hindbrain
hipp..... hippocampus
IPC ..... Intermediate progenitor cell
IZ ..... intermediate zone
LGE ..... lateral ganglionic eminence
LMS ..... lateral migratory stream
N..... notochord
NC..... neural crest
NE ..... neuroepithelium
NT ..... neural tube
MB ..... midbrain
Mesen..... mesencephalon
Meten..... metencephalon
MGE..... medial ganglionic eminence
Myelen ..... myelencephalon
MZ ..... marginal zone
PNS..... peripheral nervous system
PP..... preplate
Prosen ..... prosencephalon
RG ..... radial glial cell
Rhomben ..... rhombencephalon
RMS ..... rostral migratory stream
SC..... spinal cord
SPL..... subplate layer
SVZ ..... subventricular zone
TCA..... thalamocortical axons
Tel/telen ..... telencephalon
vlctx ..... ventrolateral cortex
vTh ..... ventral thalamus
VZ..... ventricular zone

Molecular Terms

ADAM..... a disintegrin and metalloprotease
ADF ..... actin depolymerizing factor
BMP..... bone morphogenic protein
CAM ..... cell adhesion molecule
cAMP ..... cyclic adenosine mono-phosphate
Cdk..... cyclin dependent kinase
cGMP ..... cyclic guanosine mono-phosphate
CRMP ..... collapse response mediator protein
CSPG..... chondroitin sulfate proteoglycan
CXCR..... alpha chemokine receptor
dcc..... deleted in colorectal cancer
DCX ..... doublecortin
DiI ..... 1,1',dioctodecyl-3,3,3',3'-tetramethyl-indocarbo-cyanine perchlorate
DNT1..... Drosophila neurotrophic
dpp..... decapentaplegic
ERM ..... "ezrin, radixin, moesin"
F-actin ..... filamentous actin

FGF ..... fibroblast growth factor
GABA ..... γ-aminobutyric acid
GDP ..... guanosine diphosphate
GPI ..... glycosylphosphatidylinositol
GSK-3 ..... glycogen synthase kinase-3
GTP ..... guanine triphosphate
HGF ..... hepatocyte growth factor
HSPG ..... heparan sulfate proteoglycan
Ig ..... immunoglobulin domain
Lis1 ..... lissencephaly-1
MET mesenchymal-epithelial transition factor
MLC ..... myosin light-chain
MLCP ..... myosin light-chain phosphatase
MLCK ..... myosin light-chain kinase
MMP ..... matrix metalloproteinase
MRS ..... MET-related sequence
MT ..... microtubule
MT-MMP ..... membrane-type MMP
NARC ..... neural apoptosis-regulated convertase
NGF ..... nerve growth factor
NP ..... neuropilin
PAK ..... p21 related kinase
Pax6 ..... paired-box transcription factor-6
PC ..... proprotein convertase
PCSK ..... PC substilin-kexin-like
PDZ ..... "post-synaptic density-95, discs large, zona occludens-1" domain
PI3K ..... phosphoinositide 3-kinase
PKA ..... protein kinase A
PSI ..... "plexin-semaphorin-
..... integrin"
Robo ..... roundabout
ROCK ..... rho-activated kinase
RTK ..... receptor tyrosine kinase
Ryk ..... related to receptor tyrosine kinase
Sema ..... semaphorin
SH ..... sarcoma homology domain
shh ..... sonic hedgehog
SF ..... scatter factor
SFR ..... scatter factor receptor
TAG-1 ..... transient axonal guidance-1
TGF-β ..... transforming growth factor-β
trk ..... tropomyosin-related kinase
TSP ..... thrombospondin
TSR ..... thrombospondin repeat
Wnt ..... wingless
Unc ..... uncoordinated

Preface

Semaphorins are typically abbreviated as "Sema", but the capitalization changes depending on the organism, and if it is necessary, the organism of origin is indicated by the first letter of the organism's name. Invertebrate semaphorins are not capitalized; therefore, *Drosophila* semaphorin 1a would appear as d-sema-1a, for example. Vertebrate semaphorins are capitalized in the first letter. For example, *murine* semaphorin 5B would be abbreviated as mSema5B. For simplicity's sake, I have simply referred to mSema5B as Sema5B throughout the text herein.

Please not that all figure drawings/diagrams in this thesis (including adaptations) are **original** designs under copyright © 2009 by Robyn Lynn Mwuese Lett, and are not to be used or reproduced without permission of the author.

Acknowledgements

First and foremost I want to thank Tim O'Connor for letting me take up space in his lab for two whole degrees. I really appreciate all your input and effort, particularly in these last months; it has been a pleasure. One of the things I have always enjoyed, for better or worse, is the candor between us and the faith that you have shown in my intellect. I have striven in this, my second degree in your lab, to *really* earn respect, earn my place, and to become a real scientist. I would like to think that under your tutelage I have managed this. I hope I have made you proud; for I am proud to be a graduate of the O'Connor lab.

Thank you also to my committee, Drs Vanessa Auld, Wolfram Tetzlaff, and Alaa El-Husseini. Alaa's unfortunate passing on Dec 23, 2007 was a shock, and all who knew him have felt his loss. I was glad to have had him on my committee. Vanessa and Wolf, you have been most indispensable as counselors and mentors. Thank you so much for your help, guidance, and the second shot that I really and truly needed, even if it wasn't deserved until now.

To my lab mates present and past – thanks for being my colleagues and friends. Ana, you were an amazing mentor and friend to me. I am a different scientist for having worked with you. You are the kind of scientist anyone could aspire to and I have immense respect for you. Kenny, you are a great friend and colleague that I could always count on for a non-emotional view, and I hope to know you for years to come. Winnie; you did everything and managed everything. You have my endless gratitude for your endless help during my time in the O'Connor lab. I would like to mention Kristen B(ond) as an indispensable additional collaborator for my second paper. It has been a pleasure working with you. Finally, Andrew Tait; we've come a long way from the time you declared your grade 7 crush... You are one of my oldest friends and a wonderful colleague, and I love our coffee break discussions.

Dad, Mom, Ryan, Tara, and Grandma, I swear I almost quit science for art three times. But each one of you kept me going and made sure I never gave up. I put you all through so much, and you were all there for me, always. You have supported me emotionally, physically, financially, and intellectually. Dad, you were like a second supervisor. I sometimes found it easier to take criticism from you than anyone else in science – and as it turns out, you were right! Mom, I loved your visits and our wonderful conversations on work, life, and love, especially in the last two agonizing years when I was so down and needed you so much. Grandma, I always look forward to our exclusive book club meetings – I think I'm becoming more like you with each passing year. Ryan and Tara: I am so fortunate to be living in the same city as my sibs. It has been awesome! Ryan, my partner in killing time and aliens; I love that I have gotten to spend so much quality time with you recently. It has been nice to know I can always count on you for anything! Tara, I feel like I owe you so much…tu es ma petite soeur et ma grande soeur a la meme fois. N'oublie-pas qu'il saurait jamais personne qui peut tu remplacer. You might be a cancer but you are also a rock (star!). And to the new addition, Lana; I hope you can read this one day! Mmmm-ahhh! I love you all so, so much!!!

Theunis my lieflung; Ek weet dit was n lang en moeilike pad en soms baie stresvol tussen ons, nietemin, jy was my minnaar en my beste vriend. Elke pot het sy dacsl; jy is mein. My liefde is vir jou, vir altyd.

This thesis is dedicated to my grandfather,

Glen Charles Lett.

I think you would have been proud as punch.
I miss you.

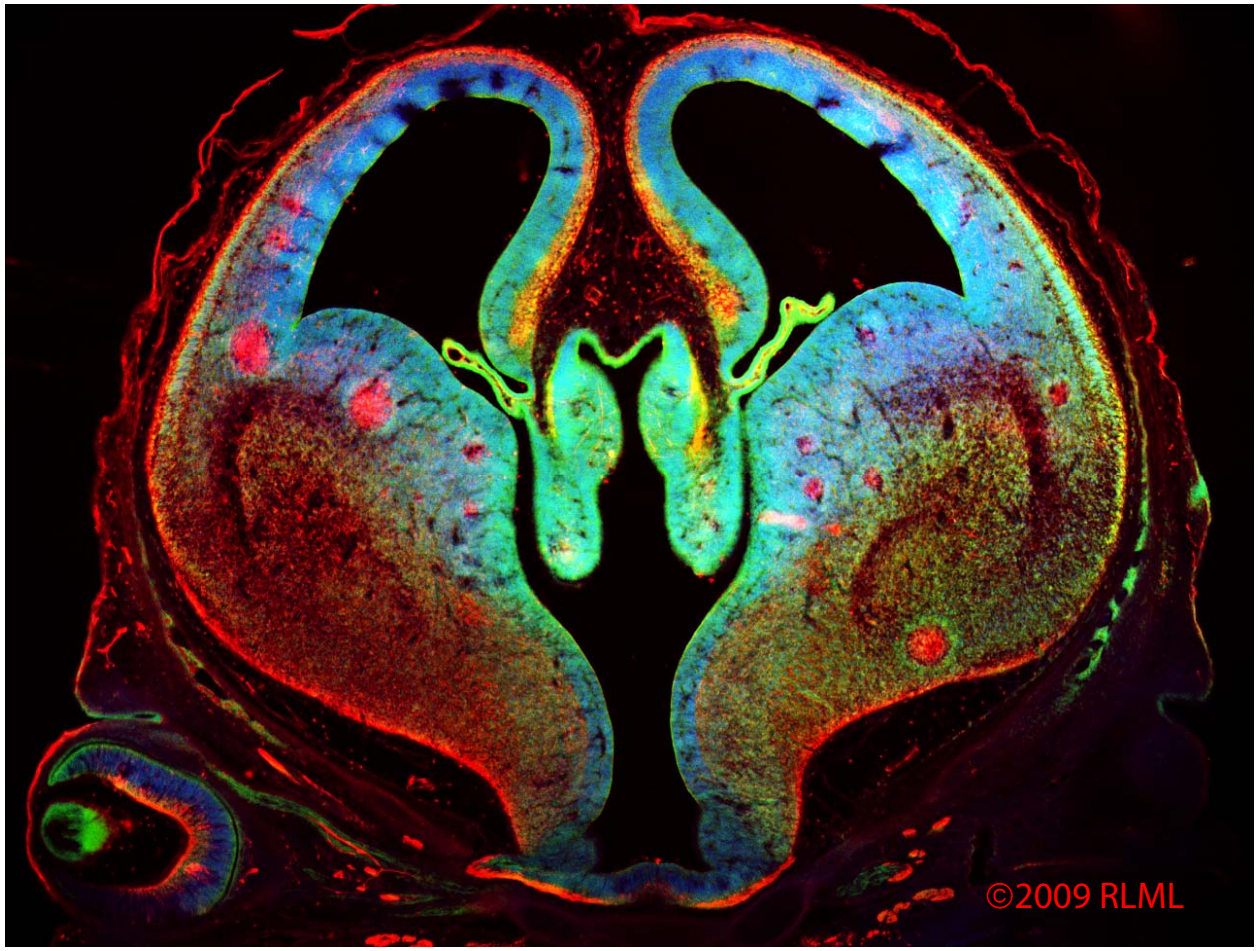

Figure 0.1 Coronal section of E14.5 mouse head
Probes with antibodies to Sem5B (green), β-III tubulin (red), and counterstained with DAPI (in blue).

Co-authorship Statement

Other than the following specific contributions, I performed all experiments and data analysis, prepared all the figures, and wrote the manuscripts. Concepts and experimental design were also my responsibility, with guidance and editing provided by my supervisor, Dr. O'Connor, and a former colleague (not a co-author), Dr. Ana Mingorance-Le Meur.

Chapter 2 and 3 are versions of manuscripts that include authors in addition to me and the primary investigator, T.P. O'Connor. Chapter 2 is published at Cerebral Cortex, vol 19, pp1408-1421. The second author, Dr. Wenyan Wang, was responsible for sub-cloning mSema5B from pBK-CMV into pDisplay, and for generating and maintaining the stable cell lines associated with the paper. She also prepared the shRNA vectors which I had designed, and validated their knock down efficiency in heterologous cells, giving rise to figure 2.6 panels A-C, all of which she provided.

In chapter 3, a version of a second submitted manuscript, Dr. Wenyan Wang was the second author. In conjunction with Dr. O'Connor, Dr. Wang devised, produced, and purified the N-terminal and C-terminal antibodies to Sema5B. Kristen Browne, the third author, performed the western blots for figure 3.2, panel A and did the MMP inhibition experiments resulting in figure 3.2 panel H, and prepared all the concentrated media for collapse assays as well.

1. Introduction

1.1 The problem of constructing a mammalian brain

One of the greatest evolutionary developments for mammals has been the addition of a neocortex to the central nervous system. It is the neocortex and the massive degree of connectivity that underlies the advanced cognitive capabilities of human (and primate) brains, such as: planning and execution; various forms of memory and recall; reading, writing, and comprehension of language; abstract thought; art and music, and not least of all, the ability to ask questions, investigate, and make discoveries about ourselves and the world around us.

1.1.1 Why study development of the cerebral cortex?

The mammalian neocortex is incredibly and elegantly organized, containing billions of neurons arranged into domains relating to sensory, motor, and integrative function, specifically and precisely localized within a well-defined cytoarchitecture and networked via trillions of functional connections. The many pathways and tracts bewteen neurons and their targets must be established in a coordinated and mutually unimpeding manner in order to ensure the appropriate formation of these connections. There are therefore complex developmental processes entailed in order to generate this complex system from a simple single cell-layered tube.

Development can be broken down into a few component processes: proliferation and cell fate determination, cell migration, axonogenesis and pathfinding, synaptogenesis, pruning

and apoptosis. The list may be simple, but there are obvious complexities as some, if not all, of these developmental steps can be occurring simultaneously, and often within the same local embryonic environment. It is not surprising then that many disorders of the nervous system are often found to be a result of procedural errors during development (reviewed in (Sheen and Walsh, 2003).

1.1.2 The neural tube

The entire mature nervous system originates from the ectodermal layer at the trilaminar stage of embryogenesis (Kintner and Lumsden, 1994). With gastrulation and the creation of the transient mesodermal notochord, this exclusively early embryonic structure secretes signals that change the molecular profile of the overlying ectoderm (Fig 1; Smith and Schoenwolf, 1997). This neural plate, as it is then called, folds, invaginates and seals off from the ectoderm to form the neural tube (Gallera, 1971; Miyatani et al., 1989; Juriloff and Harris, 2000). The neural tube is composed of a simple epithelium of columnar neuroepithelial (NE) cells surrounding an inner lumen or ventricle and is the earliest anlage of the entire central nervous system. These NE cells span from lumen (apex) to meninges (base), dividing symmetrically to increase the number of cells within the neural tube that will eventually divide asymmetrically to produce a committed progeny, neurons (Anthony et al., 2004; Haubensak et al., 2004; Götz and Huttner, 2005). Later in development, these stem cells will also generate the glial support cells: oligodendrocytes and astrocytes (Kessaris et al., 2007).

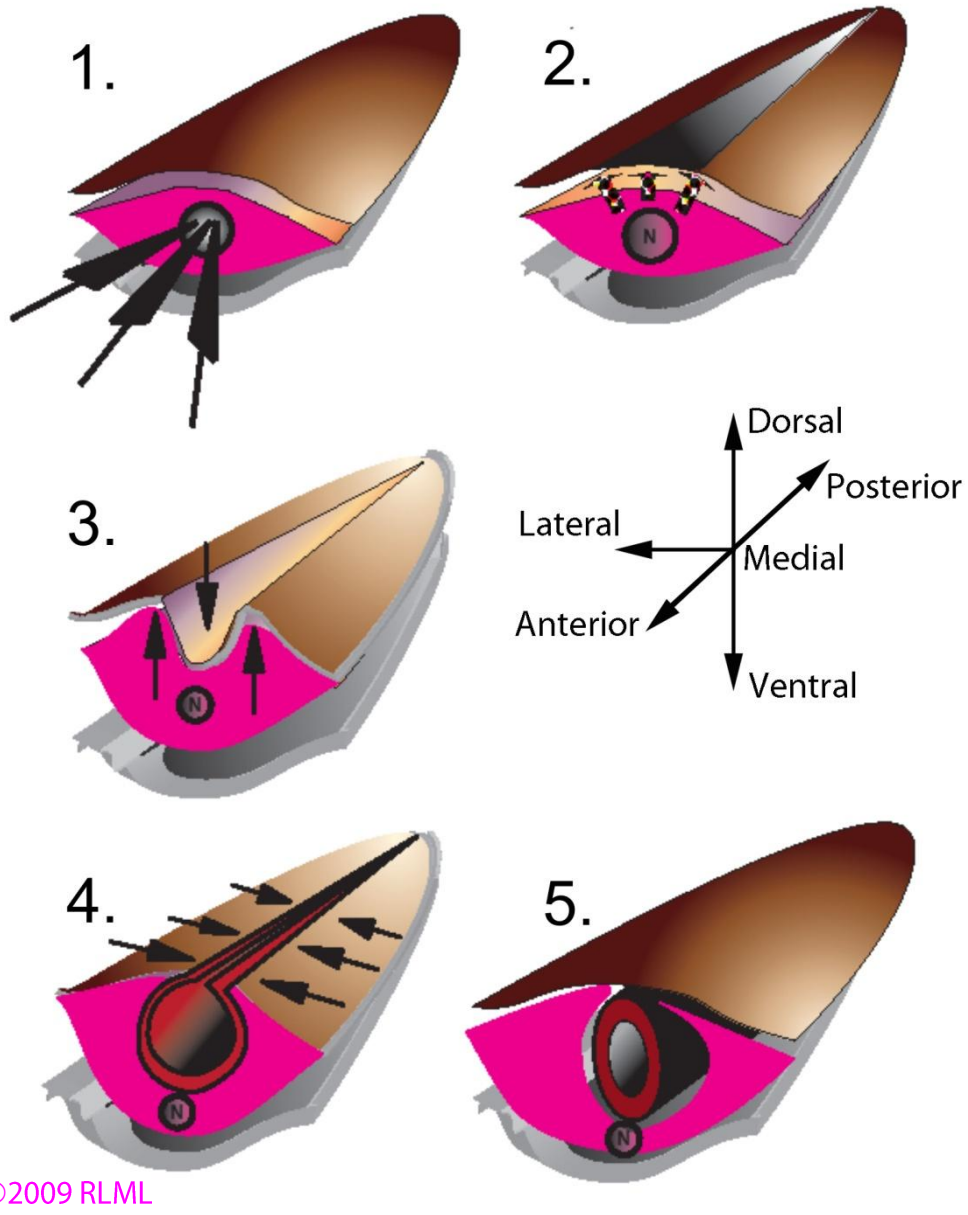

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Figure 1.1 Neurulation - formation of the neural tube.
1. After gastrulation the embryo has three primary layers: ectoderm, mesoderm, and endoderm. The notochord is a specialization of the mesoderm that runs the anteroposterior length of the developing embryo. 2. The notochord signals to the overlying ectoderm, driving among many other events a switch in expression from the cell adhesion molecule E-cadherin to N-cadherin and N-CAM - this is the neural plate. 3. Changes in cell shape of neural plate cells causes bending and involution of the midline of the plate (the midline hinge point), while the outer edges expand upwards as the neural folds. 4. The neural folds at the dorsolateral hinge points migrate to the midline to meet and begin the process of sealing and separating the neural tube away from the ectoderm, as shown in 5. The neural crest occupies the dorsal regions of mesoderm on either side of the neural tube.

At this primitive stage, the neural tube is composed of four segmental expansions or bulges: the prosencephalon (forebrain); the mesencephalon (midbrain); the rhombencephalon (hindbrain); and the spinal cord (Fig 2; Wada and Satoh, 2001). These subdivisions continue expanding from three divisions into five (Wada and Satoh, 2001). The forebrain becomes further partitioned into the telencephalon and diencephalon, as is the hindbrain, which is split into the more rostral metencephalon and the caudal myelencephalon. The metencephalon is the precursor of the pons and cerebellum, whereas the myelencephalon will develop into the medulla. The midbrain does not become further partitioned, and the spinal cord is segmented into levels along the body axis. The diencephalon will develop into the hypothalamus, mammillary bodies, and the thalamus, which is also the origin of the optic vesicles that will form the retina. The telencephalon undergoes massive proliferations and

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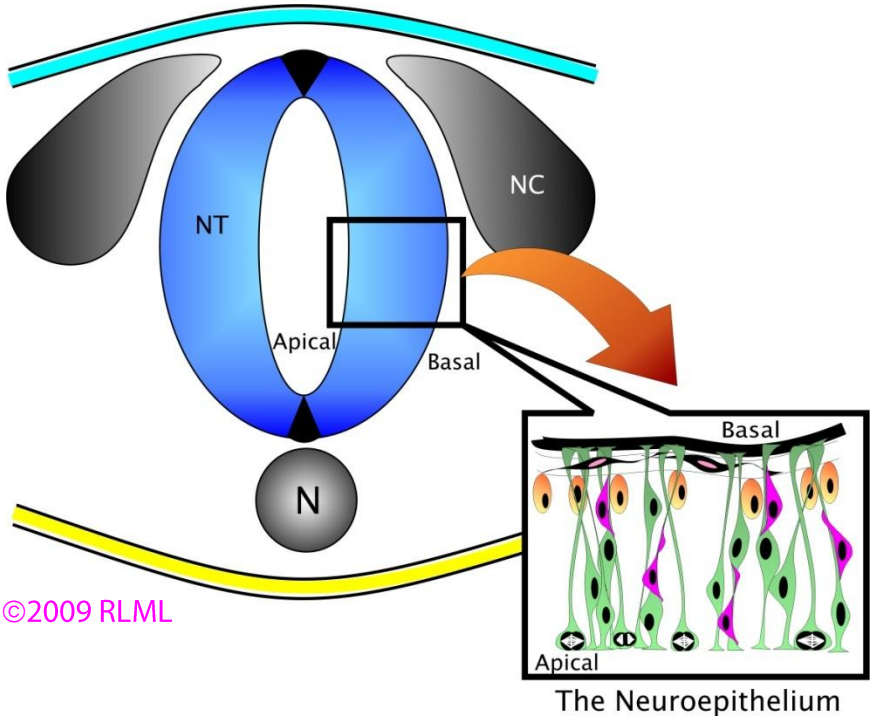


Figure 1.2 The neural tube in cross-section. Shown in blue below the overlying ectoderm (in light blue), the neural tube (NT) surrounds an inner lumen, or ventricle. Dorsolateral collections consist of neural crest cells (NC), and the notochord (N) remains ventral to the NT and above the endoderm (in yellow). The most dorsal and ventral aspects of the neural tube were the hinge points of the neural plate prior to neural tube closure, and will go on to become the roof and floor plates. The boxed area is enlarged to the right to show the pseudostratified nature of the initial neural tube.

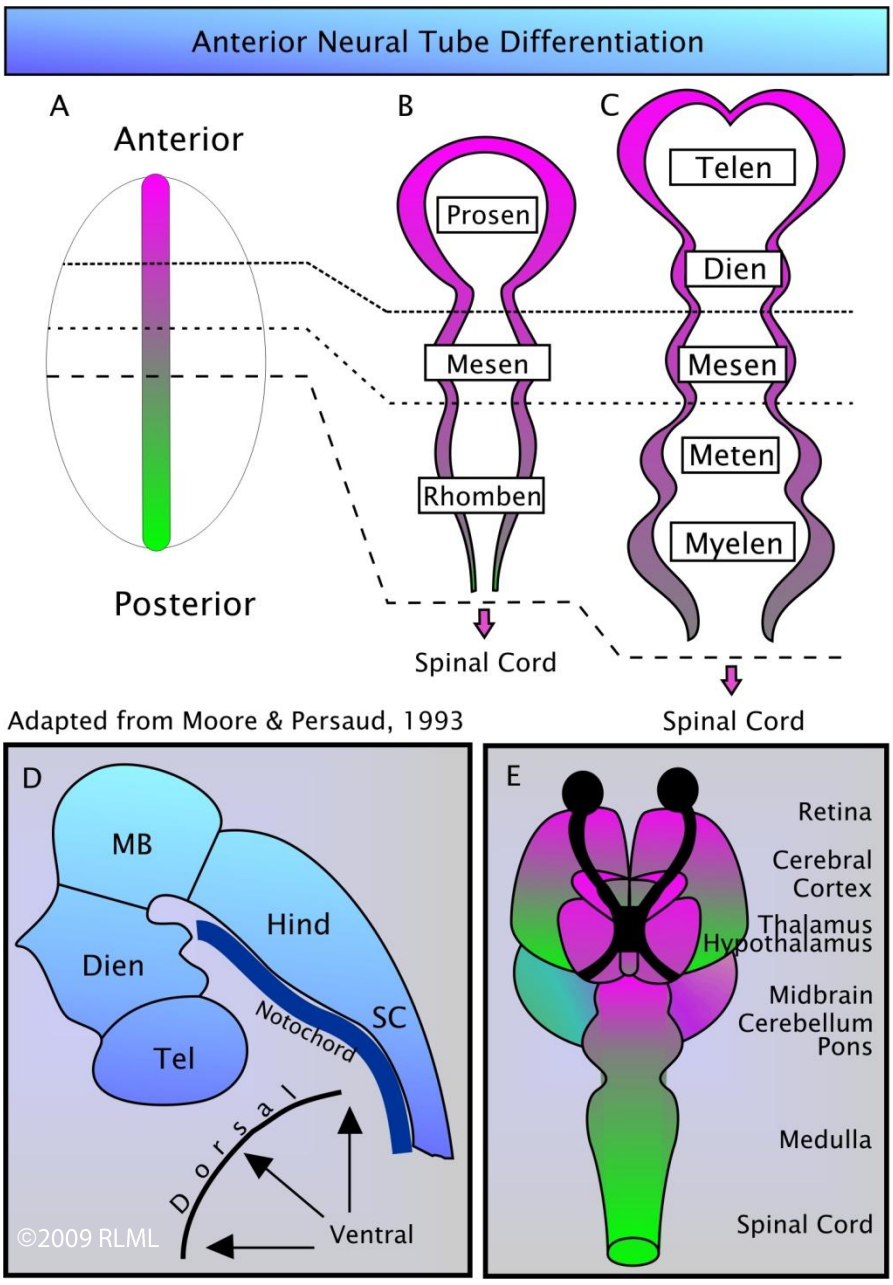
waves of neurogenesis to create the cerebral hemispheres that are further segmented into the dorsal pallium (or cortex), which includes neocortex, archicortex such as piriform and insular cortex, olfactory bulbs and hippocampus, and the subpallium or subcortex. The subcortex during development is composed of the medial, lateral and caudal ganglionic eminences (MGE; LGE; CGE), and the limbic basal forebrain nuclei - including parts of the claustralamygdalar complex (Moore and Persaud, 2008).

The final mature central nervous system is composed of two cerebral hemispheres containing superficial grey matter of the neocortex and hippocampus over an inner subcortical grey matter, the diencephalon that comprises the thalamus, (and retina), hypothalamus, and mammillary bodies, the midbrain, pons and medulla (together – the brainstem) that contain the cranial nerve nuclei and control centers for important house-keeping functions. The cerebellum, behind the pons, acts as a second brain for motor function and memory. Major fiber tracts course through the CNS to connect the cortex, subcortex, and diencephalon to the brainstem and spinal cord and ultimately the peripheral nervous system and effectors.

1.1.3 Cortical malformations: early developmental and migration defects

Very early in development errors can lead to embryonic lethality, or if not, a reasonably severe problem such as incomplete neural tube closure, which results from an incomplete segregation of the neural tube from the remaining ectoderm. When the closure error is at the posterior end of the nervous system it leads to what is commonly known as spina bifida, and

Figure 1.3
Differentiation of the anterior neural tube in schematic.



A) The neural tube runs from anterior (pink) to posterior (green) along the length of the early embryo. The three lines crossing the neural tube are to demonstrate boundary continuity as the NS is expanding. B) The anterior most portions become segmented into three basic expansions, which are (from anterior to posterior) the prosencephalon (forebrain), the mesencephalon (midbrain), and the rhombencephalon (hindbrain). The remainder of the neural tube will become the spinal cord. C) Further into development, the early segments of the anterior neural tube become further partitioned: the prosencephalon is now the telencephalon and diencephalon; the mesencephalon remains the same; the rhombencephalon becomes divided into the metencephalon and myelencephalon. Meanwhile the spinal cord is undergoing its own segmentations along its anteroposterior axis (after Moore & Persaud, 1993). D) Sagittal view of the anterior neural tube, around the same stage as in C. The location of the notochord along the ventral surface is also illustrated. Tel: telencephalon, dien: diencephalon, MB: midbrain, hind: hindbrain, SC: spinal cord. E) Anterior/ventral view of the anterior neural tube after development is complete.

when the closure defect is anterior the region may not develop leading to anencephaly (lack of a brain) or exencephaly where the brain essentially develops outside of the head (Juriloff and Harris, 2000). Later as the anterior neural tube differentiates, a host of genes are responsible for patterning, arealization, and migration, and disorders in any of these factors may lead to cortical malformations. Sonic hedgehog (Shh) and its signaling components are involved in telecephalic patterning, and their disruption causes holoprosencephaly, where the midline separation of the cortical hemispheres fails (Roessler et al., 1996; Brown et al., 1998; Golden, 1998). Misspecification of pro-neural genes or particular cell fate determinants can result in disorders like schizencephaly, where there are inappropriate clefts in the cerebral walls, or tuberous sclerosis, where improperly differentiated cells accumulate into ventricular nodules and a thick, disorganized cortex.

Migration disorders arise from the altered functionality of the machinery responsible for cell motility including cytoskeletal regulators, motors, stabilizers, and anchors (Lammens, 2000; Walsh and Goffinet, 2000; Lambert de Rouvroit and Goffinet, 2001; Bielas and Gleeson, 2003). The loss or defective expression of these proteins, such as LIS1, Nud, and DCX, to name a few, has resulted in serious human cortical dysplasias (Reiner et al., 1993; Matsudaira, 1994; Dobyns and Truwit, 1995; des Portes et al., 1998; Gleeson et al., 1998. Partial migration defects create a variety of lissencephalies (meaning "smooth brain" owing to the lack or reduction in sulci/gyri), whereas a complete loss of migration due to loss of filamin causes periventricular heterotopias (accumulated/aggregated but differentiated neurons at the ventricular surface (Fox et al., 1998; Morris et al., 1998). Other migration disorders include Fukuyama muscular

dystrophy (Fukuyama et al., 1981; Kobayashi et al., 1998), Muscle-Eye-Brain disease, Kallman syndrome (Barkovich et al., 1998). Hallmark symptoms of most of these disorders and malformations include moderate to severe seizures, moderate to severe mental retardation, dyskinesia, and sensory deficits. As cytoskeletal-related proteins are important for cell movement and tissue development elsewhere in the body, these patients will often present with additional diminished organs or organ dysfunction (Walsh, 1999; Golden, 2001).

1.1.4 Disorders of axon guidance and connectivity

It is not surprising that with so many connections to be made in such a precise and specific manner that errors do happen. Many of these human problems, whether mild of severe, can be traced to mutations and malfunctions amongst the cues that guide axons to their tissue targets (Yaron and Zheng, 2007). Impairments of connectivity may also be a secondary feature owing to a primary migration error, and often the secondary axon guidance defect will manifest in epileptic/seizure symptomatology. To model and study these scenarios a vast range of transgenic mice have been developed, often these involve multiple genes to account for the remarkable amount of redundancy and compensation that occurs normally during development. In nature, however, there remain instances of significant disturbance of brain function.

Human disorders of connectivity may present with less dramatic morphological phenotypes than earlier developmental disorders (such as the previously described migration disorders), or indeed those of the transgenic mice developed to model these guidance defects; however,

the resultant deficiencies can be equally crippling. When centered in the neocortex, loss of guidance cue function can result in psychiatric and mood disorders, as well as epilepsy and major multi-factorial congenital disorders. Schizophrenia is a moderate to severe psychiatric disorder shown in some cases to have a molecular basis in axon guidance defects, based on such cues as Sema3A (Eastwood et al., 2003; Miyoshi et al., 2003). Autism and a congenital brain disorder, coined Cri-du-chat, have been linked to the down-regulation of Sema5A (Simmons et al., 1998; Melin et al., 2006). Furthermore, Sema3F and other class 3 semaphorins are associated with heritable forms of epilepsy (Barnes et al., 2003; Holtmaat et al., 2003), and may underlie epileptogenesis in more broad disorders as fragile-X syndrome that include seizure development, (Darnell et al., 2001; Berry-Kravis, 2002). Another very severe condition called CRASH syndrome (corpus callosum hypoplasia retardation, adducted thumbs, spastic paraplegia and hydrocephalus) is caused by mutations in L1 (Kamiguchi et al., 1998; Castellani et al., 2000), a cell adhesion molecule that also acts in a receptor complex for Sema3A (Castellani et al., 2002).

Axon guidance disturbances may also result in peripheral manifestations of motor dysfunction. Inhibition of ephrin/EphA4 signaling causes significant errors in the guidance of ocular motor axons (Wegmeyer et al., 2007) resulting in Duane's retraction syndrome (Miyake et al., 2008). Mutations in guidance cue receptors important for midline crossing of axons can bring about the serious disorder of horizontal gaze palsy with progressive scoliosis (Robo-3; (Jen et al., 2004; Chan et al., 2006); or increased susceptibility to the comparatively minor human-specific disorder, dyslexia (Robo1; (Hannula-Jouppi et al., 2005; McGrath et al., 2006).

Progressive neurodegenerative disorders including motor dysfunction, like Parkinson Disease and amyotrophic lateral sclerosis (ALS), have been genetically linked to multiple factors, including EphB2, Sema5A, Sema3A, L1, and many of their downstream signaling components (Lesnick et al., 2007; Lesnick et al., 2008). The etiology of other neurodegenerative disorders such as Alzheimer's disease/dementia is proposed to also have a basis in defective axon guidance systems.

One cannot discuss axon guidance molecules and their relevance to human disorders and dysfunction without mentioning the efforts in the field of spinal cord repair. Spinal cord injuries cause a host of novel issues in the adult, including axonal degeneration and cell death, immunological upheaval and macrophage infiltration, reactive gliosis and scarring by astrocytes, release of myelin-associated inhibitors from damaged oligodendrocytes, cavitation of the primary injury site, and of course, the up-regulation of inhibitory developmental guidance cues (e.g. semaphorins) or their respective receptors (Skaper et al., 2001; Spinelli et al., 2007). Though it would seem an insurmountable task, steps are being made to overcome or bypass these various barriers for re-establishment of connectivity between upper and lower central nervous system components, and most importantly, renewed function in affected regions.

1.2 The cellular basis of cortical development

The nervous system is composed of two main types of cells: neurons and glial (Peters and Jones, 1984; Ramon y Cajal, 1995). There are 3 kinds of glia: astrocytes, oligodendrocytes, and microglia. Neurons exist in a wide variety of morphologies, electro-conductive properties, neurotransmitter and receptor profiles, and type of incoming and outgoing contacts. However, in the cerebral cortex, neurons can be grouped into two very general types. Projection neurons are born in the local neocortical ventricular zone (VZ) and project an axon to other regions of the cortex, subcortical regions in the telencephalon, diencephalon, midbrain, brainstem, and spinal cord (Noctor et al., 2002). Figure 1.4 displays rostral to caudal cross-sections depicting the anatomical layout of the thalamus, subcortical structures (LGE, MGE, and CGE), ventrolateral cortices, and basal nuclei, with respect to the neocortex and hippocampus. Interneurons originate from distant progenitor zones (Fig. 1.6) and once established in the cortical layers, are responsible for extending within the cortical layers to connect neurons locally (Corbin et al., 2001). In the CNS, projection neurons are glutamatergic and have excitatory output, whereas interneurons are GABAergic and inhibitory (Tan et al., 1998; Anderson et al., 2002; Wonders and Anderson, 2006).

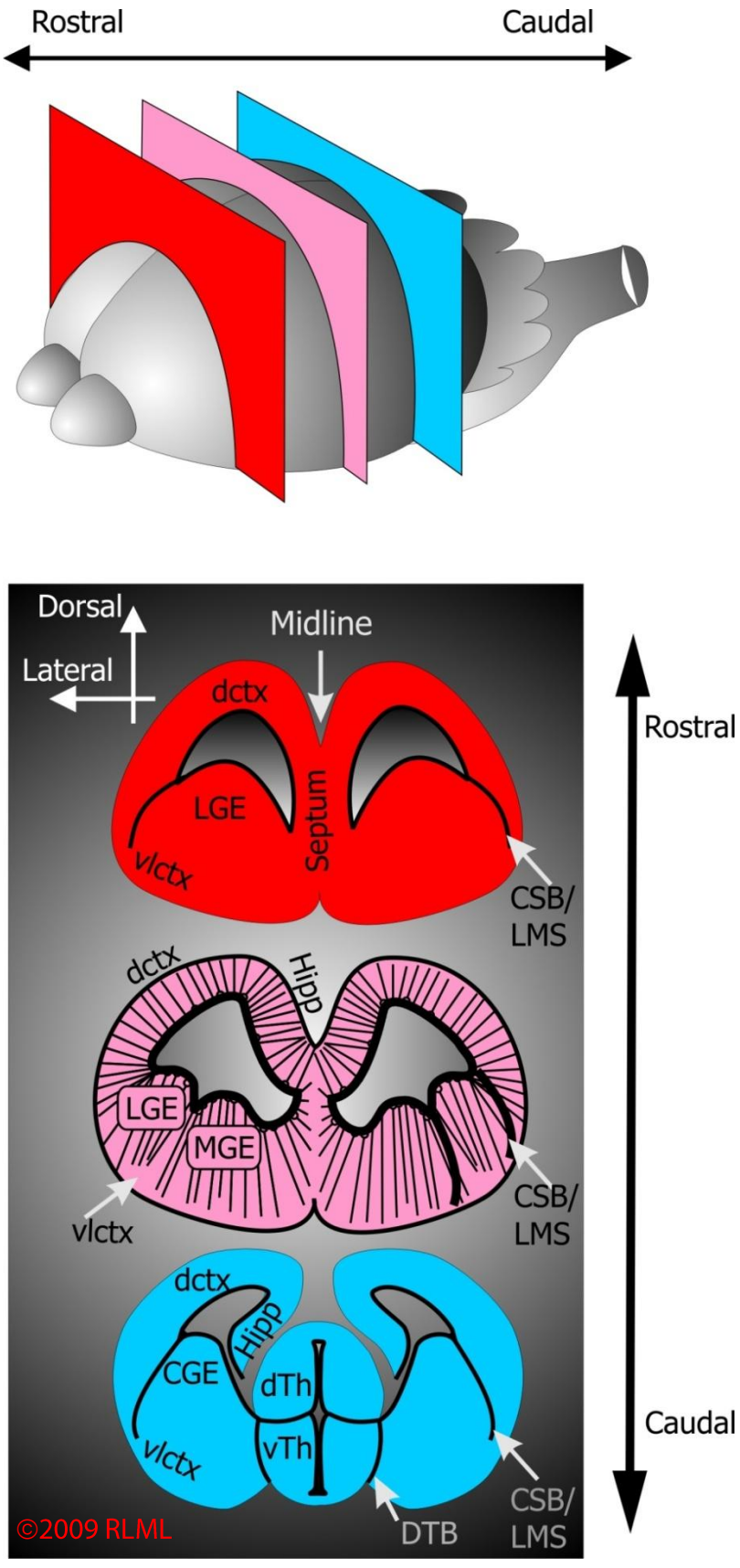


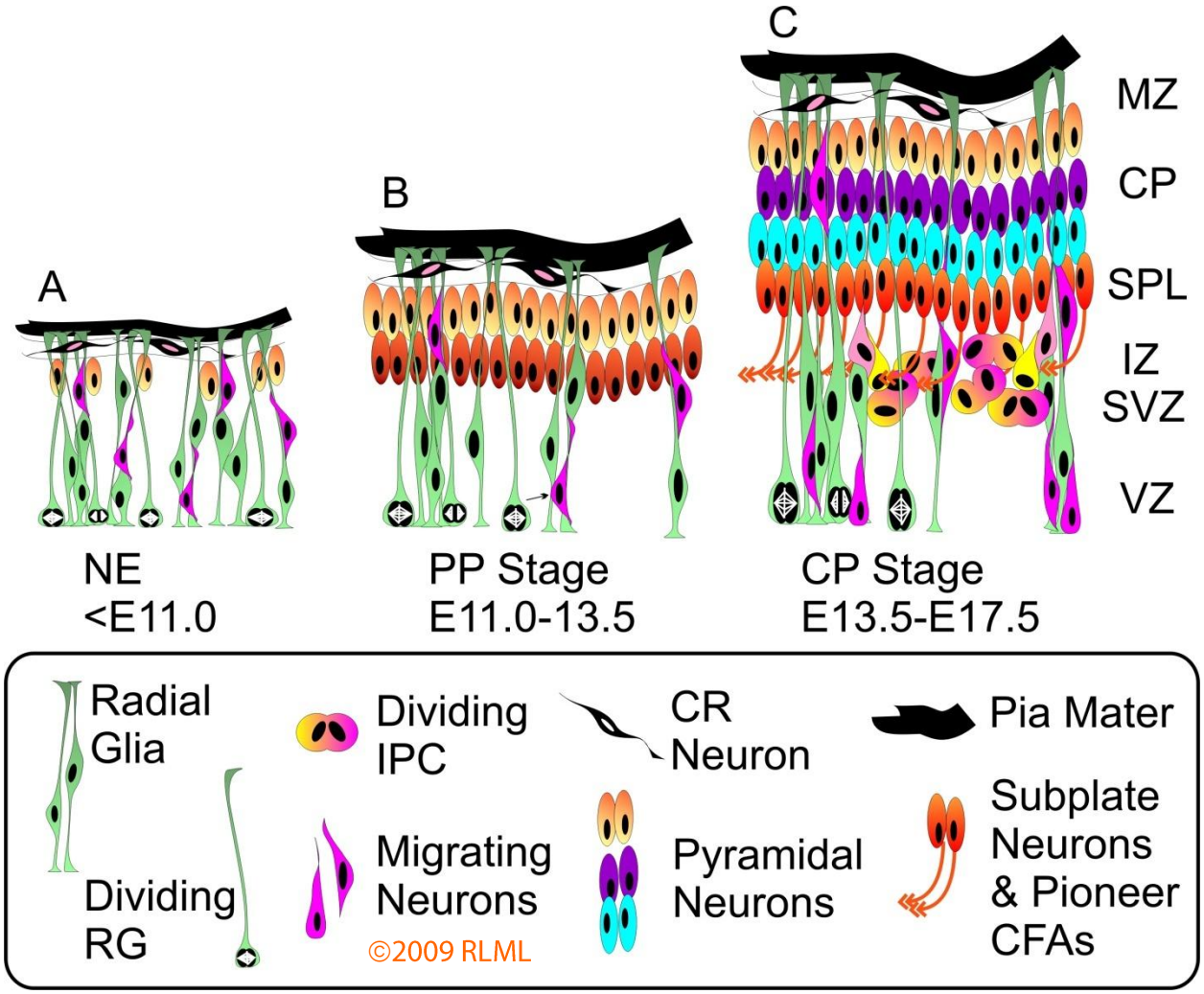
Figure 1.4 Cross-sectional anatomy of the telencephalon Schematic illustrations of coronal sections of the E14 mouse telencephalon at three rostral-caudal positions, illustrating the relative positions of the overlying neocortex (dctx: dorsal cortex), ventrolateral cortex (vlctx), hippocampus (hipp), the lateral, medial and caudal portions of the gangionic eminence (LGE, MGE, CGE), and the dorsal and ventral thalamus (dTh, vTh). In the second coronal view, a simplified layout of the radial fiber pattern is included. Also depicted are histogenic boundaries: the corticostriatal boundary (CSB, also known as the lateral migratory stream - LMS) separating cortex from subcortex; the diencephalic-telencephalic boundary (DTB) separating diencephalon from telencephalon. Also shown is the boundary between the the dorsal and ventral thalamic nuclei, commonly called the thalamic reticular nucleus. These boundaries differentiate two CNS regions and often coincide with molecular differences. Boundaries not shown include those that partition the hindbrain.

1.2.1 Layers of the developing neocortex

During development, the basic layout of the neocortex is that of a progressively layering tissue built from the inside-out (Fig 1.5). The ventricular zone is the primary local germinal region, housing the neural progenitors of the cortex. Radial glial cells are the main progenitors for all cells in the telencephalon (Anthony et al., 2004). At the onset of the neural tube stage, they are identified as NE cells in a purely proliferative state to initiate amplification of cell number (Hartfuss et al., 2001). Around E11.5, NE cells being making a transformation from a proliferative state into a neurogenic state, after which they are referred to as radial glial cells (Smart, 1973; Chenn and McConnell, 1995; Götz and Barde, 2005; Götz and Huttner, 2005). They may divide symmetrically to produce two daughter radial glial cells or asymmetrically to maintain a single radial glia while generating either a single neuron or an intermediate progenitor (IPC; Fishell and Kriegstein, 2003; Miyata et al., 2004; Götz and Barde, 2005). IPCs arise simultaneously with the advent of neurogenesis (Fig. 1.5C; Guillemot, 2004). They compose the secondary germinal zone located just basal to the VZ (Haubensak et al., 2004), the subventricular zone (SVZ), and can divide up to 3 more times to produce post-mitotic neurons (Noctor et al., 2004; Englund et al., 2005). Generally it is thought that the deep layers of the cortical plate are generated from the single divisions of radial glia and that IPCs generate the upper layer neurons of the cortical plate, although this is still controversial (Pontious et al., 2008).

Figure 1.5 Development of the neocortex

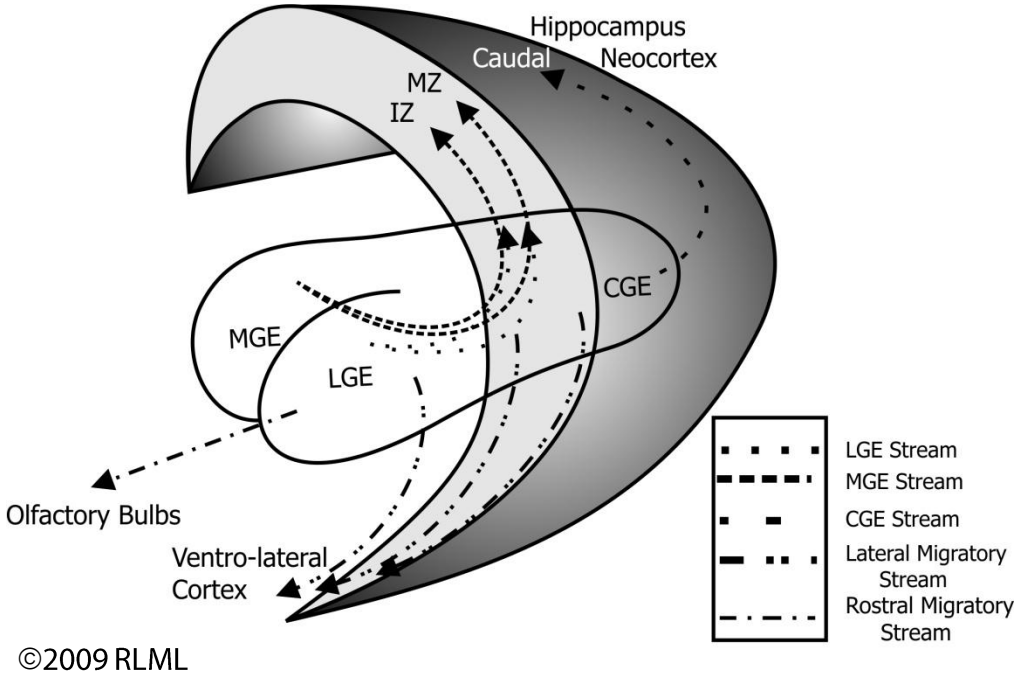
A) Prior to E11 in the mouse, the cortical anlage is a simple pseudostratified columnar epilthelium (NE) composed of proliferating neuroepithelial cells (in green). Cajal-Retzius neurons (in black) migrate to occupy the regions nearest to the pia mater at the outer-most (or lateral) aspect of the neural tube (the marginal zone - MZ). B) NE cells become radial glia and begin dividing to produce pyramidal neurons (shown migrating in pink) that form the preplate (PP; shown as orange layers at the pial interface). C) Radial glia continue to generate neurons that split the preplate and create the cortical plate (CP), between the MZ and the subplate layer (SPL) - formerly part of the PP. During this period of increased neurogenesis, basal progenitors, or intermediate progenitor cells, occupy the subventricular zone (SVZ) and divide to produce neurons destined for the cortical plate as well, particularly for the upper layers. While the cortical plate is forming above, the SPL also projects axons that create a region between itself and the underlying SVZ. These fibers, and those of thalamic axons arriving from the subcortex, as well as GABAergic interneurons migrating from the medial ganglionic eminence, comprise this region, called the intermediate zone (IZ). RG: Radial glia; IPC: Intermediate progenitor cell; CR: Cajal-Retzius; CFA: Corticofugal axons.



Cortical formation is initiated first by the creation of the pre-plate (PP), a transient structure composed of presumptive sub-plate neurons and marginal zone neurons (Fig. 1.5B). Following formation of the preplate, the next set of neurogenic divisions produces the deepest layer 6 pyramidal neurons, which migrate the short distance to the preplate by somal translocation (Kriegstein, 2004). Their arrival splits the PP into a marginal zone (MZ) at the pial interface, and a sub-plate layer (SPL) beneath. The subplate, like the preplate, is also transient and has a role in pioneering the corticofugal projection (Allendoerfer and Shatz, 1994). Each subsequent wave of newly post-mitotic neurons must target the outermost layer before the MZ (Luskin and Shatz, 1985). They migrate along the radial glia scaffold (illustrated in Fig. 1.4 and 1.5) past previous groups of neurons, where they detach from their radial guide and complete differentiation within the cortical plate. This is the basic mechanism of inside-out cortical plate formation. With each completed layer, subsequent groups of neural progeny are progressively restricted in the type of neuron they may become (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996; Desai and McConnell, 2000; Fukumitsu et al., 2006). In this manner, neurons in each layer express a different set of marker proteins and have different roles in cortical function (McConnell, 1995).

The marginal zone is the outer-most layer of the developing cortex interfacing with the non-neural connective tissue of the pia mater. Until the very elegant study by Borrel and Marin (Borrell and Marín, 2006), it was incorrectly assumed that Cajal-Retzius (CR) cells, like other preplate cells destined for the marginal zone, were generated by the first wave of neurogenesis by radial glia. CR cells arise from the cortical hem, the ventral corticostriatal VZ,

Figure 1.6 Tangential migration in the developing telencephalon.
The ganglionic eminences are the source for interneurons in the dorsal neocortex and hippocampus. At E12.5 neurons from the medial and caudal ganglionic eminences (MGE and CGE) migrate into the cortex. The MGE stream supplies largely the antero-lateral portions of the cortex, while the CGE supplies the caudo-medial portions of the cortex and the entire hippocampus (Corbin or Nery et al, 2003). Later, around E14.5 the lateral ganglionic eminence (LGE) also begins to supply interneurons destined for the neocortex. However, the main target for LGE-derived neurons is the olfactory bulb via the rostral migratory stream or RMS. The lateral migratory stream (LMS) runs along the cortico-striatal boundary. Neurons in the LMS originate in the VZ at the corticostriatal angle and migrate to the ventrolateral cortex to populate basal nuclei and archicortex.



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and the caudal ganglionic eminence and migrate tangentially between the pia mater and the neuroepithelium/ventricular zone to create the basal-most neuronal layer (Bielle et al., 2005; Borrell and Marín, 2006). These cells express a secreted protein called reelin, which is responsible for regulating the inside-out formation of the cortical plate. In *reeler* mice lacking reelin (D'Arcangelo et al., 1995) or one of its downstream pathway constituents (the *scrambler*, or *yotari* mutations), the cerebral cortex in nearly inverted, having been established in an outside-in fashion instead. It is thought by some that reelin mediates a stop signal

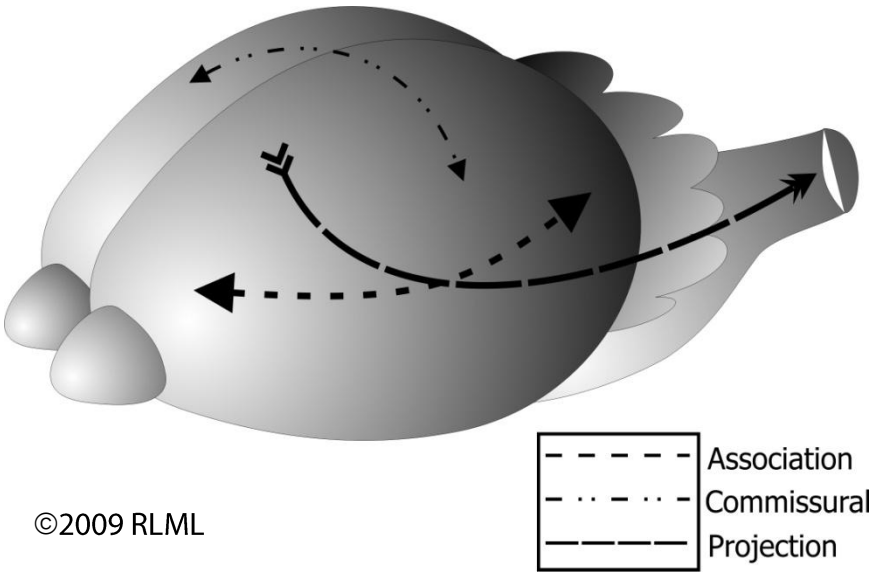
(Dulabon et al., 2000; Hack et al., 2002), but the exact nature of this mechanism is still debated.

The inhibitory interneurons that modulate the output of pyramidal neurons of the neocortex are born of radial glia as well, but of not those in the dorsal cortex. Early in neurogenesis, interneurons arise from the VZ of medial and caudal ganglionic eminences (Wichterle et al., 1999; Nery et al., 2002), with contributions from the lateral ganglionic eminence and the septal region at around E14.5. Starting around E12.5 these cells migrate tangentially (or parallel to the pial surface) from these ventral telencephalic regions to destinations within the cortical layers by way of the MZ or IZ. Interneuron migration to the cortex is complete around E18.

1.2.2 Fiber tracts in the developing brain

As the lamination of the cortex proceeds, and layers of neurons finish differentiating in their appropriate location, they begin to project axons (Fig 1.7). The first neurons to begin pathfinding to a target are called "pioneers". Association tracts connect one part of the cortex with a different part of the cortex in the same hemisphere, often bringing related information together for integrative processing of external information. Commissural tracts connect a structure of one hemisphere with the same structure in the contralateral hemisphere, thus allowing the two halves of the brain to communicate. The largest commissural tract is the corpus callosum which connects the entire neocortex of one hemisphere with the contra-

Figure 1.7 Types of tracts in the telencephalon.



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There are three basic categories of tracts in the CNS. Association tracts connect within a hemisphere, for example between the left frontal lobe and left parietal lobe. Commissural tracts connect structures across the midline from one hemisphere to the other, for example between the two halves of the hippocampus. Projection tracts are long distance tracts connecting one part of the CNS with other distant regions, for example, connecting the cortex and the spinal cord.

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lateral neocortex. Projection tracts connect one part of the CNS with another distant part of the CNS. The first tracts initiated from within the neocortex are the corticofugal projection tracts. Typically the nomenclature for CNS tracts includes first the structure where the tract begins (i.e. the cortex) followed by the structure in which the tract terminates (i.e. the thalamus or spinal cord). Therefore, corticofugal terminations refer to the collective set of lower CNS structures to which these cortical fibers project.

1.3. Molecular control of axon guidance

1.3.1 The growth cone

One of the distinctive aspects of neuronal differentiation is the formation of the dendrites and axons. It is these axons that form the basis of the CNS white matter tracts. In order for tracts to be formed, axons must grow and find their way through the embryonic environment, to find

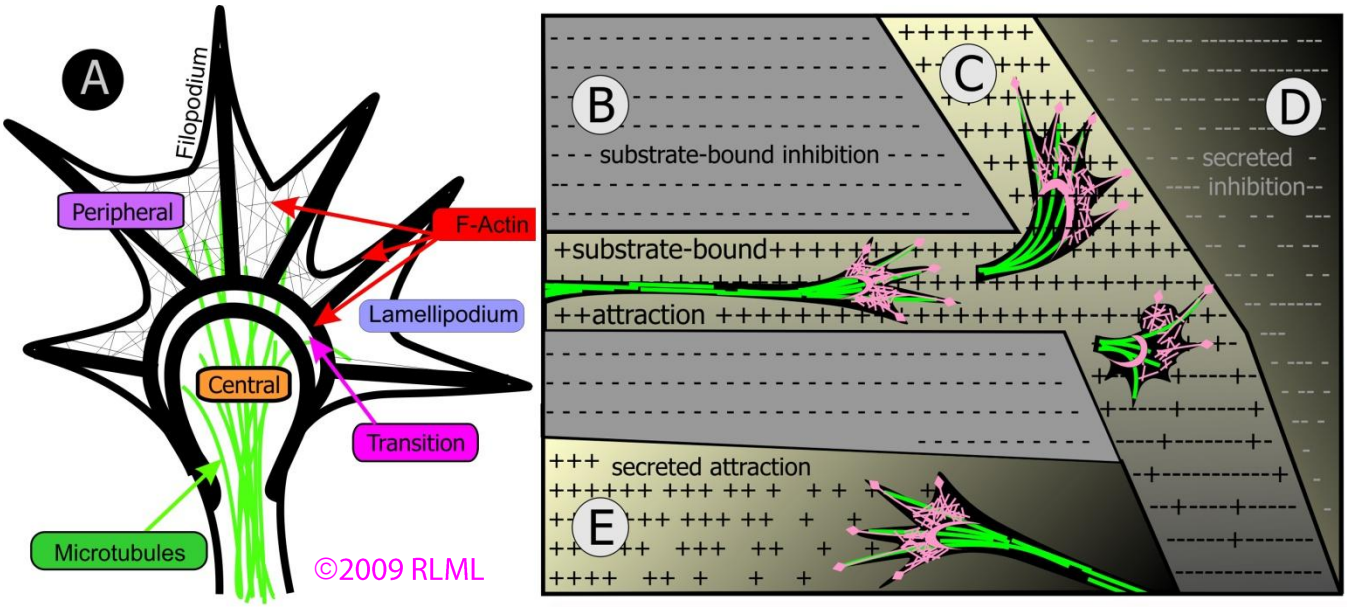
their final target. This is the process of axon pathfinding, and owing to the complexity of the embryonic environment is likewise inherently intricate.

The motile tip is the growth cone, a specialized sensory apparatus at the tip of a growing axon or dendrite (Ramon y Cajal, 1995). Originally called a "battering ram" by Ramon y Cajal, this expansion of the neurite terminal is underlain by specific organizations of actin fibers and microtubules (Fig 1.8A). The growth cone is responsible for sensing instructive and trophic extracellular molecules and to integrate the isolated information into a cohesive response, ultimately resulting in directional change and control of the extent of outgrowth (Lin and Forscher, 1993; Bagnard et al., 2000; Huber et al., 2003; Lowery and Van Vactor, 2009). A given type of growth cone will only respond to a specific set of cues based upon the complement of receptors and receptor complexes it expresses at the time of pathfinding (Goodman et al., 1983; Kapfhammer and Raper, 1987b). Therefore, a growth cone can be completely blind to a particular molecule of great abundance, providing it does not express the appropriate receptors for that cue. In this manner, many types of neuronal growth cones can navigate through the same environment and receive independent instruction as though they were in an isolated environment (Kapfhammer et al., 1986).

The growth cone is divided into three regions; the peripheral, transitional and central domains (Cheng and Reese, 1985; Lowery and Van Vactor, 2009). The peripheral zone is rich in filamentous actin; when densely bundled it forms the skeletal basis of the spike-like

Figure 1.8 The growth cone and its guidance

A. Schematic of a growth cone. The peripheral domain is shown containing actin bundles protruding to form filopodia, and an actin meshwork underlying intervening lamellipodia. The central domain consists largely of bundled microtubules emerging from the consolidated axon which are shown as occasionally extending into the periphery. The transition zone contains the actin arc, and is the region of force production and actinomysosin contractility for peripheral F-actin. B-E) For the navigating growth cone in the embryonic environment, guidance cues create domains of attraction (C and E) and repulsion (B and D) which can be short range (substrate bound - B and C) or long range (secreted - D and E). The schematic illustrates growth cones guidance along permissive substrates and avoidance of inhibitory substrates, as well as gradient sensing of attractive and repellant signals. In many ways, axon pathfinding is a similar process to cell migration.



protrusive filopodia (Fournier et al., 2000; Jay, 2000), or when organized into a specifically-interconnected meshwork creates the the sheet-like lamellipodia that are often seen as web- or veil-like extensions between filopodia (Lewis and Bridgman, 1992). The transition zone is at the interface of the peripheral and central domains(Lowery and Van Vactor, 2009). It contains actin arcs that lie semi-circumferentially in the growth cone and perpendicular to the bundles projecting into filopodia. Actin arcs are the hub of the actomyosin apparatus that contracts the motile peripheral actin structures in response to negative cues (Jay, 2000; Zhang

et al., 2003; Medeiros et al., 2006). The central domain is predominantly filled with stable microtubules, like those within the axon shaft trailing the growth cone (Tanaka et al., 1995; Lee and Suter, 2008). Some of these microtubules are inherently dynamic at their plus-ends and frequently invade filopodia, which is likely to underlie subsequent stabilization and direction of outgrowth in response to incoming directional information (Buck and Zheng, 2002; Zhou and Cohan, 2004).

The highly dynamic filopodia and lamellipodia are constantly extending and retracting. One school of thought is that growth cone dynamics are underlain by a balance between stochastic behavior (random sampling) and deterministic behavior (continual axonal outgrowth) that permit proper sensation of specific instructions from the local microenvironment (Maskery and Shinbrot, 2005). However it is more widely accepted that vertebrate growth cones have an absolute requirement for neurotrophic input via tropomyosin-related kinase (Trk) receptors for survival, which also drive outgrowth (Lentz et al., 1999; Goldberg et al., 2005). It is not apparently an absolute requirement, however, *Drosophila* neurotrophin (DNT1) expression correlates with neural survival (Zhu et al., 2008), and though somewhat elusive, the general mechanisms of promoting invertebrate axon extension and survival are under investigation (Blackshaw et al., 2004). Regardless, instructional cues interact with plasma membrane-expressed cognate receptors, and the resultant downstream cascades of signals ultimately impinge upon the actin and microtubule cytoskeletons (Lowery and Van Vactor, 2009).

Axon outgrowth depends upon growth cone as a vehicle. The propulsion is driven by combination of dynamic F-actin formation, contractility and substrate adhesion coupling the actin cytoskeleton to the extracellular environment via receptor complexes (Lin and Forscher, 1993; Lin et al., 1994; Jay, 2000; Suter and Forscher, 2000). Actin filaments are thought to treadmill in preparation for incoming instructive signaling; LIM kinase controlled actomyosin contraction pulls actin filaments toward the central of the growth cone (Aizawa et al., 2001), with de-polymerization occurring at the minus end at the transition zone by ADF/cofilin (Matsudaira, 1994), and concomitant plus-end re-polymerization at the interface of the plasma membrane (Suter and Forscher, 2000; Medeiros et al., 2006). Incoming attractive and repulsive signaling then impinges upon the actin treadmill and modulates various aspects to control the direction of growth cone advancement (Fig 1.8 B-E; Bard et al., 2008; Lee and Suter, 2008).

Net growth cone advancement is due to a change in the balance and increases forward protrusion over retrograde flow. In order for this forward movement to occur, actin bundles must be stabilized against a growth promoting substrate by focal contacts – the equivalent of focal adhesions in migrating non-neuronal cells (Lowery and Van Vactor, 2009). In pioneering growth cones, such as those of subplate axons, focal contacts are complex aggregates of transmembrane and membrane-associated proteins linking the actin cytoskeleton to the external extracellular matrix, and are composed of integrin, talin, vinculin, and ERM family proteins (Jay, 2000). In contrast, follower axons rely upon permissive interactions and adhesion with the pioneer axons, often employing cadherins, integrins and other cell

adhesion molecules such as TAG-1 and L1-CAM (Malhotra et al., 1998; Itoh et al., 2004; Maness and Schachner, 2007; Bard et al., 2008; Wolman et al., 2008). Retrograde flow due to actomyosin contractility against substrate tethered actin filaments leads to forward propulsion of the growth cone (Lin et al., 1996), and Ena/Vasp proteins competing with capping proteins at the plus end promote continued actin polymerization causing increased filopodial protrusion (Lowery and Van Vactor, 2009).

Attractive and repulsive cues drive rearrangements of the actin cytoskeleton, therefore directing the path of growth cone protrusion. They control direct regulators of F-actin through Rho GTPase acting proteins (Rho GAPs), Rho guanine exchange factors (Rho GEFs), and Rho GDP dissociation inhibitors (Rho GDIs) and their downstream activities upon Rho GTPases (Fig 1.9; Watabe-Uchida et al., 2006). Attractive and permissive cues promote the formation of focal contacts, and continued plus-end actin polymerization, thereby enhancing forward movement (Robles and Gomez, 2006). The encounter of a negative cue leads to inhibition or removal of adhesive complexes and the increase of retrograde actin flow over and above any forward polymerization (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987a, b; Lowery and Van Vactor, 2009). Inhibitory substrates may lead to stalling, where reverse flow equals forward protrusion and therefore no net movement can be achieved (Jay, 2000; Suter and Forscher, 2000). If the repellant is strong enough, or acting in relative isolation from permissive cues, actin severing and depolymerizing proteins, as well as plus end capping proteins become active, leading to de-adhesion and uncoupling from the extracellular matrix resulting in catastrophic loss of F-actin and the collapse of the growth

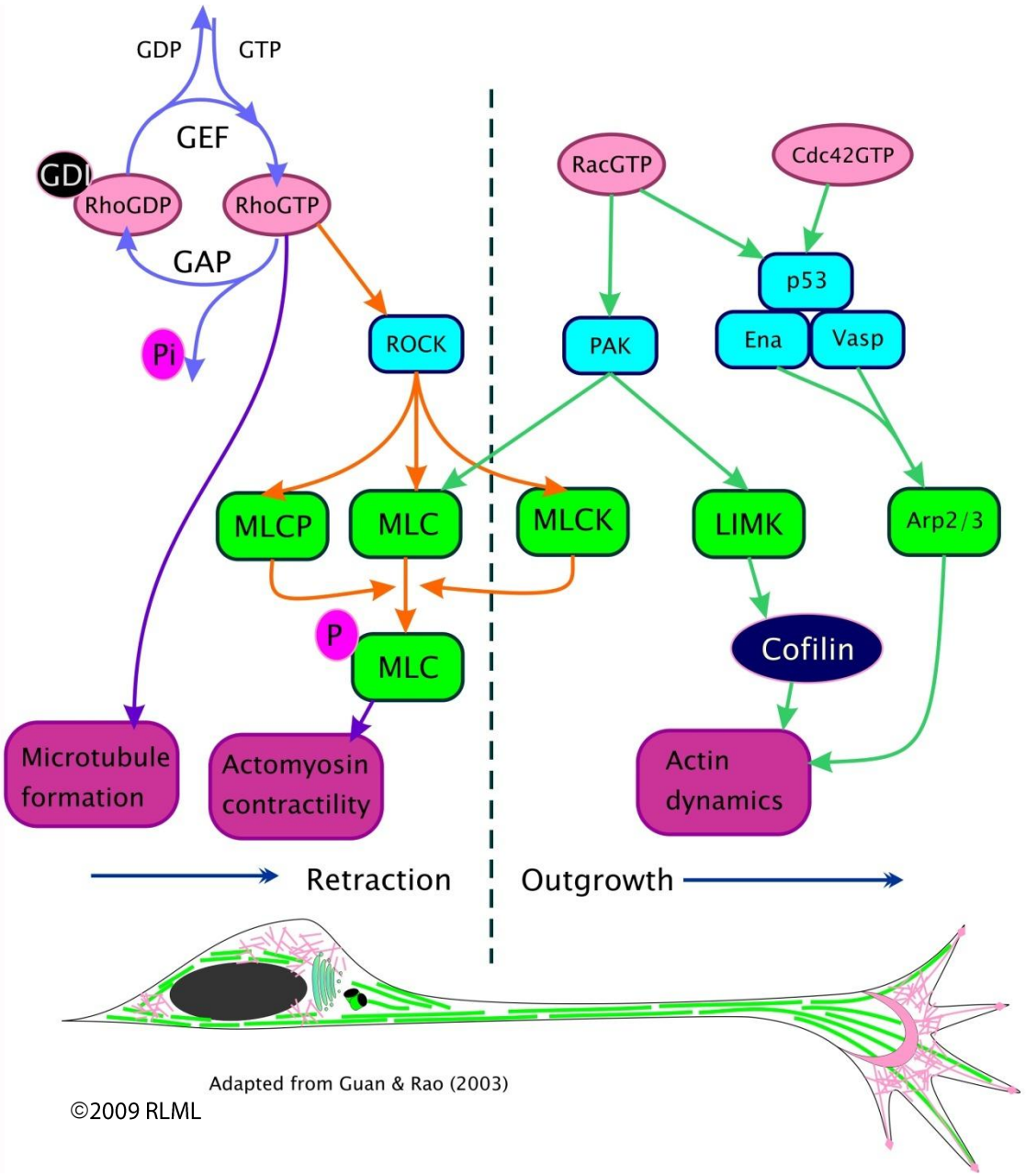


Figure 1.9 Control of outgrowth is mediated by the Rho family GTPases
In a migrating cell, Rho is responsible for actin stress fiber formation and the actomyosin contractions that cause retraction at the rear of the cell. Rac1 and Cdc42 are responsible for lamellipodia and filopodia at the leading edge, and promote actin filament polymerization and meshwork formation. In a growth cone, attraction and repulsion are said to employ similar mechanisms. Rho activation causes retraction, underlain by actin depolymerization, loss of adhesion, and collapse of the growth cone cytoskeleton. Attraction via Rac1 and Cdc42 is said to increase adhesion through formation of focal contacts and engagement to the cytoskeleton, as well as increased actin polymerization. There are indications of more complex interactions, such as the requirement for activated Rac1 for the subsequent activation of RhoD, which like Rho A, is responsible for repulsive signaling from plexins.

cone skeletal structure (Arimura et al., 2000; Aizawa et al., 2001; Atwal et al., 2003; Ito et al., 2006; Schmidt and Strittmatter, 2007; To et al., 2007). This event is generally referred to as growth cone collapse, and is a common measure of assessing repulsion/inhibition.

The microtubule cytoskeleton is necessary for steering of the growth cone (Tanaka et al., 1995; Gordon-Weeks, 2004). In response to an attractive/permissive cue, microtubules are uncoupled from the actin bundles and are free to explore the cue-bound regions of the growth cone periphery (Lee and Suter, 2008). Microtubules become tethered to the actin cytoskeleton in exploring filopodia due to inhibitory/repulsive signaling resulting in their retraction by cue-directed increases in retrograde actin flow (Zhou and Cohan, 2004). Microtubules are thought to direct transport of signaling molecules for the continued outgrowth and stabilization of the growth cone is the direction of substrate adhesion – i.e. toward the positive cue or opposite the negative cue – for the establishment of the axon in a novel direction (Buck and Zheng, 2002).

1.3.2 Guidance Cues, Receptors, and Effectors

Guidance cues can exist as secreted gradients or tethered to a substrate. Secreted repellants and attractants can direct growth cones from a distance, whereas substrate-bound positive and negative cues create corridors and barriers for axon outgrowth (Fig 1.9). Regardless of the mode of expression of a guidance cue, their instruction lies in the composition of the receptor complex and the downstream effectors of that receptor (Grunwald and Klein, 2002; Meyer and Feldman, 2002; Huber et al., 2003; Shrahna et al., 2006). Equally important is the overall

expression of guidance cues in the surrounding environment, for the growth cone must also integrate all the incoming signals and make a single choice of direction. This is accomplished at the level of molecules governing the cytoskeleton. (Kapfhammer and Raper, 1987a; Gallo and Letourneau, 2004)

In general, external guidance cue signals converge upon the Rho family of GTPases, which are intimately involved in regulating the specific organization and conformation of the F-actin skeleton (Huber et al., 2003). Three classical Rho GTPases (Rho A, Rac1, and Cdc42) are well described in their roles regulating specific morphologies of the actin cytoskeleton for cell motility, and have also been shown to mediate similar activities in growth cones (Meyer and Feldman, 2002; Govek et al., 2005). Attractive signaling leads to activation of Rac1 and Cdc42 activation, frequently via PI3 kinase and protein kinase A (PKA) while inhibiting Rho A. Often this results in slowed contractility of actin filaments while activating PAK (p21-activated kinase), the actin nucleating factors Arp2/3 and WASP, as well as Ena/Vasp and focal contact/adhesion proteins leading to increased substrate coupling, stabilization, and extension of actin filaments. Generally inhibitory guidance signals increase Rho A activation and decrease the activation of Rac1 and Cdc42 (Zhang et al., 2003) (Meyer and Feldman, 2002). Rho A specifically interacts with and activates ROCK (Rho-activated kinase) which inactivates myosin light chain phosphatase (MLCP) thereby promoting phosphorylation of myosin light chain by MLC kinase and activation of actin contractility (Zhang et al., 2003; Govek et al., 2005; Yuan et al, 2003).

The diversity of molecules that are involved in directing the guidance of axons is broad. Here I will review the major molecular group that act as guidance cues and their primary receptor complexes, with examples of some significant in vivo axon guidance functions. There are four families of "traditional" guidance cues: netrin, slit, ephrin, and semaphorin (FIg 1.10 and 1.11; Yu and Bargmann, 2001; Grunwald and Klein, 2002; Guan and Rao, 2003; Huber et al., 2003). As research progresses, we are beginning to see examples where the so-called traditional guidance cues may act as morphogens by creating patterns and aiding in tissue/cell specification (Hinck, 2004), while canonical morphogens have been found in critical axon guidance roles (Schnorrer and Dickson, 2004; Charron and Tessier-Lavigne, 2007; Zou and Lyukvyutova, 2007).

1.3.2.1 Morphogens

There are several examples of morphogens with very important axon guidance functions. Bone morphogenic protein (BMP) is expressed by the roof plate of the spinal cord and induces the development of dorsal tissues, such as commissural neurons, and also acts as a repulsive signal to initiate the direction of commissural axons toward the ventral spinal cord (Augsburger et al., 1999). Likewise, sonic hedgehog (Shh) expression in the notochord drives the formation of the floor plate that subsequently secretes Shh and induces motor neuron differentiation. Shh also provides an attraction in addition to the netrin signaling that attracts commissural axons to their first intermediate target, the floor plate (Serafini et al., 1996; Charron et al., 2003; Okada et al., 2006). Until recently, the guidance cue that governed the anterior turn made by post-crossing commissural axons was unidentified. We now know it is

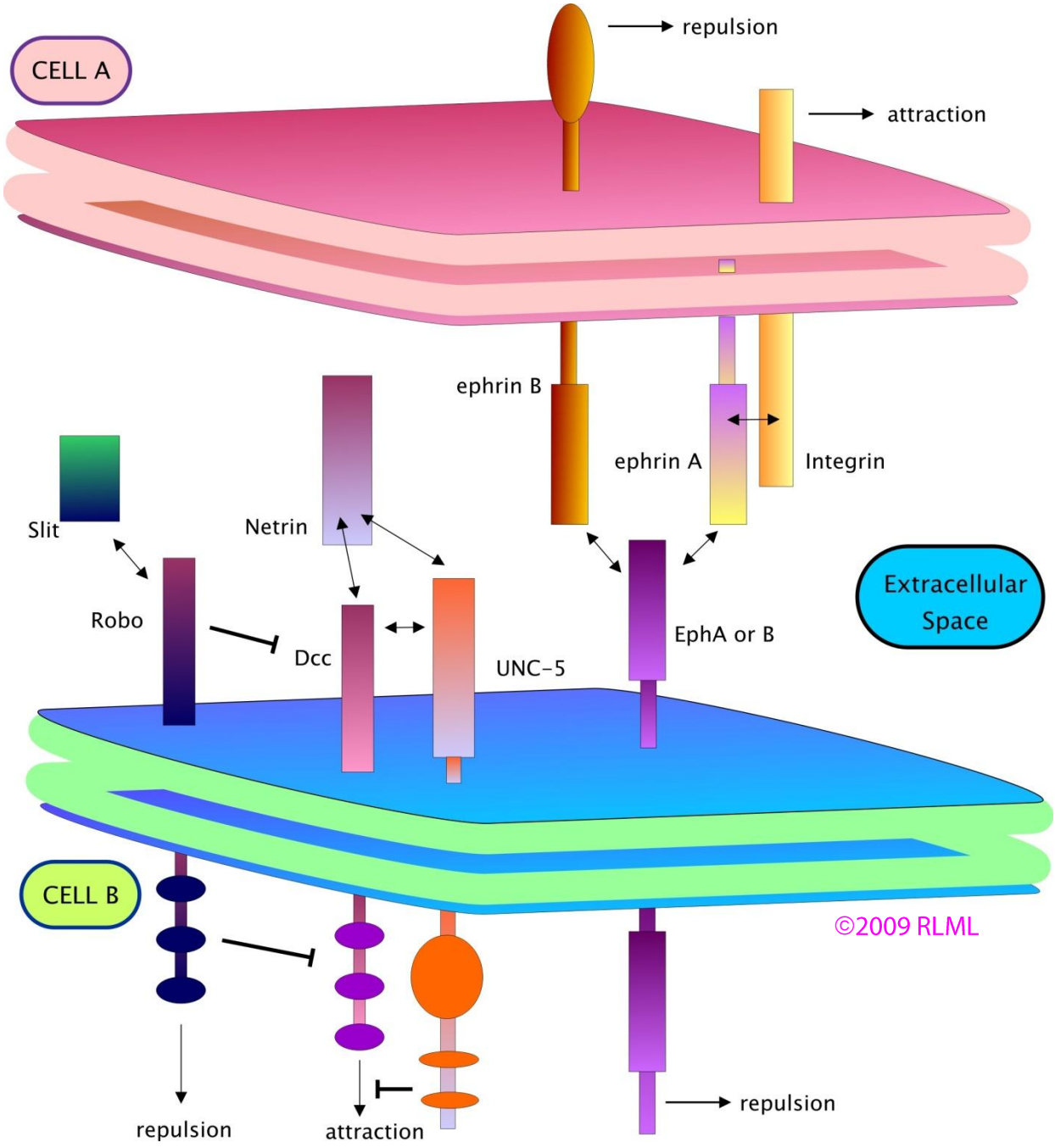


Figure 1.10 The classical guidance cue families and their receptors.
Other than semaphorins, the classic guidance cues are Slits, Netrins, and ephrins. Slits act through Robos, Netrins acts through DCC/neogenin and sometimes Unc-5, which reverses netrin attraction to repulsion. Robo is also said to block the attractive DCC signal as well as mediating its own repellant effects. Ephrins interact with Ephs, which transduce repulsive signals. Eph-to-ephrin reverse signaling can be repulsive or attractive, and to this end, may modulate the activity of other transmembrane proteins such as integrin or CXCR complement receptors.

actually mediated by two Wnt gradients. Commissural axons are not only pushed toward higher CNS structures via Wnt5A-Ryk-mediated repulsive signaling (Keeble et al., 2006), but also pulled by Wnt4-Frizzled mediated attraction (Lyuksyutova et al., 2003; Wacker et al., 2008).

1.3.2.2 Netrins

Netrins are a small family of secreted glycoproteins (vertebrate netrins 1-4), and though originally found to be attractive, can elicit repulsion under certain circumstances. *Deleted in colorectal cancer* (DCC) is a non-catalytic receptor (Keino-Masu et al., 1996) regulating actin dynamics and directing axon outgrowth toward an increasing netrin gradient. Netrin may also act as a repellent agent when the Unc-5 receptor is also present. Originally found in the nematode *C. elegans* (Leung-Hagesteijn et al., 1992), UNC-5 in mammals also interacts with DCC through intracellular domains in a ligand-dependent fashion to convert attraction to repulsion (Leonardo et al., 1997).

Netrin is one of the fundamental players in one of the most common models for segmental vertebrate axon guidance. It was shown that netrin was expressed by cells of the spinal cord floor plate (Serafini et al., 1996), providing attraction to DCC-expressing commissural axons descending from the dorsal spinal cord (Fazeli et al., 1997). Attractive netrin/DCC signaling is also a critical guidance component for the reciprocal tracts that form between the developing neocortex and thalamus. Specifically, netrin attracts pioneering corticofugal axons toward the lateral ganglionic eminence from beyond the cortical striatal boundary (Métin et al., 1997),

and are also attractive to dorsal thalamic axons coursing their way to the cortex (Braisted et al., 2000).

1.3.2.3 Slit and robo

There are three vertebrate Slits that act through three Robo receptors. Slit and Robo are also crucial players in mediating spinal commissural axon guidance. In this case, however, the effects are inhibitory. Axons that have been attracted to the floor plate by netrin must then be repelled if they are to ascend to the thalamus. This repulsion is delivered by Slit expression from the floor plate cells. As axons pass the floor plate, they become responsive to slit by insertion of Robo into the growth cone membrane (Long et al., 2004; Zou et al., 2000; Mambetisaeva et al., 2005). Robo also binds and inhibits DCC activation by netrin, which further aids in the rapid switch in attraction to repulsion in the floor plate region.

In the cortex and thalamus, Slit and Robo are likewise important inhibitors of inappropriate midline crossing by corticofugal axons, and mediators directing appropriate midline crossing by callosal axons (Bagri et al., 2002). Slit-2 exists in a medial to lateral gradient throughout the cortical layers while Slit-1 has uniform expression in the developing cortical plate, together ensuring a lateral projection of Robo+ cortical fibers into the intermediate zone (Bagri et al., 2002; Andrews et al., 2006; López-Bendito et al., 2007).

1.3.2.4 Ephs and ephrins

Ephs are the largest family of receptor tyrosine kinases, having eight type-A isoforms and six type-B isoforms that bind to the five type-A ephrins and the 3 type-B ephrins, respectively, to initiate both "forward" (Eph-mediated) and "reverse" (ephrin-mediated) signaling (Bruckner et al., 1997). Forward Eph signaling and reverse B ephrin signaling can both lead to growth cone collapse and repulsion (Cowan and Henkemeyer, 2001; Elowe et al., 2001). In contrast, A ephrins, though GPI-linked, can signal through lipid-raft associated tyrosine kinases, to increase cell adhesion and attraction in part though integrin activation (Davy and Robbins, 2000).

Eph/ephrin interactions are best recognized for their role in vertebrate retinal-tectal targeting. A and B ephrins exist in perpendicular anterior-posterior and medial-lateral gradients in the tectum; whereas complementary nasal-temporal and dorsal-ventral gradients of Eph A and B and ephrin A and B pattern the retina. Together these expression patterns lead to a very precise map of the retina represented by the terminations of retinal axons on their tectal targets (Bonhoeffer and Huf, 1982; Walter et al., 1987; Cheng and Flanagan, 1994; Drescher et al., 1995). In the cortical layers, ephrin/Eph activity is likewise required for the patterning of cortical regions and layers (Yun et al., 2003; Uziel et al., 2006). Once thalamocortical axons have reached the internal capsule and invade the cortical plate, both forward and reverse signaling are responsible for the appropriate regional termination and arborization of axons in the presumptive layer 4 (Dufour et al., 2003; Torii and Levitt, 2005).

1.4 Semaphorins are the largest family of guidance cues

There are more than 30 members in eight classes of semaphorins expressed in invertebrates, vertebrates and viruses, with 20 individuals found in each of the human and mouse genomes. Each one contains a conserved semaphorin domain which is the N-terminal modular hallmark of all semaphorins. Other than their guidance cue roles, semaphorins are involved in many physiological processes, including immune function, apoptosis, organogenesis (of lung, kidney, liver, and heart, for example), and have oncogenic and tumor suppressor roles in the etiology of several cancer types (Roth et al., 2009). Evidence also has suggested that semaphorins have functions even in cell cycle regulation and morphogenesis of CNS and other tissue. The eight classes of semaphorins are divided upon sequence similarity and the addition of protein domain specializations C-terminal to the semaphorin domain. I will describe each class briefly here, except class 5, which I will discuss in greater detail to follow.

The class 1 and 2 semaphorins are found exclusively in invertebrates. Class 1 are transmembrane semaphorins with a potential attractive or permissive function for growing axons (Wong et al., 1999; Godenschwege et al., 2002; Cafferty et al., 2006), whereas class 2 molecules are secreted, bearing an immunoglobulin domain C-terminal to the sema domain, and have been shown to create *in vivo* repulsive gradients in both flies and grasshoppers (Isbister et al., 1999; Bates and Whitington, 2007; Zlatic et al., 2009). Class 3 to 7 semaphorins, with one notable exception, are exclusively vertebrate-expressed. Class 3 semaphorins are secreted, bearing an immunoglobulin domain C-terminal to the sema domain and terminate in a basic tail that is thought to tether them within the extracellular matrix. Except Sema3C

which is attractive (Bagnard et al., 1998), class 3 semaphorins (A-G) are generally repellent cues (Luo et al., 1995; Raper, 2000). Sema3A is the prototypic repellant semaphorin; studied extensively in terms of receptors, co-receptors, and down-stream signals (Castellani and Rougon, 2002; Antipenko et al., 2003; de Wit and Verhaagen, 2003; Kruger et al., 2005; Yazdani and Terman, 2006; Zhou et al., 2008), yet the knock-out has an unremarkable phenotype (Catalano et al., 1998). Class 4 semaphorins largely act within the immune system (Kumanogoh et al., 2002; Suzuki et al., 2002a; Kumanogoh and Kikutani, 2004), though some have been shown to function within the CNS as well (Fujioka et al., 2003; Masuda et al., 2004; Burkhardt et al., 2005; Ito et al., 2006). These are transmembrane molecules with an immunoglobulin domain and a cytoplasmic portion containing a PDZ domain. Class 6 semaphorins are also transmembrane molecules with nervous system function (Kerjan et al., 2005; Bron et al., 2007; Mauti et al., 2007; Runker et al., 2008). They have no C-terminal specializations excepting a large intracellular domain bearing an SH2 domain for protein-protein interactions and potential for signaling through Src kinase. There is only one identified class 7 semaphorin. It is GPI-linked to the plasma membrane and has an attractive function based on its signaling through integrin and/or plexin receptors (Pasterkamp et al., 2003; Suzuki et al., 2007).

A growing number of factors are now known to modulate the function of semaphorins. For example, specific receptor complements and intracellular signaling components (including calcium levels and the presence of cyclic nucleotides) can modulate growth cone behavior in response to a semaphorin (Polleux et al., 2000; Falk et al., 2005; Bron et al., 2007; Ding et al.,

2007; Ben-Zvi et al., 2008; Schmidt et al., 2008; Vizard et al., 2008). Cell adhesion molecules such as α1β1 integrin and Ig superfamily members such as L1-CAM and TAG-1 can act as direct or indirect semaphorin receptors (Suzuki et al., 2007; Law et al., 2008); (Pasterkamp et al., 2003; Bechara et al., 2007; Suzuki et al., 2007). Extracellularly, the presence of chondroitin sulfate proteoglycan (CSPG) and heparan sulphate proteoglycans (HSPG) in the local environment can regulate many guidance cues including semaphorins (Laabs et al., 2005; de Wit and Verhaagen, 2007; Shipp and Hsieh-Wilson, 2007). For example, Sema5A functions as either an inhibitory or attractive guidance cue depending on the presence of CSPG or heparin sulfate respectively (Kantor et al., 2004). Similarly, sulfated proteoglycans have been proposed to potentially increase the repellant nature of Sema5B (Shipp and Hsieh-Wilson, 2007).

1.4.1 Proteolytic regulation of semaphorins

An additional and fundamental mechanism of semaphorin activity regulation is posttranslational modification by proteolysis. Cleavage of secreted guidance cues is common and among semaphorins is important for regulating their activity (Yazdani and Terman, 2006). Previously identified proteolytic enzymes that cleave semaphorins include several different matrix metalloproteinases (MMPs; Dickson, 2002; McFarlane, 2003; Basile et al., 2007; Gonthier et al., 2007), and the substillin-like proprotein convertases (PCs; Seidah et al., 2008), of which furin is a member. For example, the secreted repellants Sema3A, 3B, 3E, 3F, 3G all require a single cleavage and subsequent dimerization to elicit activity (Adams et al., 1997; Koppel et al., 1997; Klostermann et al., 1998); however, a second cleavage event results in inactivation of

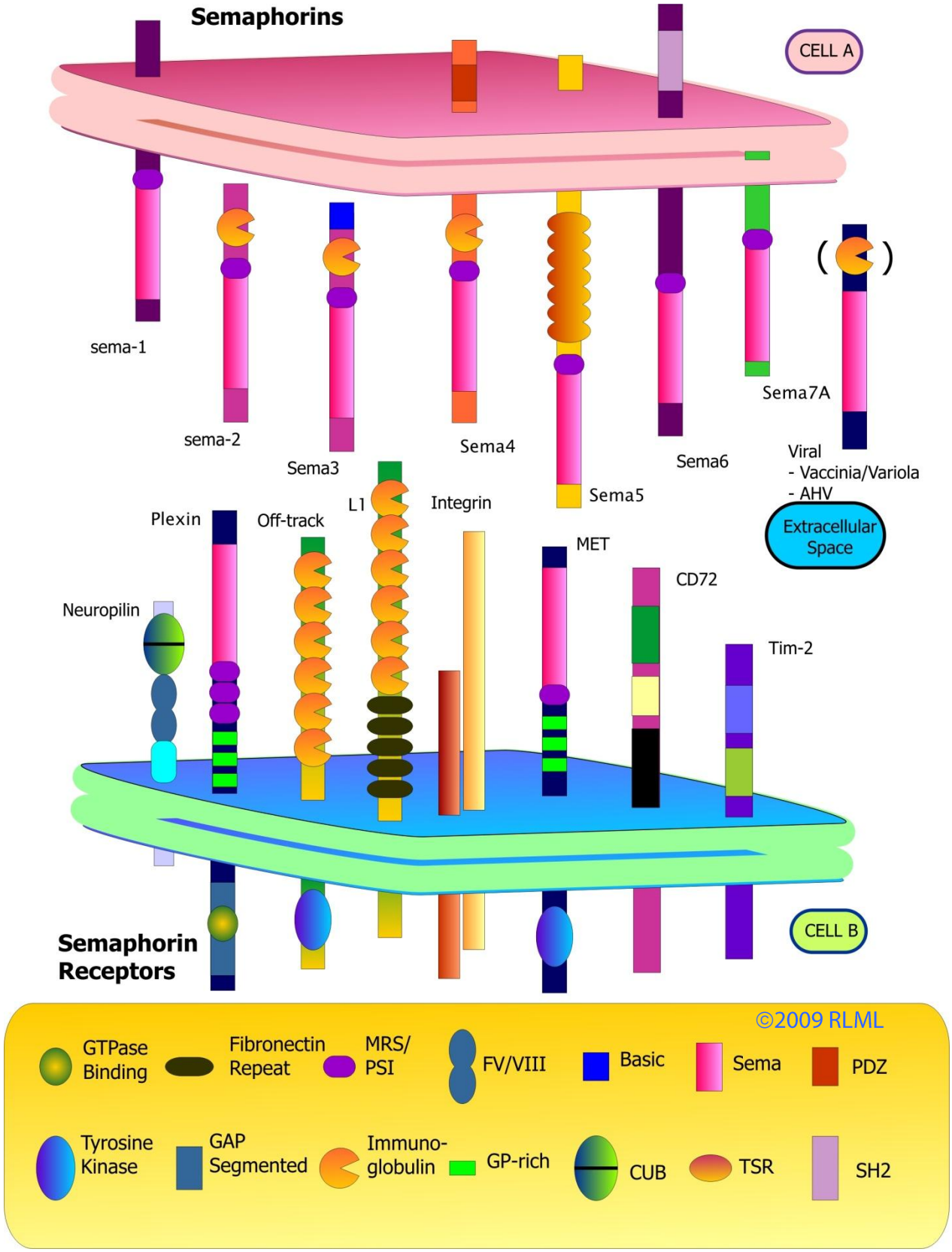
functional activity (Adams et al., 1997; Christensen et al., 2005; Kigel et al., 2008; Varshavasky et al., 2008). As a transmembrane cue, Sema4D (also referred to as CD100) is cleaved by MT1-MMP creating a secretable dimer that interacts with plexinB1 (Elhabazi et al., 2001; Basile et al., 2007). This semaphorin receptor in turn is also cleaved to permit the formation of heterodimers for improved signal transduction (Artigiani et al., 2003). Though most descriptions of the *in vivo* importance of proteolysis of semaphorins result from *in vitro* work, *in situ* studies with organotypic slice cultures are supportive of the claims of (Gonthier et al., 2007; Gonthier et al., 2009).

1.4.2 Semaphorin receptors

1.4.2.1 Neuropilins

The protein A5, now know as neuropilin (NP), labels specific axon tracts in the Xenopus and chick embryos (Takagi et al., 1987; Takagi et al., 1995). Subsequent screens in chick and mouse identified this protein as the obligate receptor for the Sema3A repellent signal (Feiner et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Neuropilin knock-outs are lethal by E12.5 (Kitsukawa et al., 1997), and contain multiple instances of severe axon mis-targeting in developing cortical tracts (Falk et al., 2005; Piper et al., 2009), illustrating a critical developmental role for the receptor. There are two neuropilins that transduce secreted semaphorin signals, whether attractive or repulsive (Kolodkin and Ginty, 1997; Kolodkin et al., 1997; Giger et al., 1998; Takahashi et al., 1998), and each neuropilin is responsible for mediating the effects of multiple secreted semaphorins (Takahashi et al., 1998; Renzi et al.,, 1999), thus explaining the disparity between the severity of the NP-1 knock-out and the

Figure 1.11 The semaphorin family and receptors.
There are 8 classes of semaphorin. Class 1, 4, 5, and 6 are transmembrane, class 2, 3 and viral semaphorins are secreted, and class 7 is GPI-linked to the plasma membrane. Secreted class 3 semaphorins act through a complex that must contain neuropilin and plexin; it may also contain L1, or, in some instances, TAG-1 (not shown here). Transmembrane semaphorins all appear to act through one of the nine plexins. Class 2 semaphorins have an unknown receptor, as do some of the class 4 - 6 semaphorins. Plexin alone may also recruit the scatter factor receptor MET, CD72, or Off-track (in flies). Some semaphorins may also act upon other receptors in the absence of plexin. These include vascular endothelial growth factor receptor (VEGFR; not shown), integrin and Tim-2. Abbreviations in the protein domain legend: MRS: MET-related sequence; PSI: plexin, semaphorin, integrin; GAP: GTPase activating protein; GP-rich: glycine and proline rich domain; CUB: C1r/C19, Uegf, BMP1 - complement-binding (domains a1 and a2); FV/VIII: coagulation factor V and VIII (domains b1 and b2); MAM: meprin, A5, Mu domain; Sema: semaphorin domain; TSR: thrombospondin repeat; PDZ: PSD-95, GLGF, ZO-1 domain; SH2: src-homology type 2 domain.


Semaphorins
CELL A
sema-1
sema-2
Sema3
Sema4
Sema5
Sema6
Sema7A
Viral
- Vaccinia/Variola
- AHV
Extracellular Space
Plexin
Off-track
L1
Integrin
MET
CD72
Tim-2
Neuropilin
CELL B
Semaphorin Receptors
©2009 RLML
GTPase Binding
Fibronectin Repeat
MRS/ PSI
FV/VIII
Basic
Sema
PDZ
Tyrosine Kinase
GAP Segmented
Immuno-globulin
GP-rich
CUB
TSR
SH2

relatively minor axon guidance defects in the non-lethal Sema3A knock-out (Catalano et al., 1998). The intracellular domain of neuropilin has a PDZ binding domain that likely determines the receptor's subcellular localization (Cai and Reed, 1999). Neuropilin, however, has no intracellular motifs capable of downstream signaling; so it was presumed that a co-receptor must be required to enable semaphorin activity (Takagi et al., 1987; Takagi et al., 1995; He and Tessier-Lavigne, 1997). There is no evidence that non-secreted semaphorins interact with neuropilins.

1.4.2.2 Plexins

Other than semaphorins, there are several membrane bound molecules that bear the semaphorin domain, including the HGF receptor MET, the SF-2 receptor RON, and a series of related proteins – SEX, OCT, NOV – which have all come to be called plexins (Maestrini et al., 1996; Artigiani et al., 1999). Having been reclassified, plexins were then first identified as binding proteins for the transmembrane *Drosophila* semaphorin-1a and a secreted pox-virus encoded semaphorin (Comeau et al., 1998; Winberg et al., 1998). It became evident that plexin, a semaphorin binding protein, and neuropilin, the receptor for secreted semaphorins, actually created complexes for the transduction of Sema3A, where binding with neuropilin resulted in their clustering of NP with plexin (Takahashi et al., 1999; Rohm et al., 2000). Plexins also serve as receptors for transmembrane semaphorins without the aid of neuropilins (Tamagnone et al., 1999; Perälä et al., 2005; Suto et al., 2005).

There are nine plexins divided into subfamilies A (1-4), B (1-3), C1, and D1. While they can mediate signaling in other parts of the developing organism (Brown et al., 2001), plexin A

subfamily members are capable of forming receptor complexes with neuropilins (Tamagnone et al., 1999; Cheng et al., 2001; Suto et al., 2003; Perälä et al., 2005; Suto et al., 2005; Spinelli et al., 2007), or of acting alone as transmembrane semaphorin receptors. A type plexins are the most likely receptor for orphan semaphorin ligands in the nervous system, due to their predominant expression in the developing cortical layers, particularly. Plexins from subfamilies B-D have different specificities, often mediating effects within the immune system from class 4 semaphorins and Sema7A.

1.4.2.3 Other Receptors

Although neuropilins was identified as a necessary component of semaphorin signaling, plexins are actually the most commonly used semaphorin receptor and, except in the immune system, it is generally assumed that if a receptor for a semaphorin is unknown, the receptor complex likely contains one of the nine plexins (Tamagnone et al., 1999). In addition to these two prominent receptors, semaphorins receptors may be functionally coupled to other transmembrane proteins. In the fly, plexin interacts with Off-track, a transmembrane protein with a catalytically inactive kinase domain, for the mediation of d-sema-1a repulsion (Winberg et al., 2001).

Occasionally, semaphorins may interact with a completely atypical protein without the necessity of a plexin. For example, in the immune system, Sema4D triggers a plexin B1-dependent activation of the MET scatter factor receptor, though it may interact with CD72 alone (Kumanogoh et al., 2000; Kumanogoh and Kikutani, 2004). Sema4A also has plexin-

independent effects on T cells via Tim-2 (Kumanogoh et al., 2002). Similarly, Sema7A may bind plexinC1 (Scott et al., 2008), but enhances integrin function in a plexin-independent manner also (Pasterkamp et al., 2003; Suzuki et al., 2007). Repulsive semaphorin signaling via NP/plexin complexes leads to integrin inhibition and de-adhesion from a substrate. NP also recruits L1 cell adhesion molecule to a plexin complex, and many of the mechanistic aspects of secreted semaphorin-mediated repulsion rely upon intact L1 function.

On an interesting note, transient axonal guidance molecule (TAG-1, also known as axonin-1 in chick; (Furley et al., 1990; Karagogeos et al., 1991), also interacts with the L1/NP/plexin complex. TAG-1 is normally associated with positive effects on neurite outgrowth, interaxonal adhesion, and neuronal migration (Felsenfeld et al., 1994; Milev et al., 1996; Denaxa et al., 2001; Denaxa et al., 2005; Chatzopoulou et al., 2008; Wolman et al., 2008), largely through interaction and modulation of other membrane proteins (Kuhn et al., 1991; Malhotra et al., 1998). A blockade of TAG-1 with antibodies, or siRNA, results in a loss of semaphorin-mediated repulsion from Sema3A, as well as other unknown repellant cues in the ventral spinal cord (Masuda et al., 2003; Law et al., 2008). Therefore, TAG-1 may well be responsible for mediating the effects of some of the other semaphorins that are expressed as inhibitors in the ventral spinal cord.

1.4.3 Semaphorin receptor signaling

The intracellular domain of plexins bears homology to RasGAPs, though they have no intrinsic GAP activity. Plexin B activity is dependent upon the binding of active Rac1 and leads to

recruitment of PDZ-RhoGEF and leukemia-associated RhoGEF, leading to the activation of Rho A and its downstream effectors of inhibition, ROCK, LIMK, and cofilin (Driessens et al., 2001; Aurandt et al., 2002; Perrot et al., 2002; Swiercz et al., 2002). Other plexin members likely rely upon Rac1, as secreted semaphorin-induced repulsion has been shown to require its activation as well (Jin and Strittmatter, 1997; Fournier et al., 2000; Jurney et al., 2002), although the primary Rho GTPases employed by plexin A have been shown to be Rnd1 and RhoD (Zanata et al., 2002).

Repulsive semaphorin signaling requires the intact behavior of many specific interactions. Upon ligand binding, tyrosine kinases such as Fes, Fyn, Cdk5, and the serine/threonine kinase GSK-3β become released from neuropilin-mediated inhibition to phosphorylate not only plexin A but some of its downstream targets (Mitsui et al., 2002). This includes PAK, which is inactivated upon phosphorylation, thereby promoting LIMK- and cofilin-mediated actin retraction and depolymerization (Maekawa et al., 1999). Redox signaling through flavoprotein monooxygenases and lipoxygenases is also important (Mikule et al., 2002; Suzuki et al., 2002b; Terman et al., 2002; Schmidt et al., 2008), as oxidation of signaling components may modulate their functions and in particular promotes actin depolymerization (Dalle-Donne et al., 2001).

GSK-3β and Cdk5 are known to regulate microtubule dynamics and plus-end MT associations with adhesion complexes. In concert with the inhibitory phosphorylation of collapsing response mediator protein (CRMP; Arimura et al., 2000; Mitsui et al., 2002), which also has microtubule binding and assembly functions (Fukuta et al., 2002), this suggests a direct

regulation of MT downstream of repulsive semaphorin signaling in addition to the effect upon that of F-actin (Eickholt et al., 2002). Attractive semaphorin signaling for secreted semaphorins remains to be elucidated (Pasterkamp and Kolodkin, 2003), although interaction with MET and phosphorylation of plexin B is said to be required for Sema4D-mediated adhesion (Giordano et al., 2003).

Cyclic nucleotides are also major modulators of growth cone responses to semaphorins (Song and Poo, 2001). Though cAMP is employed in other guidance systems, cGMP is the dependent factor in semaphorin signaling, whereby increased cGMP levels through activation of guanylyl cyclases converts Sema3A-induced repulsion into attraction (Song et al., 1998) , and this likely relates to activity of the cGMP-dependent protein kinase G (Polleux et al., 2000). RhoD, one of the Rho GTPases required for Sema3A repulsion, is responsive to a cGMP-responsive GEF and the presence of L1 can reduce cGMP levels to further promote repulsive growth cone responses (Castellani et al., 2000).

1.4.4 Class 5 semaphorins

The fifth class of semaphorins is unique for several reasons. In addition to the conserved semaphorin domain, class 5 molecules bear seven type-1 (completely conserved) and type-1-like (partially conserved) thrombospondin repeats (TSRs; Adams et al., 1996). TSRs are capable of conferring a whole additional complexity to the function of a protein, as they can mediate a host of activities, including: permissive neurite outgrowth (Adams and Tucker, 2000); extracellular matrix carbohydrate and proteoglycan interactions (Krutzsch et al., 1999;

Li et al., 2002); modulation of integrin-based adhesions; enhancement of synaptogenesis (Christopherson et al., 2005), and; binding and activation of transforming growth factor – β1 (TGF- β1) for mitotic cycle arrest and subsequent cellular differentiation (Schultz-Cherry and Murphy-Ullrich, 1993). Secondly, class 5 semaphorins are represented not only in vertebrates (Sema5A and 5B in primates, rodentia, and aves), but in invertebrates as well (sema-5c in *Drosophila;* Yazdani and Terman, 2006). An additional Sema5D protein was said to be found in humans, however, by my analysis, and that of others (Roth et al., 2009), this is a truncated version that is exactly composed of 597aa from the N-terminal of Sema5B and may simply reflect incomplete sequencing or a putative proteolytic fragment.

Semaphorins 5A and 5B (originally called SemF and SemG) were discovered in 1996 by Püschel and colleagues (Adams et al., 1996; Püschel et al., 1996). They showed that SemF has a great deal of mesodermal expression, but in the CNS was expressed in the dorsal neocortical (dorsal pallial) VZ, with more broad expression in ventral pallial regions (Adams et al, 1996; Püschel et al, 1996). Sema5A was subsequently shown to have expression reflecting a role in claustroamygdalar development. Its expression is found in the ventral and ventrolateral pallial VZ, the ventral migratory stream (which is the ventral correspondent of the lateral migratory stream), and the subsequent differentiating field of the ventral basal amygdalae and claustrum (Pineda et al, 2005; Medina et al, 2004). Sema5A has also been implicated in OPC/oligodendrocyte-mediated inhibition of retinal ganglion cell axons along the optic nerve, and interestingly in this system does not vary in its inhibition based upon the presence of other cues that often thought to be modulatory – such as netrin, laminin and L1-CAM

(Goldberg et al, 2004; Oster et al, 2003). However, the original SemF studies did show a high amount of mesodermal expression, the importance of which may be reflected in a knockout where resultant cranio-vascular defects are lethal as early as embryonic day 11 (Fiore et al, 2005). As an axon guidance molecule, Sema5A has been shown to provide both attractive and repellant signals to the fasciculus retroflexus, a tract of axons from the habenular nucleus that must extend between the dorsal and ventral thalamus on a course through the septal nuclei (Kantor et al., 2004). The two roles of Sema5A come from modulation by the thrombospondin repeats in interaction with sulfated proteoglycans in the extracellular matrix (Kantor et al, 2004). Other roles for Sema5A have not been demonstrated.

Sema5B was shown originally to have broader CNS expression, including the VZ and spreading into the SVZ, but rather than restricted to a part of the pallium, this expression is found throughout the extent of the developing neural tube (Adams et al, 1996). Later in development SemG in the spinal cord was additionally found in developing ventral-lateral motor pools (Püschel et al 1996). In the rat telencephalon, Sema5B was found along the lateral migratory stream (in the lateral pallium) to expressing regions in ventrolateral cortex, including piriform cortex (distinguishable by its hippocampus-like three-layered organization rather than the six-layers of the neocortex), insular cortex, and lateral portions of the basal nuclei of the claustroamygdalar complex (Skaliora et al., 1998). Through never suggested, it is plausible that Sema5A and Sema5B cooperate in this aspect of basal nuclei development. However, until the following work was completed, there was no published evidence for a functional role of Sema5B in the developing nervous system.

Table 1 Percent Sequence Identities Between Class 5 Semaphorins

	mSema5A	mSema5B	Human	Amino Acids
mSema3A		35% ID, 55% +ve		772
mSema5A			94% ID, 96% +ve	1074
mSema5B	58% ID, 74% +ve		94% ID, 96% +ve	1093
d-sema5c	36% ID, 53% +ve	38% ID, 41% sema54% +ve		1093

In 2000, a fly homologue (or paralogue) of vertebrate class 5 semaphorins was cloned and described – d-sema-5c (Khare et al, 2000). This class 5 member is only overall 35-40% identical to either Sema5A or Sema5B. However, d-sema-5c is exactly the same size as Sema5B and has conserved domain structure of the class 5 type – i.e. the sema domain followed by a PSI linker and seven type-1 and type-1-like thrombospondin repeats on a single pass transmembrane molecule with a short intracellular domain. The transcript was described as diffuse in early embryonic development (thereby having maternal contributions), with progressive segmented mesodermal expression corresponding with muscle attachment sites, midgut and cardiac development (Khare, et al, 2000). Expression was also seen in the larval head region, though no axon guidance role has been proposed or evaluated. However, it was subsequently determined that d-sema-5c was unnecessary for development as homozygous mutants were not only viable, but normal (Bahri, et al, 2001). The sole elucidated activity of sema-5c to date is a TSR-based binding of the Drosophila relative of TGF-β, *decapentaplegic* (*dpp)*, in its latent, inactive form and its activation through a specific domain present on d-sema-5c. No function has been ascribed to the semaphorin domain.

Class 5 semaphorins are therefore likely to mediate several potentially unrelated activities in nervous system development. The thrombospondin repeats of class 5 semaphorins are evidently biologically and physiologically relevant, conferring functional modulation of both d-sema-5c and Sema5A, and potentially of Sema5B as well. The broad germinal zone expression of Sema5B over this critical neurogenic period of nervous system development points to an important and elaborate functional regulation.

1.5 Corticofugal pathfinding as a model of complex axon guidance

The reciprocal connections between cortex and thalamus are among the longest of the mammalian forebrain, requiring many intermediate targets and guidance decisions (Fig. 1.12). The following describes the development of these projections in the developing mouse. The subplate layer, though transient, is responsible for pioneering the initial projection of descending cortical fibers (McConnell et al., 1989), which are soon followed by the axons of layer 6, then of layer 5, of the newly forming cortical plate. Layer 6 axons project exclusively to the thalamus, while layer 5 axons project to the midbrain, hindbrain, and spinal cord (O'Leary and Koester, 1993). Axons are initially repelled from the cortical plate by the secreted semaphorin, Sema3A (Bagnard et al., 2001), and Slit-1 (Bagri et al, 2002), while being attracted to the lower intermediate zone/upper subventricular zone by Sema3C (Bagnard et al., 1998; Polleux et al., 2000). Simultaneously, medial sources of Slit-2 are thought to be responsible for directing subplate axon outgrowth away from medial structures (Bagri et al., 2002; López-Bendito et al., 2007). Corticofugal axons clearly must not, and do not, enter the germinal zones as they navigate toward the CSB. It was claimed at one point that Sema3A was

mediating this repulsion (Bagnard et al., 2000), but this has since been refuted by the evidence that Sema3A in fact acts from the cortical plate (Polleux et al., 2000; Bagnard et al., 2001). The class 5 semaphorin, Sema5B, is expressed in regions of the cortex and subcortex flanking the projection of and avoided by descending cortical axons. I will show in chapter 2 that the Sema5B expression in the VZ and SVZ is responsible for this corticofugal repulsion.

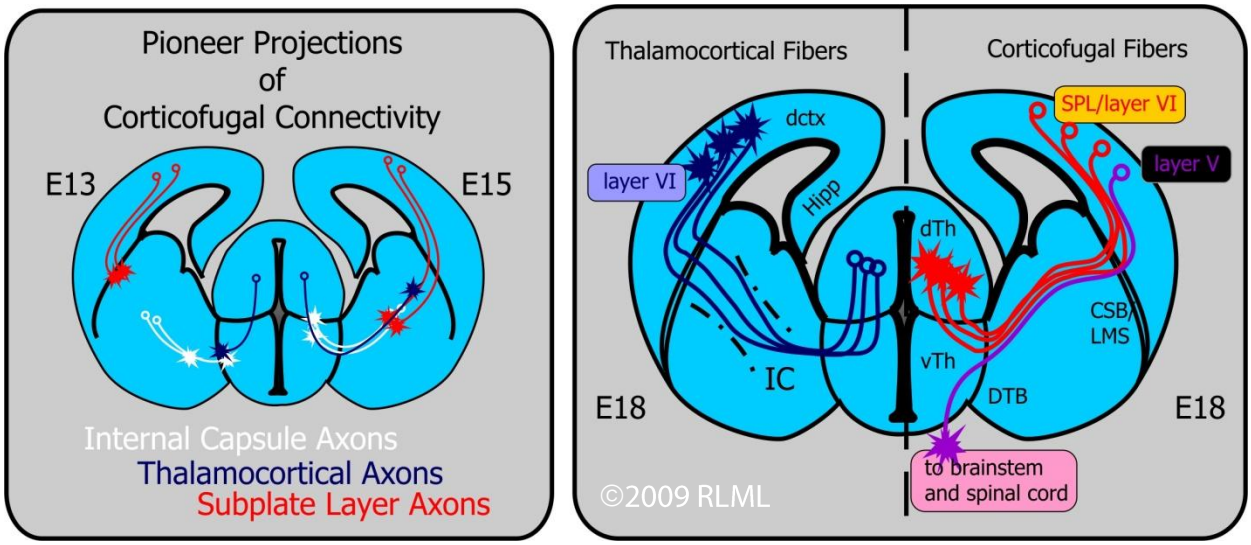


Figure 1.12 Corticofugal and thalamocortical connectivity.
Around E13.5 in the mouse, pioneer axons have been initiated and have encountered their primary intermediate targets. Thalamic and internal capsule axons have reached the diencephalic-subpallial boundary and subplate axons have reached the corticostriatal boundary. LGE cells expressing neuregulin migrate along the internal capsule cells to create a permissive passageway through the MGE which would otherwise be inhibitory for thalamocortical axons. At about E15 cortical axons are attracted by netrin into the LGE, and are also said to follow internal capsule and thalamic axons as a scaffold, projecting first into the ventral, and then the dorsal thalamus. Corticothalamic pathfinding is complete around E18. At this time, cortical axons not designated for the thalamus have turned ventrally at the ventral thalamus and thalamic eminence (not shown - is ventral to vTh) toward the brainstem and spinal cord.

The initial targeting of subplate axons is toward the cortical striatal boundary (CSB). The CSB separates the dorsal telencephalon (i.e. neocortex and ventrolateral cortex) from the ventral telencephalon (Chapouton et al., 2001; Rallu et al., 2002). It is characterized by the expression of Pax6, the loss of which results in defective formation of reciprocal corticothalamic connections (Stoykova et al., 1997; Jones et al., 2002). R-cadherin has been shown to be important in regulating adhesion along, and therefore maintaining the integrity of, the CSB (Inoue et al., 2001). However, there are likely additional factors mediating a boundary-type function for axons and migrating neurons at the CSB (Chapouton et al., 1999; Zaki et al., 2003), and as Sema5B is expressed along this region as well, I will discuss the inhibitory function it might serve along the CSB.

Dorsal cortical axons are the first to reach the CSB around E13.5, pausing approximately 24 hours as axons accumulate (Molnár and Cordery, 1999; Bellion et al., 2003; Bellion and Métin, 2005). Once accumulated, cortical projections coordinately penetrate the lateral ganglionic eminence (LGE), in part due to netrin-1 attraction (Métin et al., 1997; Richards et al., 1997), where they intermingle with thalamocortical axons that are thought to serve as a scaffold for cortical axons as they pathfind to the thalamic reticular nucleus and the dorsal thalamus (Molnár and Blakemore, 1991; Braisted et al., 2000; López-Benito et al., 2006). The presumptive internal capsule passes ventral to the germinal zones of the ganglionic eminences and dorsal to the differentiating field of the striatum and globus pallidus. I will demonstrate as well that these flanking regions express Sema5B, and by expanding these domains ectopically, there is a novel gain of function associated with increased inhibition of

corticofugal axons. The axons of internal capsule neurons, as well as a population of LGE-derived migrating neurons, also serve as a pioneering bridge from within the ganglionic eminences through to the dien-telencephalic boundary (DTB; López-Bendito et al., 2006; Molnár and Cordery, 1999).

The reciprocal pathfinding between the cortex and the thalamus is complete by E18 in the mouse. At this time, corticofugal axons destined to form long range connections, such as the corticospinal tract, have turned ventrally after passing the DTB to extend into the brainstem and are in the midst of a Sema6A-dependent crossover from the ipsilateral medulla to the contralateral spinal cord (Runker et al., 2008). However, the trajectory from the cortical plate to the dien-telencephalic boundary is shared by all corticofugal axons, regardless of final target. Therefore the Sema5B expression to be described in chapter 2 is ideally positioned to regulate inhibition for all descending cortical fibers.

1.6 Thesis hypotheses and objectives

Hypothesis 1: Semaphorin 5B is a necessary inhibitory cue for corticofugal axons.

1.1 Sema5B is an inhibitory cue for cortical and thalamocortical axons

Aim: To determine the effects of recombinant HA-tagged Sema5B on explanted E13 cortical and thalamic neurons in 2-dimensional co-cultures.

1.2 Sema5B is sufficient to create novel domains of inhibition

Aim: To use overlays of Sema5B-expressing cells for the creation of ectopic domains of expression to test the potential gain of function for cortical axons.

1.3 Sema5B is necessary for maintaining endogenous domains of inhibition

Aim: To knock down Sema5B expression in the cortical ventricular zone for the examination of the loss of function on cortical axons.

Hypothesis 2: Semaphorin 5B is proteolytically processed

2.1 Sema5B is processed *in vivo* to generate at least one secreted N-terminal fragment, and C-terminal transmembrane fragment(s).

Aim: To perform western analysis and immune-histochemistry using antibodies to the N- and C-terminals of Sema5B for the verification of multiple Sema5B fragments and differential tissue localization.

2.2 Recombinant HA-tagged Sema5B is also cleaved in heterologous cells in a matrix metalloproteinase-dependent fashion.

Aim: To use western analysis of expressing cell media concentrates to determine the extent of processing recombinant Sema5B, and to disrupt this processing with inhibitors.

2.3 The secreted N-terminal domain of Sema5B is capable of causing collapse and inhibition of cortical axons

Aim: To test the collapsing ability of the Sema5B-expressing cell media upon cultures of dissociated cortical neurons, and to use antibodies to disrupt collapse.

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2. Semaphorin 5B is a novel inhibitory cue for corticofugal axons[1]

2.1 Introduction

The reciprocal connections between cortex and thalamus are among the longest of the mammalian forebrain, requiring many intermediate targets and guidance decisions. The pioneer projections to the thalamus and other subcortical targets are formed by axons of subplate neurons, subsequently followed by axons from layer VI and V of the newly forming cortical plate (Clascá et al., 1995; De Carlos and O'Leary, 1992; McConnell et al., 1989). The initial targeting of subplate axons is toward the cortical striatal boundary (CSB). Axons are repelled from the cortical plate by Sema3A, while being attracted to the lower intermediate zone/upper subventricular zone by the semaphorin, Sema3C (Bagnard et al., 2001; Bagnard et al., 1998; Polleux et al., 2000; Skaliora et al., 1998). Cortical axons also avoid the lower subventricular and ventricular zones (SVZ/VZ), a repulsion originally thought to be mediated by Sema3A (Bagnard et al., 2000), although this remains controversial (Polleux *et al.*, 2000). In addition, a medial to lateral gradient of slit proteins is thought to be responsible for directing subplate axon outgrowth in a lateral direction away from medial cortical structures (Bagri et al., 2002; López-Bendito et al., 2007).

Dorsal cortical axons are the first to reach the CSB around E13.5, pausing approximately 24 hours as axons accumulate (Bellion and Métin, 2005; Bellion et al., 2003; Jacobs et al., 2007; Molnár and Cordery, 1999). Having accumulated, cortical projections coordinately penetrate

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the lateral ganglionic eminence (LGE), in part due to netrin-1 attraction (Métin et al., 1997; Richards et al., 1997), where it has been proposed that they physically interact with thalamocortical axons that may serve as a scaffold for cortical axons as they pathfind to subcortical targets (Braisted et al., 2000; Hevner et al., 2002; López-Bendito et al., 2006; Molnár and Blakemore, 1991). Likewise axons from the cortex are also believed to serve as scaffolding for thalamic axons as they target layer IV (Molnár and Cordery, 1999).

Semaphorins are known to play a role in nervous system development generally (Fiore and Püschel, 2003), and axon guidance specifically (Luo et al., 1995; Nakamura et al., 2000; Püschel, 1996). They are characterized by the presence of a conserved "sema" domain and individual members are grouped into classes based upon other domain specializations (Committee, 1999). The sema domain confers the inhibitory activity of the molecule, whereas additional domains may modulate this activity (Gherardi et al., 2004; Kantor et al., 2004; Koppel et al., 1997). Class 5 semaphorins are transmembrane proteins that in addition to the typically inhibitory semaphorin domain possess 7 thrombospondin repeats (Adams et al., 1996; Püschel et al., 1996). This is intriguing as thrombospondin repeats are permissive for axon outgrowth and can modulate synaptogenesis (Christopherson et al., 2005), suggesting a complex developmental role for class 5 semaphorins (Adams and Tucker, 2000). Indeed Sema5A has been shown to be permissive or inhibitory to axon outgrowth depending on whether matrix proteoglycans are present (Goldberg et al., 2004; Kantor *et al.*, 2004; Oster et al., 2003) and it has been reported from glycosaminoglycan microarray studies that Sema5B

also possesses the ability to interact with both heparan and chondroitin sulfate proteoglycans (Shipp and Hsieh-Wilson, 2007).

Previous observations in the developing rat indicated that Sema5B is expressed in regions of the basal telencephalon coincident with the corticofugal projection into subcortical regions (Skaliora *et al.*, 1998). We were therefore interested in the function of Sema5B during corticofugal development in the mouse. In our examination of the developing mouse cortex, we found that Sema5B expression flanks the presumptive internal capsule during its formation. Through gain-of-function experiments we show that Sema5B is repulsive to cortical axons, but not to dorsal thalamic axons. In addition, using shRNA to knock down Sema5B we show that it is necessary as a source of repulsion for the appropriate guidance of cortical axons away from structures such as the ventricular zone as they navigate toward and within subcortical regions.

2.2 Methods

2.2.1 Animals

Timed pregnant CD1 mice were lethally anesthetized by chloral hydrate injection (0.25mg/kg, i.p.). E10-E18 CD1 mouse embryos were dissected into ice-cold PBS. For protocols requiring fixed tissue, mouse embryos (whole or dissected, depending on their size) were placed in 4% paraformaldehyde (PFA; in PBS, pH 7.0) and rocked at 4°C overnight. Fixed tissue was cryoprotected in 15%, then 30% sucrose/PBS, for at least 4 hours on a rocker at 4°C.

Cryoprotected tissue was then embedded in OCT (TissueTek) and stored at -80°C until sectioning.

2.2.2 Stable cell lines

Mouse semaphorin 5B (Adams *et al.*, 1996; Püschel *et al.*, 1996) was subcloned into pDisplay (Invitrogen) for the purpose of stably expressing Sema5B in cell lines. This full-length semaphorin 5B was transfected into HEK 293 cells with Lipofectamine 2000 (Invitrogen). Clonally derived stable cell lines were maintained with Geneticin (Gibco) selection, in the culture medium (DMEM-F12 Ham supplemented with 5% fetal bovine serum). Expression of Sema5B was confirmed by immunocytochemistry (with and without detergent; Fig 2.3A-C) using a monoclonal antibody (Sigma) against the hemagglutin (HA) tag located at the N-terminus of Sema5B.

2.2.3 *In situ* hybridization

Semaphorin 5B in pGEM-T (Promega) was linearized by restriction enzyme digestion with SacII (antisense direction) or SalI (sense direction), and the probe was transcribed from the SP6 (antisense) and T7 (sense) promoters using the Roche Dig-RNA labeling kit. Probes were boiled at 80°C for 10 minutes to fragment the full-length transcripts, and cooled in ice water for 5 minutes prior to their dilution to 1µg/ml in hybridization solution. Smaller embryos (E10-E13) were processed for wholemount ISH (as per Shen et al, 1997) and animals from E11-E18 were sectioned at 20µm on a cryostat at -20C. In situ hybridization of frozen sections was performed with some minor modifications of the Roche In Situ Hybridization manual (Dijkman et al., 1995).

In situ hybridization on electroporated slice cultures was conducted as per De Lecea et al (1997), with some modifications. In brief, slices were fixed in 4% paraformaldehyde for 48 hours at 4°C, then embedded in grade IV-V bovine albumin (Fisher Scientific), mounted on a solution of grade I-II chicken albumin and gold gelatin, then re-sliced on a Vibratome at speed 5 and amplitude 5 at 50µm for processing as floating sections. Hybridization with digoxygenin-labeled probes proceeded at 60°C in a solution of 50% deionized formamide, 10% dextran sulphate, 5x Denhardt's solution, 0.62M NaCl, 10mM EDTA, 20mM PIPES-Na, 0.2% SDS, 250µg/ml heat-denatured salmon sperm DNA and 250µg/ml heat-denatured yeast tRNA.

2.2.4 *In vitro* co-cultures

E13.5-E14 brains were dissected into neurobasal media containing B27 supplement and 1% pen/strep. The pia mater was removed and the brains were separated into hemispheres. Dorsal cortex, lateral cortex and dorsal thalamus were isolated, cut into small pieces with a scalpel (approximately 0.25mm^2) and left for at least 2 hours at 37°C (5% CO_2) in fully supplemented neurobasal medium (5% FBS, B27, 1% pen/strep, 1mM L-glutamine, 0.065% glucose, $NaCO_3$). Explants were then plated on coverslips previously coated with poly-L-lysine (100µg/ml) and laminin (100ng/ml) and allowed to recover and adhere for 24 hours before vector- (pDisplay) or semaphorin 5B-transfected HEK293 cells were added to explant cultures. Prior to their addition, cells were concentrated by centrifugation and re-suspended in neurobasal medium. These co-cultures were incubated for ~40 hours to permit adequate outgrowth and interaction between axons and HEK293 cells, and then fixed in 4% PFA in PBS

(pH 7.0) at room temperature for 30 minutes. Axons were visualized by the Tuj1 antibody to neuron-specific βIII-tubulin (Covance) and goat-anti-mouse Alexa 586 (Molecular Probes), and entire co-cultures were counterstained for F-actin using phalloidin Alexa 488 (Molecular Probes). Images were captured with a Zeiss Axioplan2 epifluorescent microscope and Northern Eclipse imaging software. Degree of repulsion between axons and cell islands was determined by selecting the area of a cell island, and counting the number of axons or axon bundles that grow toward the cell island, followed by the number of axons that make contact with that cell island. This is demonstrated in figure 3L, which shows cell islands selected under the merged channel, and tuj1 labeling alone, under which channel axon contact with the islands was determined. Cell islands were identified as groupings of HEK293 cells, and were only scored if they were within reach of axon outgrowth from the explant. Growth cone collapse was identified with phalloidin staining as the compaction of actin-based structures into a compact tip (see Fig 2.3M, N). Percent growth cone collapse was determined as the number of collapsed growth cones over the total number of axons growing within close proximity of cell islands. Significance of the main effects of cell type, explant type, or their combined effect upon percent contact and percent collapse was determined by analysis of variance (ANOVA – SPSS).

2.2.5 Organotypic slice cultures

In order to assay the affects of Sema5B on developing tracts of the telencephalon, organotypic slice cultures were employed, as per Marin et al (2001) and Flames et al (2004), with some modifications. Briefly, E14 brains were embedded in bacterial-grade agar (Fisher),

sectioned at a 45 degree angle into 250µm slices, and placed on polycarbonate organotypic culture supports (BD) that fit into a 6-well plate. Only those slices corresponding to regions of the dorsal cortex that project to the internal capsule were utilized. Slices were cultured for at least two hours in DMEM-F12 Ham (Sigma), supplemented with 5% heat-inactivated FBS, N2 (Gibco), 0.1% pen/strep, 50mM L-glutamine, 0.06% D-glucose, 50µg/ml gentamicin. This media was replaced with neurobasal medium, supplemented with B27 (Gibco), 0.1% pen/strep, 2mM L-glutamine, 0.06% D-glucose, 50µg/ml gentamicin. Descending projections in both hemispheres of a slice were labeled with a crystal of 1,1',dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes). Confluent dishes of Sema5B-expressing or control HEK293 cells were labeled with cell tracker green (Molecular Probes), and centrifuged into a pellet. The pellet was washed twice with DMEM, and centrifuged a final time at 4000rpm for 5 minutes. Approximately 0.5µl of condensed labeled cells was pipetted ipsilaterally directly onto the slice over the ganglionic eminences to cover the route of the internal capsule. Slices were cultured with the cells for approximately 40 hours, and examined with an inverted Nikon fluorescence microscope. DiI and cell tracker green images were captured in the exact same field using Metaview digital imaging software, and then merged into a single red/green image using Adobe Photoshop. To quantify the effects of Sema5B on corticothalamic axon pathfinding in slice cultures, merged images of individual organotypic slices (examples of which are seen in Fig 2.4) were assessed and grouped as normal or abnormal using the following criteria. Abnormal axon growth included growth into inappropriate cortical regions (ventrolateral cortex), and halting of outgrowth at the border of HEK293 cells compared to that of untreated and control slice cultures. One

image was taken per organotypic slice and slices were pooled by experiment and the number of slices displaying abnormal pathfinding was compared to the total number of slices to produce a percent error value. Significance of the differences in error rate was determined statistically using t-test for independent means (SPSS). An FV1000 confocal microscope was used to image examples of both control and experimental slices. To assess the guidance of thalamocortical axons in slice culture, the DiI label was instead placed in the dorsal thalamus; cells were labeled with cell tracker blue. Slices were cultured for 4 days prior to fixation to ensure growth of the thalamocortical fibres into the cortex, then mounted in Mowiol (Sigma) and imaged with a FV1000 confocal microscope. Figure 2.5F and G show organotypic slices under a single channel to clearly visualize the DiI labeled fibres. The area of cell placement was circumscribed by a green outline.

2.2.6 Preparation of shRNA vectors and validation

pSico oligomaker v1.5 software was used to scan the mouse semaphorin 5B gene for sequences ideal for RNA interference, and to design the oligoduplex palindromes required for hairpin loop formation with the XhoI overhangs necessary for insertion into pLentilox 3.7 (Reynolds et al., 2004). The following sequences were targeted: shRNA1 (561) = 5′-GGACTATTGAGAAGATC AA; shRNA2 (959) = 5′-GAGGTCCCCTTCTACTATA; shRNA3 (1664) = 5′-GAAGACAGTT CCAACATGA. One targeted sequence generated an incorrect final insert sequence with no homology to Sema5B and was therefore used as a scrambled control. A second control was designed toward the luciferase gene, which has no sequence similarity to anything within the mouse genome. Oligoduplex palindromes were cloned into the

XhoI/HpaI restriction sites of pLentilox 3.7, which contains an IRES GFP sequence downstream of the cloning site and confers ampicillin resistance. Resultant shRNA vectors were tested for their ability to knock down Sema5B expression by Lipofectamine transfection into our HEK293 cells stably expressing HA-tagged Sema5B. Cells were then analyzed for reduction of Sema5B expression by staining for HA and imaging for GFP, which identifies the shRNA-transfected cells. Cells positive for GFP but negative for HA were considered to be knocked down. By immunohistochemistry, knock down of Sema5B was quantified by determining the proportion of total cells that were both HA and GFP positive – the greater the proportion, the less the efficacy of knock down. Knock down was also confirmed by western blot for HA and GFP with a β-actin loading control.

2.2.7 *Ex vivo* electroporation

Ex vivo electroporation of E13 mouse embryos was performed as per Hand et al (2005) with the following modifications: embryos were not decapitated prior to electroporation. 1µl of trypan blue/DNA (2.24µg/µl) was injected unilaterally and electroporation parameters were 5 unipolar pulses of 55V of 50ms duration at 500ms intervals. The paddles were placed such that DNA would be taken into the lateral ventricular zone of the neocortex, including both dorsal and lateral pallium, especially the corticostriatal boundary. Brains were removed, embedded in 3% agar, sliced on a Vibratome 1500 at ~300µm and cultured for 24-30 hours prior to fixation (Polleux and Ghosh, 2002). Culture media are described under "organotypic slice culture". Slices were imaged using both epifluorescence (Zeiss Axioplan2, Northern Eclipse) and confocal (FV1000) microscopy. Guidance errors were quantified in two ways. The

first was by counting the number of axons within a given GFP-positive region per each individual slice, to give the average number of axons misprojecting into the ventricular zone under each condition. Controls were pooled and compared against both shRNA1 and shRNA3. shRNA2 was only used in the initial testing of the knock down that was performed under different parameters, and therefore could not be included in these measures. A second method of quantification pooled each experiment to allow determination of the percentages of slices within a given experiment that displayed errors in pathfinding – defined as >10 DiI-labeled cortical axons aberrantly penetrating the GFP-positive VZ. Variance of each value was calculated as standard error of the mean. Significance of the knock down effect was determined by analysis of variance (ANOVA-SPSS).

2.3 Results

Previous studies examining early expression of semaphorins in the nervous system have suggested that Sema5B may play a role in telencephalic development (Adams *et al.*, 1996; Skaliora *et al.*, 1998). To explore its potential functions we first examined Sema5B expression throughout cortical development, and particularly during the establishment of telencephalic connectivity.

2.3.1 Semaphorin 5B expression during telencephalic development

Sema5B is expressed throughout the entire embryonic neuraxis as early as E10.5. When hybridization was performed in wholemount embryos (Fig 2.1A-C), it was apparent that the pattern of expression was greatest in the CNS and levels of expression in other tissues were

below detection. At E10.5 Sema5B appeared to be expressed homogenously throughout the CNS and on closer examination of cryosections, cells expressing Sema5B were found spanning the width of the neuroepithelium (Fig 2.1D). Ventricular Sema5B expression was retained along the length of the CNS at E12.0 but appeared to be stronger and more defined within the forebrain (Fig 2.1B). Examinations of cryosections at this stage revealed that Sema5B was expressed in the ventricular zone where the cell bodies of radial glia are located, but not in the emerging preplate (Fig 2.1E, black arrowheads). Expression was also seen in the ventricular zones of the ganglionic eminences and the septum, which appeared as a stripe of increased staining in the forebrain of the wholemount preparations (Fig 2.1B, arrow). At E13.5, Sema5B signal along the spinal cord had diminished (Fig 2.1C and data not shown), and the emergence of expression in the developing olfactory bulbs (Fig 2.1C, black arrowhead; Fig. 1F, black arrow) and ventrolateral cortex was observed (Fig 2.1C). In sagittal sections, strong Sema5B expression was apparent within the ventricular zones of the ganglionic eminences and septum, and the developing hippocampus (Fig 2.1F, white asterisk), whereas little expression was detected in the emerging cortical plate (Fig 2.1F, arrowheads).

Examination of coronal sections of E14.5 brains showed strong labeling of Sema5B throughout the entire rostral-caudal extent of the ventricular zone, with a slight reduction in intensity at the medial and caudal hippocampal ventricular zone (Fig 2.2). Rostrally, the olfactory bulb mantle and the ventricular zone exhibit robust Sema5B expression (Fig 2.2A, B).

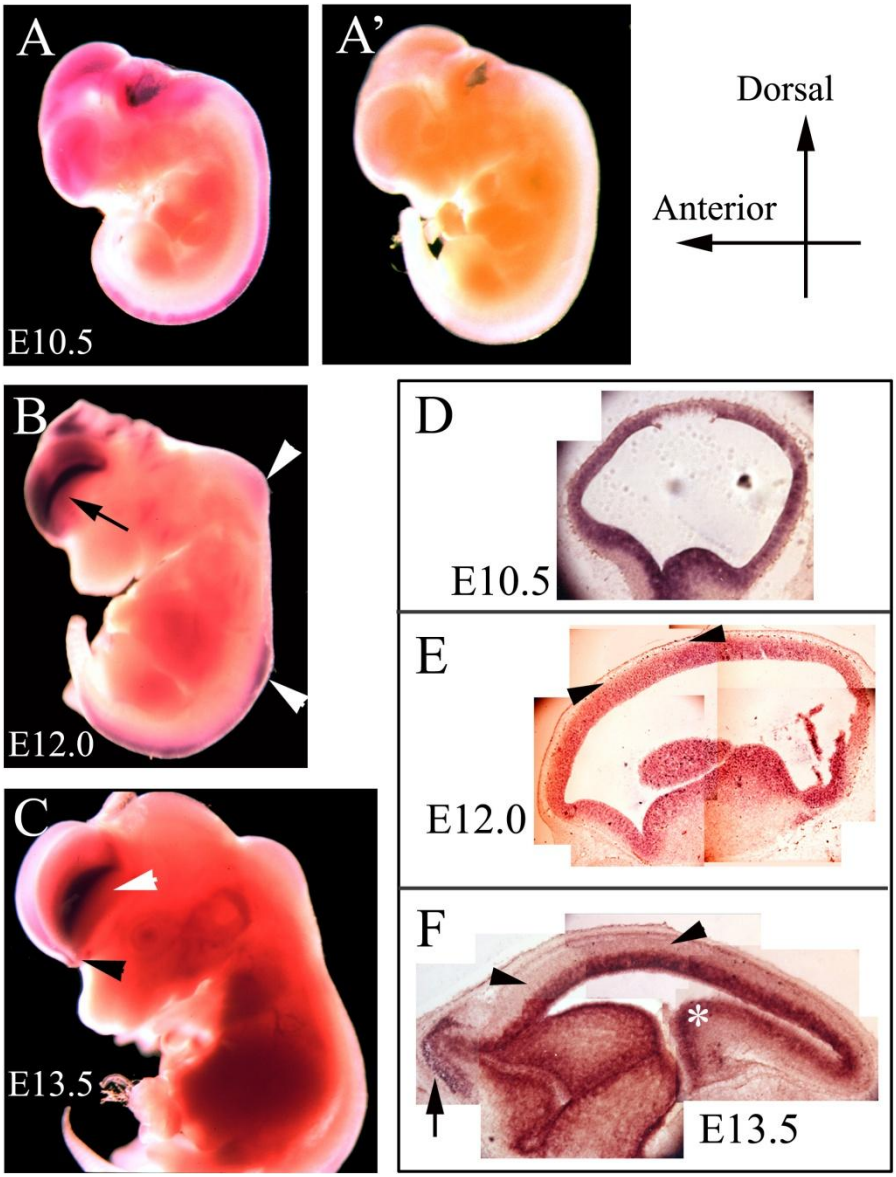


Figure 2.1 Early embryonic expression of Sema5B. A) At E10.5 expression is evident along the length of the neuraxis. A') Sense control at E10.5. B) Expression of Sema5B at E12.0. The ganglionic eminences (GE) can be seen through the cortical wall as a dense stripe of label (black arrow) and expression remains fairly robust within the spinal cord (arrowheads). C) At E13.5, in addition to the GE, there are patches of staining along the ventrolateral aspect of the telencephalon, best seen caudally (white arrowhead), and a developing layer of staining in the newly emerging olfactory bulbs (black arrowhead). D-F) In situ hybridization labeling on sagittal sections corresponding to ages of the embryos in A-C. The ventricular epithelium is intensely labeled while the developing cortical plate is negative (black arrowheads; E, F). Expression in the ventricular zone of the hippocampus (white asterisk; F) and within the developing olfactory bulb is also evident (black arrow; F).

By this stage, regions of Sema5B expression have expanded to include the piriform cortex, particularly in the vicinity of the lateral olfactory tract, which is surrounded by Sema5B expression (Fig 2.2B, C), the subventricular zone (SVZ) of the medial ganglionic eminence (MGE), and the differentiating field of the MGE-derived globus pallidus (Fig 2.2C, D), and the

dentate migratory path in the hippocampus (dmp; Fig 2.2E). In the ventrolateral regions of the telencephalon, Sema5B was expressed in the endopiriform nucleus (En), the basolateral amygdalar complex (BLC), in addition to the expression in the piriform and insular cortices (Fig 2.2D, E). Also, some Sema5B expression was apparent along several boundary regions of the forebrain; the lateral migratory stream (or cortical striatal boundary (CSB); arrows in Fig 2.2C-E), the border between the LGE and MGE (Fig 2.2C), and the thalamic reticular nucleus at the border between the dorsal and ventral thalamus (Fig 2.2E).

A similar pattern of expression was maintained over the period of cortical neurogenesis (E13.5 until E16.5), but by E17 Sema5B expression in the dorsal cortex was sharply downregulated to a very restricted region at the ventricular zone. Sema5B continued to be expressed robustly in ventrolateral cortex (including the piriform cortex) and hippocampus, at diminished levels in the olfactory bulb, and was generally no longer expressed in any of the subcortical regions or migratory streams as seen during neurogenesis. At postnatal ages Sema5B expression is only maintained in the hippocampus and piriform cortex (data not shown). At the time corresponding to the establishment of connectivity between the cortex and thalamus, Sema5B was highly expressed in regions of the dorsal and ventral telencephalon that are avoided by corticothalamic axons, and largely absent from the route of the internal capsule (between the arrowheads; Fig 2.2D, G). This suggested a possible role for Sema5B in corticothalamic axon guidance through the ventral telencephalon.

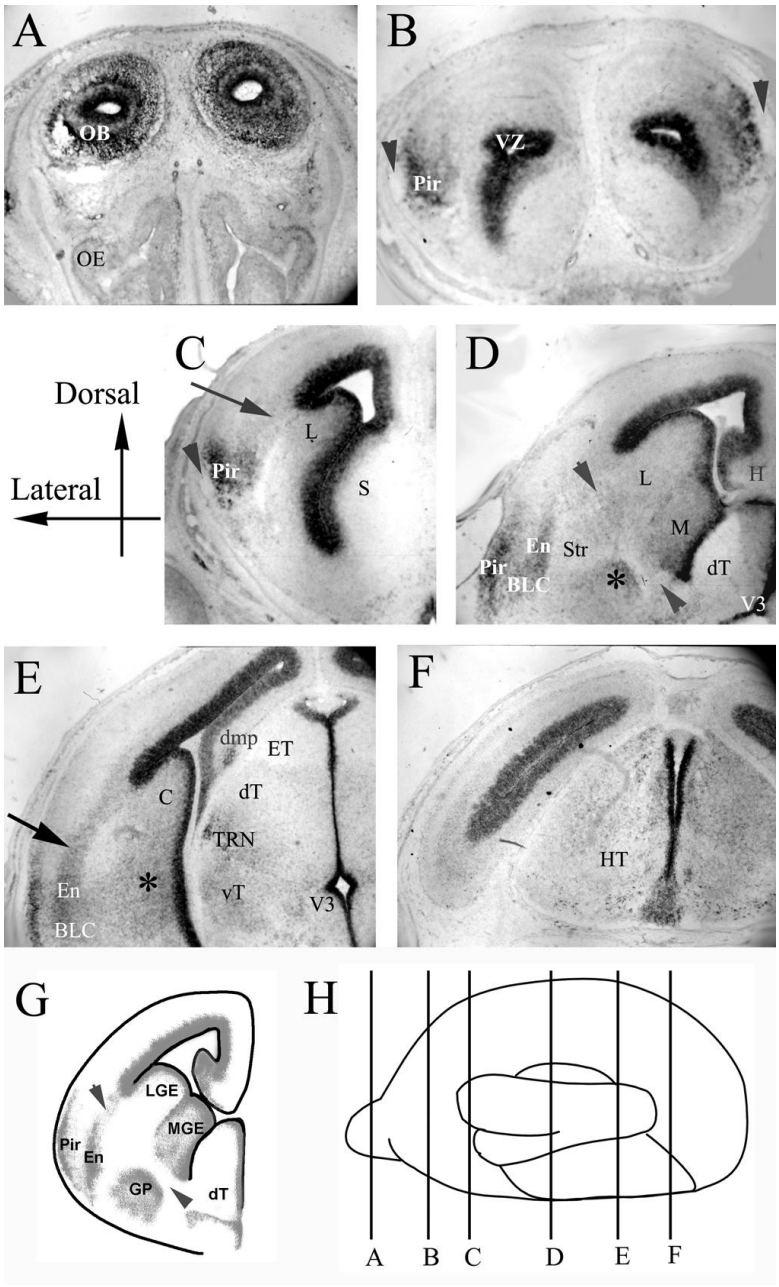


Figure 2.2 Sema5B is expressed in regions of the telencephalon avoided by corticothalamic axons. (A-F) Rostral to caudal series of coronal cryosections of a representative E14.5 brain. A) Sema5B expression is evident in the VZ and a layer within the mantle of olfactory bulbs (OB); while there is little expression in the olfactory epithelium (OE). B) Sema5B expression is evident in the VZ of anterior forebrain, and the piriform cortex (Pir). The lateral olfactory tract can be seen surrounded by Sema5B label (arrowheads in B and C). C) Sema5B expression can be seen in the VZ of the dorsal cortex, lateral ganglionic eminence (L), septum (S) and piriform cortex. D) Expression of Sema5B is also apparent in the thalamic VZ (at V3 – the third ventricle) and hippocampus (H). Also labeled is the piriform cortex (Pir), the endopiriform nucleus (En), basolateral portions of the amygdalar complex (BLC in D, E), the lateral migratory stream/corticostriatal boundary (arrow in C and E), subventricular zone (SVZ) of the medial GE (M), and the globus pallidus (*). Of note is the lack of Sema5B expression in a corridor between the SVZ of the MGE and the globus pallidus (arrowheads). This corridor connects the dorsal thalamus (dT) with the differentiating field of the striatum (Str), neither of which express Sema5B. E) LMS/CSB (arrow) labeling is more evident, the ventrolateral label continues, as well as the caudal (c) GE and caudal medial GE (*) and the migratory path of the dentate gyrus (dmp) and the thalamic reticular nucleus (TRN). F) Cortical VZ label appears less intense than at rostral levels and strong VZ expression continues caudally through the brainstem toward the spinal cord. G) Schematic of Sema5B expression as seen in D. Arrowheads are to indicate the routes of the internal capsule which lacks Sema5B expression. H) Schematic illustrating the approximate rostral-caudal location of coronal sections shown in A-F.

2.3.2 Sema5B is repulsive to cortical projections in vitro

Pioneer axons extending from the cortical plate follow a trajectory that avoids and/or pauses at several Sema5B-expressing regions in the developing telencephalon. First they avoid the strongly Sema5B-expressing neocortical subventricular and ventricular zones, and stop short of the ventrolateral cortical domains that also express Sema5B. When they reach the CSB, a region where a subset of cells express Sema5B (Fig 2.2C-E), axons from the dorsal cortex pause until more lateral cortical axons arrive (Bellion and Métin, 2005; Bellion *et al.*, 2003; Molnár and Cordery, 1999). Cortical axons then coordinately extend through subcortical regions bordered by Sema5B expression in the MGE and its derivative, the globus pallidus (Fig 2.2C, D). We therefore sought to determine whether Sema5B might constrain corticothalamic axons to their correct projection by functioning as an inhibitory cue to cortical axons. In addition, because dorsal cortical axons pause at the CSB for longer than lateral cortical axons, we wished to separately examine dorsal and lateral cortical axons to determine whether there was a differential response to Sema5B depending on the location of the cortical axon origin.

Dorsal and lateral cortical explants from an early stage of corticofugal development (E13.5) were cultured in the presence of Sema5B-expressing or control HEK293 cells. Axons growing from dorsal cortical explants displayed a marked avoidance of Sema5B-expressing cell islands (Fig 2.3C, D, O; 24.7% contact ± 2.6 SEM, n=102 cells islands surrounding 28 explants) compared with those that encountered vector-only control cells (Fig 2.3A, B, O) creating a "halo" of axons around a group of cells. Of the axons that were growing toward control cell

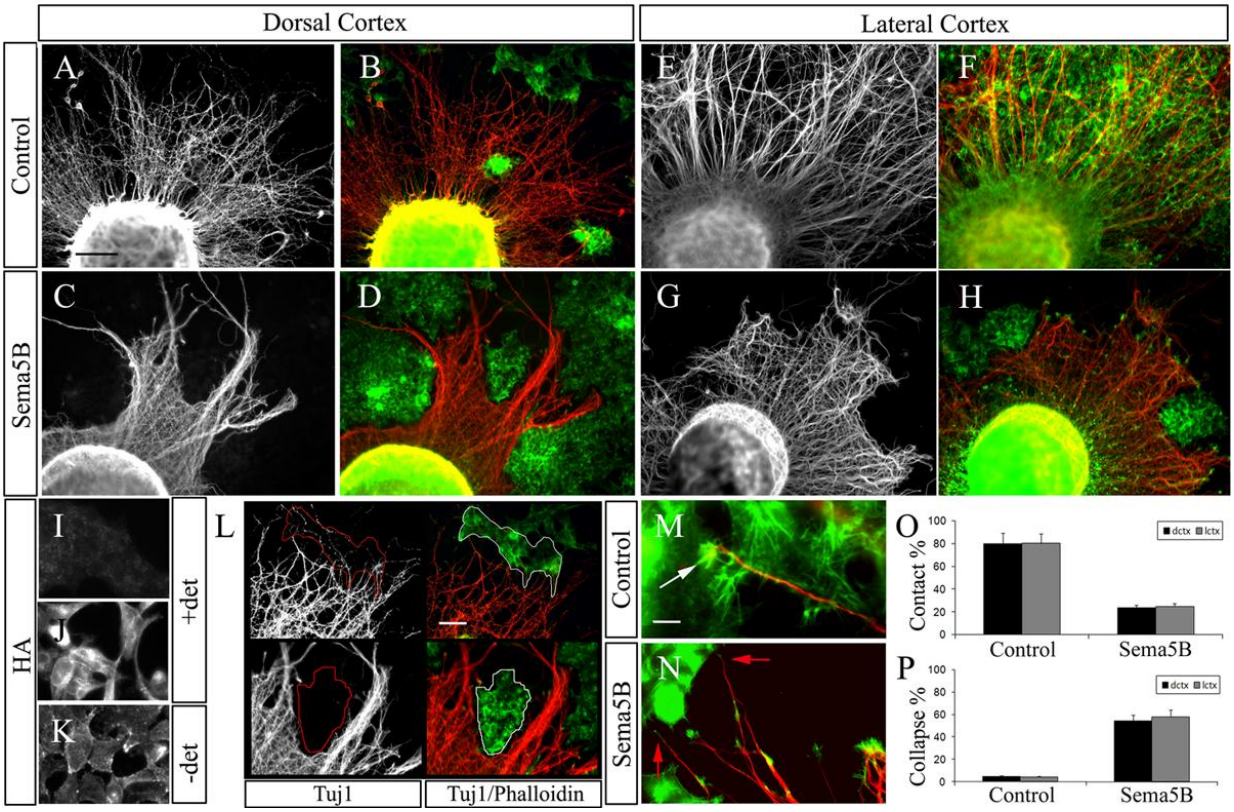


Figure 2.3 Dorsal and lateral cortical axons avoid Sema5B-expressing cells in vitro. A-D) E13.5 dorsal cortical explants cultured with control HEK293 cells (A, B) or Sema5B-expressing cells (C, D), quantified as percent of axon contact with cell islands (±SEM, black bars in O). A, C) Tuj1-labeled axons. B, D) Overlay of tuj1 (red) and phalloidin (green), showing axonal contact or avoidance of cell islands. E-H) E13.5 laterally-derived cortical explants cultured with vector-only-transfected cells (E, F) or Sema5B-expressing cells (G, H), quantified as percent of axon contact with cell islands (±SEM, grey bars in O). E, G) Tuj1- labeled axons. F, H) Merge of tuj1 (red) and phalloidin (green), showing axonal contact or avoidance of cell islands. I) Control cells stained for the HA tag. J) Immunocytochemistry for HA-Sema5B cells in the presence of detergent. K) Immunocytochemistry for HA on Sema5B-transfected cells without detergent, demonstrating that HA-Sema5B reaches the plasma membrane. L) Images representing how quantification in O was obtained. Upper two panels are higher magnification images of A and B; lower two panels are higher magnification images of C and D. A cell island would be identified (outlined in white) within an image in the RGB channel. Switching to the red channel permitted clear assessment of axons approaching and contacting or avoiding cell islands (within the red outline). M, N, P) Higher magnification images depict the morphology of growth cones as they approach control (M) or Sema5B-expressing cells (N); quantified for both dorsal (black bars) and lateral (grey bars) cortical explants as percent of growth cones collapsed contact with cells (±SEM; P). Growth cones growing on control cells are generally not collapsed (M; white arrow), whereas growth cones in contact with Sema5B-expressing cells are frequently collapsed (N; red arrows). In merged images, explant cell bodies may appear yellow. Scale bar in A represents 100µm for panels A-H. Scale bar in upper right panel of L represents 50µm for all panels. Scale bar in M represents 25µm for both M and N.

islands, 79.8% ± 9.5 (SEM) grew into or upon islands (n=72 islands, 26 explants; ANOVA: main effect of cell type, p>0.001). Similarly, axons from lateral cortical explants displayed a significant avoidance of Sema5B-expressing cells (Fig 2.3G, H, O) compared with those grown with control cells (Fig 2.3E, F, O). Lateral cortical axons contacted and grew upon control cells (80.4% ± 8.5 SEM; n=91 islands, 38 explants), whereas approximately 75% avoided contact with Sema5B-expressing cells (24.7% contact ± 2.6 SEM; n=92 islands, 21 explants; ANOVA: main effect of cell type, p<0.001). The expression of Sema5B at the plasma membrane of HEK293 cells was confirmed by labeling for HA with and without detergent (Fig 2.3J, K; vector only-transfected cells to do not express the HA tag (Fig 2.3I)). Sema5B was therefore inhibitory to axon growth upon heterologous cells, but no increase in inhibitory response was seen with dorsally-derived axons compared with laterally-derived axons.

Growth cones are enriched in polymerized bundles of actin and a dense actin filament network, which underlie filopodia and lamellipodia, respectively. When a growth cone encounters an inhibitory cue, part of the repulsive response is to disassemble and collapse these actin-based structures into a compact tip. Approximately 54.25% ± 5.40 (SEM) of the dorsal cortical growth cones were collapsed at the border of Sema5B cell islands (Fig 2.3N; n=102 islands, 28 explants; graph Fig 2.3P; red arrows highlight compact actin at axon tips) while significantly less collapse occurred in growth cones approaching control cells (4.98% ± 0.59 SEM; Fig 2.3M white arrow; graph, Fig 2.3P; ANOVA: main effect of cell type, p<0.001). Growth cones of laterally-derived cortical explants contacting Sema5B cells were similarly significantly more collapsed (58.11% collapse ± 6.09 SEM; n=92 islands, 21 explants)

compared with control conditions (4.39% ± 0.46 SEM; n=91 islands, 38 explants), at a similar rate to dorsal growth cones (Fig 2.3P).

Although qualitatively the avoidance of Sema5B cell islands by dorsal cortical axons appeared more marked, quantitative comparison between the two types of explants encountering either cell type revealed no statistically significant difference for percent contact (Fig 2.3O, ANOVA: no main effect of explant type, p=0.748; no effect of interaction between cell type and explant type, p=0.933), or for percent growth cone collapse (Fig 2.3P, ANOVA: no main effect of explant type, p=0.458; no effect of interaction between cell type and explant type, p=0.627). Sema5B is significantly repulsive to all cortical axons and not specifically more inhibitory to dorsal cortical axons.

2.3.3 Sema5B is repulsive to descending cortical projections within organotypic slices

Sema5B is expressed in a number of regions along the pathway navigated by corticofugal fibers, suggesting a guidance role for Sema5B in establishing this tract. Having shown that Sema5B is avoided by cortical axons *in vitro*, we hypothesized that Sema5B expression in the cortical SVZ/VZ, ventrolateral cortices, claustroamygdaloid complex, and parts of the emergent basal ganglia may serve to inhibit axons from invading these areas during pathfinding. To test this, organotypic slice cultures from E14.5 mice were employed to determine whether Sema5B can perturb the pathfinding of descending cortical axons in their normal environment.

Sema5B-expressing or control cells were placed directly on slices along the pathway normally established by cortical axons in the subcortex. E14.5/15 brains were chosen in order that axons would have already encountered the CSB (which does express Sema5B at lower levels) and would have begun penetrating the LGE. In this manner, we ensured that any effect we observe was due to the exogenous Sema5B added. Corticofugal axons were unaffected by the presence of control cells, growing in a normal trajectory via the prospective internal capsule (Fig 2.4D-F, L). In contrast, descending axons grown in the presence of Sema5B-expressing cells did not grow along their normal trajectory (Fig 2.4G-K, M); they instead avoided entering regions where the cells were added at the border of the bolus, regardless of proximity to endogenous histogenic boundaries. A variety of repulsive behaviors were seen among these axons. Most slices had axons that ceased growing in the region of cells expressing Sema5B (Fig 2.4G-I, K), whereas some axons turned away and began to grow inappropriately towards lateral areas (Fig 2.4J, M). Confocal microscopy further demonstrates that DiI-labeled cortical axons grow normally into the region overlaid with control cells (red arrows, Fig 2.4L; merged in L'), whereas those encountering Sema5B-expressing cells make deviations from normal pathfinding (red arrowheads, Fig 2.4M; merged in M'). Overall, a significant proportion of slices (76.8% ±4.4 SEM; n=97 slice cultures) contained abnormal cortical projections in the presence of Sema5B-expressing cells compared with 19.4% (±5.4 SEM; n=90 slice cultures) of controls (Fig 2.4N; t-test of independent means, $p<0.001$).

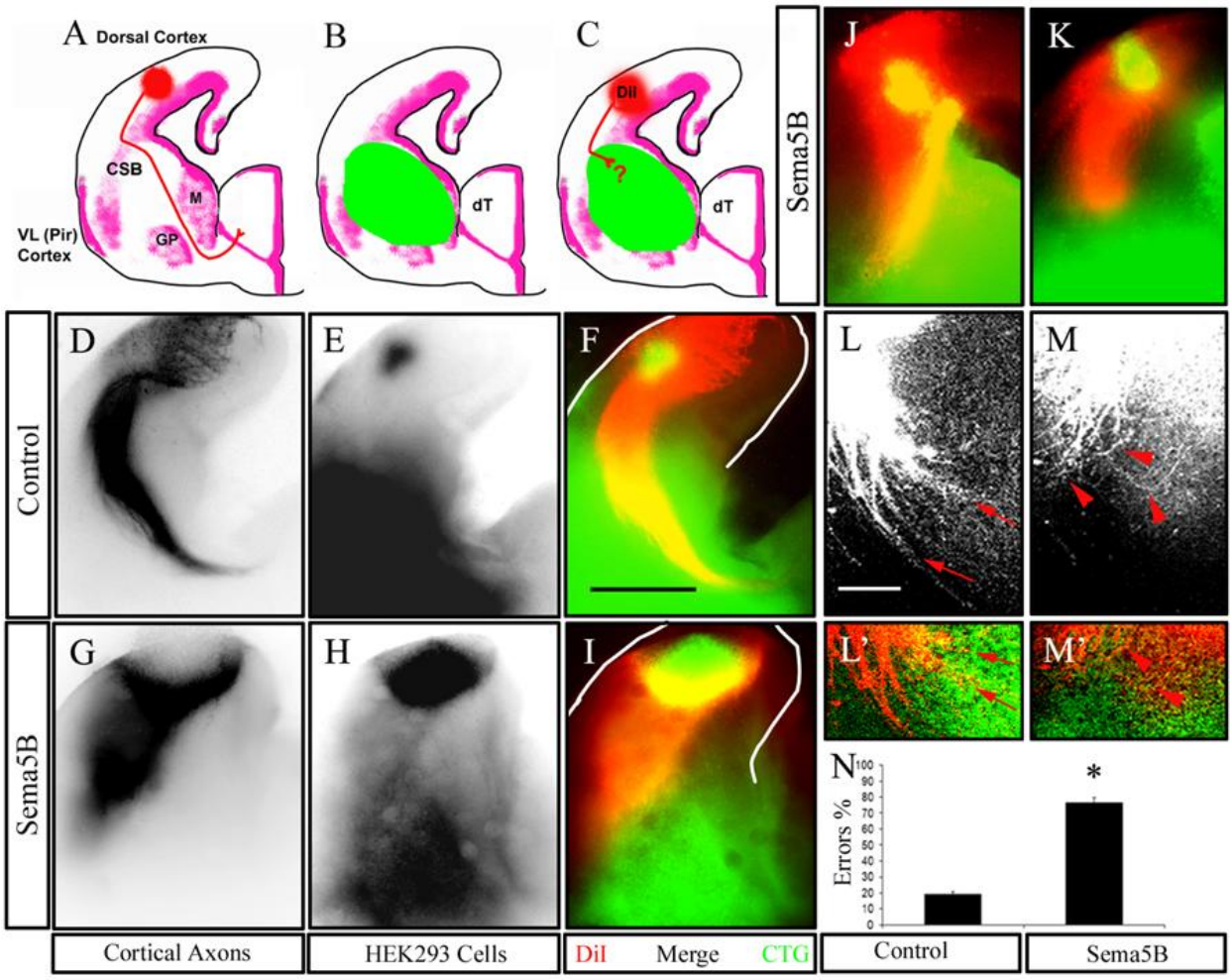


Figure 2.4 Ectopic Sema5B is sufficient to cause aberrant pathfinding of CTAs.
A-C) A schematic illustration of the experimental paradigm used to test the function of Sema5B in situ. A) The normal projection of DiI labeled cortical axons from the dorsal cortex (red) and the endogenous expression of Sema5B (pink) is shown. B) An illustration of the approximate placement of the labeled HEK293 cells (green) placed on the subcortical region of an organotypic slice (green). C) An example of a possible outcome if the Sema5B-expressing cells are inhibitory to the cortifugal axons. D, E) Inverted fluorescent images of DiI-labeled cortical axons (D) and transplanted cells (E) in a control slice. The strong DiI label bled into the green channel, appearing as a spot in the dorsal cortex in E, H, K and M. F) Merge of D and E showing that corticofugal axons extend normally. G, H) Inverted fluorescent images of DiI-labeled cortical axons (G) and Sema5B-expressing cells (H) transplanted in an experimental slice. I) Merge of G and H showing that corticofugal axons avoid the region of exogenous Sema5B-expressing cells. J, K) Two additional examples of exogenous Sema5B-induced guidance errors. J) An example of neurite extension toward inappropriate ventrolateral structures. K) An example of outgrowth arrest at the border of Sema5B-expressing cells. L) Confocal image of DiI-labeled cortical axons pathfinding normally (red arrows) into a region overlaid with control HEK293 cells. L') Merge of confocal image from L (red) and cell tracker labeled control cells (green). M) Confocal image of DiI-labeled cortical axons deviating from normal pathfinding (red arrowheads) in the region of Sema5B-

expressing HEK293 cells. M') Merge of confocal image from M (red) and cell tracker labeled Sema5B-expressing cells (green). N) Guidance errors were quantified by determining the proportion of slices per experiment binned as "aberrant" (± SEM; see also Methods). Scale bar in F represents 500µm for panels D-K. Scale bar in L represents 150µm for L-M'.

2.3.4 Sema5B is not repulsive to the dorsal thalamocortical projection

Sema5B is expressed within specific ventrolateral regions, such as piriform cortex, endopiriform nucleus and basolateral portions of the claustroamygdaloid complex, and subcortical regions including the VZ of the LGE, throughout the MGE and its derivative, the globus pallidus. As we have demonstrated that Sema5B is inhibitory to descending cortical axons, it was attractive to consider that Sema5B may be the unknown inhibitory cue expressed in MGE and MGE-derived territories which have been found to block thalamocortical axon invasion of the subcortex (López-Bendito *et al.*, 2006). As with cortex, explants of E13.5 dorsal thalamus were co-cultured in vitro with Sema5B-expressing or control HEK293 cells. Compared to control (Fig 2.5A, B, E; 77.3% contact ± 1.4 SEM, n=41), axons derived from thalamic explants had no increase in avoidance of Sema5B-expressing cells (Fig 2.5C, D, E; 77.5% contact ± 1.3 SEM, n=47; t-test for equality of means, $p<0.001$). To further examine whether dorsal thalamic axons were truly non-responsive to Sema5B, we made E13.5/14 organotypic slice cultures, labeling the dorsal thalamus with DiI. Aggregates of cell tracker-labeled control or Sema5B-expressing HEK293 cells (shown outlined in green in Fig 2.5F and G, respectively) were then placed as previously over the presumptive internal capsule within the subcortex.

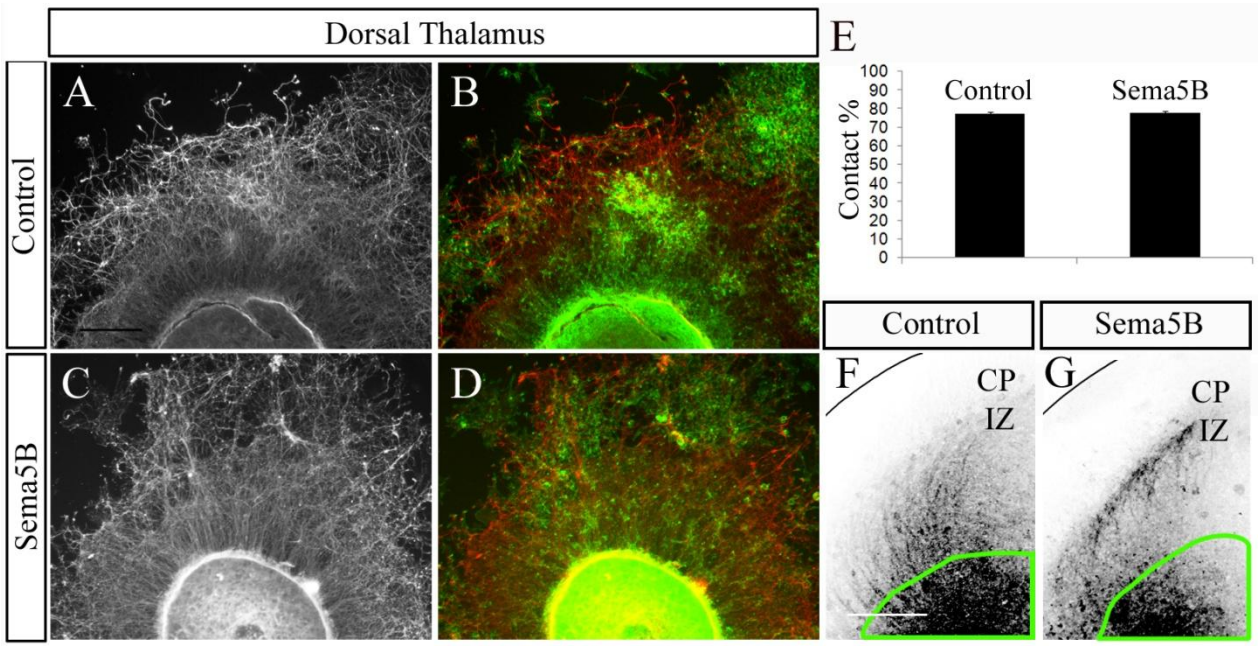


Figure 2.5 Dorsal thalamic axons do not avoid Sema5B either in vitro or organotypic slice culture. A-E) E14 dorsally-derived thalamic explants cultured with control (A, B) or Sema5B-expressing (C, D) HEK293 cells, quantified (as described in Fig. 3L) as percent of axon contact with cell islands (± SEM; E). A, C) tuj1-labeled axons. B, D) Overlay of tuj1 (red) and phalloidin (green), showing axonal contact with cell islands. F, G) Confocal images of E14.5 organotypic slice cultures showing DiI-labeled dorsal thalamic axons pathfinding normally into the intermediate zone (IZ) of the cortex through control (F) or Sema5B-expressing (G) HEK293 cells. The area of cell overlay is defined by a green outline. Scale bar in A represents 100µm for panels A-D. Scale bar in F represents 350µm for panels F and G.

We then monitored the guidance of thalamic axons en route to their targets in the cortex. We found that the thalamocortical projection in ex vivo slice culture was established normally and reached the intermediate zone and cortical plate in the presence of ectopic Sema5B in subcortical regions (Fig 2.5G). Thus, although corticofugal axons were significantly repelled (as shown in Fig 2.4), thalamocortical axons were unaffected by recombinant Sema5B (Fig. 5).

2.3.5 Loss of Sema5B expression from the neocortical VZ results in corticofugal axon pathfinding errors

Having determined that Sema5B is sufficient to inhibit cortical, though not thalamocortical, axons, we wished to investigate whether Sema5B does inhibit axons in situ. We chose to assay this possibility by ex vivo electroporation of shRNA-expressing plasmids followed by immediate slice culture and DiI-labeling of cortical axons (Hand et al., 2005).

Three target sequences within the semaphorin 5B gene were chosen for RNA interference (Reynolds *et al.*, 2004). shRNA1, shRNA2, and shRNA3 were each confirmed as properly inserted into pLentilox 3.7 with the correct desired sequence. A 4th insert containing a nonsense sequence with no similarity to the Sema5B gene, and a construct designed towards the luciferase gene, were chosen as controls for potential off-targets effects due to the other Sema5B-shRNA vectors. Each of the three shRNA vectors was tested for knock down capability by transfection into the stable Sema5B-expressing cell line. Positive transfectants were identified by GFP fluorescence, encoded by an IRES-GFP sequence on pLL3.7 downstream of the shRNA insert. Transfected cultures were also stained for HA, which tags the recombinant version of Sema5B. Cells that were positive for GFP and negative for Sema5B-HA were considered as knocked down (white arrowheads; Fig. 6A) and each of the three constructs knocked Sema5B expression down in nearly every cell into which they were successfully transfected (Fig 2.6A, B) where 1.0% (± 0.4 SEM), 0.7% (± 0.4 SEM), 0.3% (± 0.3 SEM) of cells co-express Sema5B-HA and GFP (red arrows; Fig 2.6A, upper

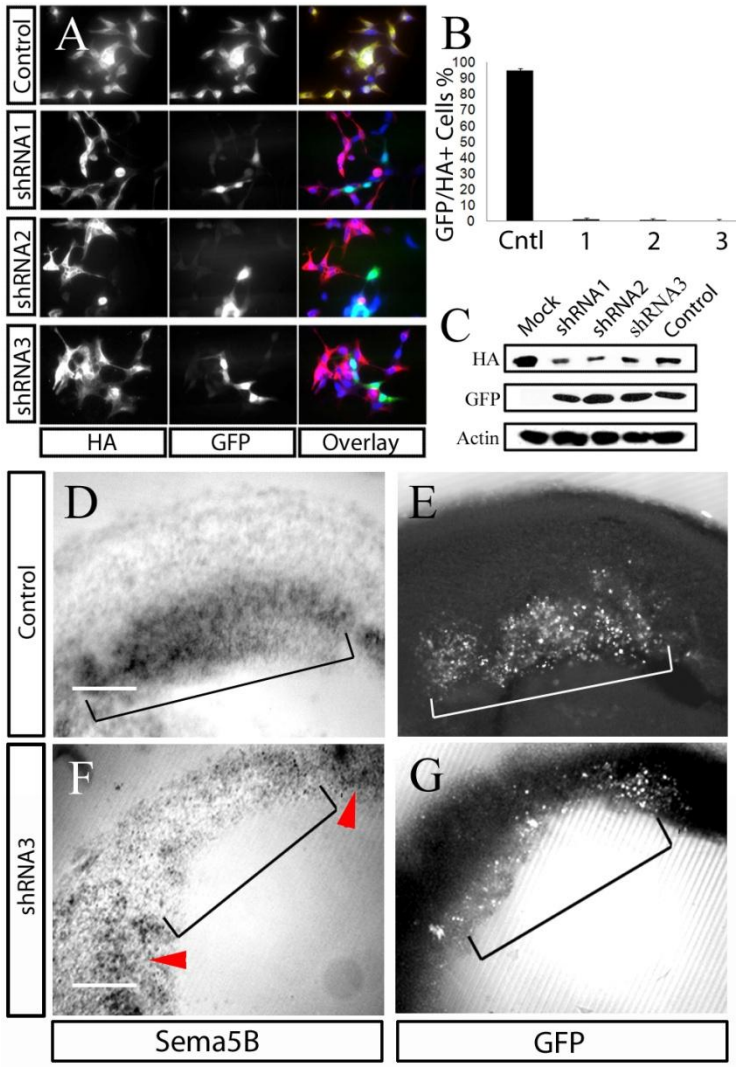
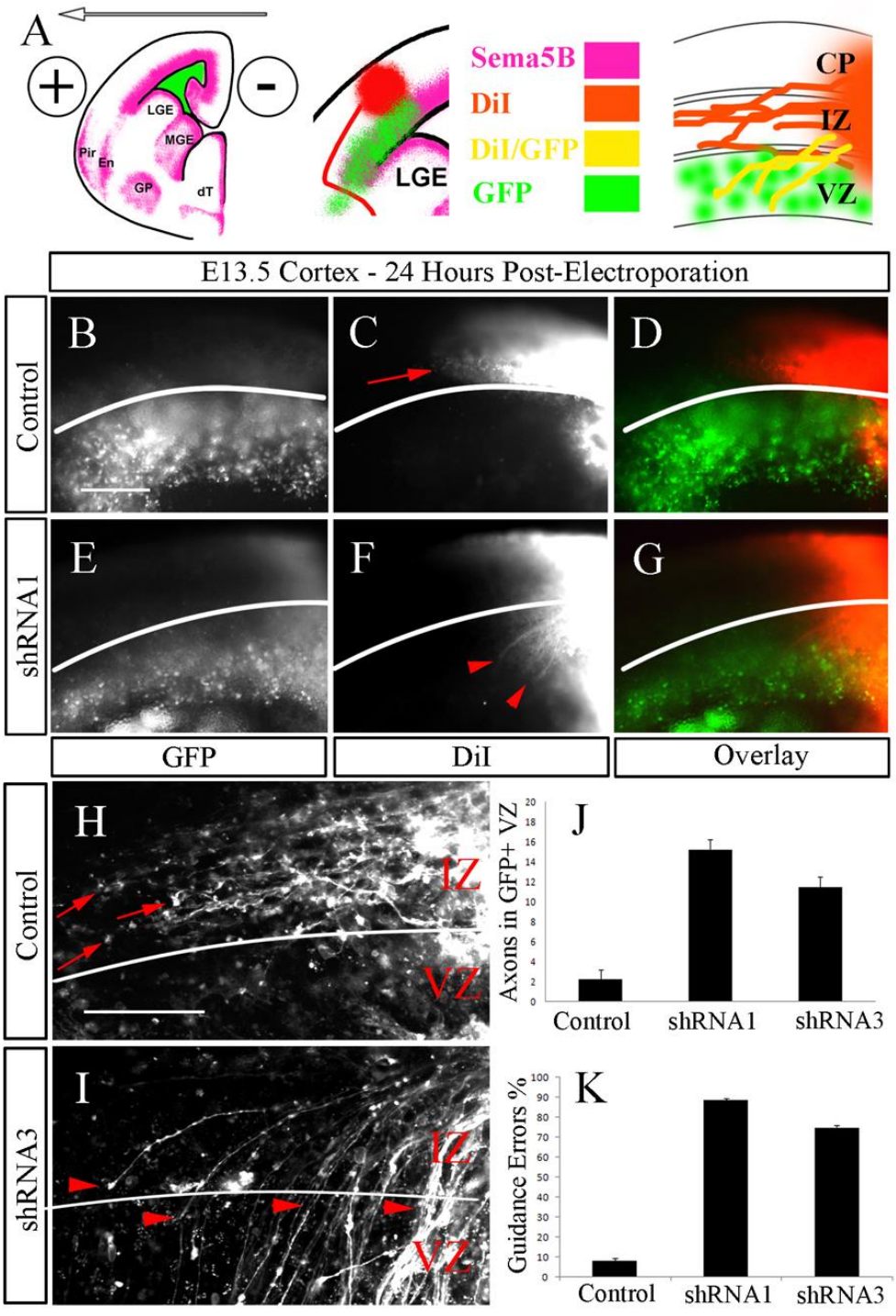


Figure 2.6 Effective knock down of Sema5B with shRNA vectors. A) HEK293 cells were transfected with control or shRNA1-3. Images are of GFP fluorescence and α-HA (red) which labels HA-tagged recombinant Sema5B. Cells were counterstained with bis-benzimide for quantification. Cells expressing the shRNA constructs showed reduced HA-Sema5B expression (white arrowheads) compared to control (red arrows). B) Sema5B knockdown was quantified by determining the proportion of total cells that were positive for both HA-Sema5B and GFP. The majority of cells expressing shRNA constructs showed little HA-Sema5B labeling (shRNAs 1-3 are represented by bars labeled 1-3, respectively), indicating Sema5B knock down (control shRNA plasmid; Cntl). C) Western analysis of HEK293 cells 24 hours after mock-transfection or transfection with control or shRNA1-3 vectors. Based on GFP expression, all three shRNA constructs transfected with similar efficiency and were effective at reducing the amount of HA-Sema5B detected in cell lysates. β-actin demonstrates that similar amounts were loaded in control and knocked down cell cultures. D-G) Organotypic slice cultures of electroporated E13.5 brains. D, F) Non-isotopic in situ hybridization for Sema5B in slice cultures electroporated with control shRNA (D) or Sema5B-shRNA3 (F). Brackets indicate regions of electroporation, seen also in adjacent sections showing GFP fluorescence of control shRNA (E) and Sema5B-shRNA (G). Red arrows in F indicate regions where Sema5B RNA is still present. Scale bar in D represents 250μm for D and E. Scale bar in F represents 200μm for panels F and G.

three panels) indicating a high degree of knock down. On the contrary, transfection of either control shRNA plasmid had no effect on Sema5B-HA levels (95.0 ± 3.5 % of cells co-express HA and GFP). We were therefore confident in having three shRNA vectors effective in producing knock down of Sema5B and valid control vectors that do not result in Sema5B knock down (quantification; Fig 2.6B). Western analysis was also performed on knock down cultures to show quantitatively that Sema5B expression was reduced compared to controls (Fig 2.6C). Not all cells were transfected with Sema5B-targeting shRNA, therefore, HA remained detectable in the cell lysate, but was significantly reduced compared to control shRNA transfected or non-transfected cells.

For the purposes of shRNA-mediated knock down of Sema5B, E13.0/E13.5 mice were chosen because at this stage axons are still growing within the intermediate zone (IZ) in proximity to the Sema5B-expressing VZ. We chose shRNA1 and shRNA3 for these experiments. Plasmids were injected unilaterally into the left lateral ventricle and electroporated into the lateral VZ at or near the CSB (see Fig 2.7A for schematic of endogenous expression, electroporation, and DiI labeling). After electroporation with control or shRNA plasmids, brains were sliced by vibratome and labeled with DiI in the cortical plate. Some control and shRNA electroporated slices were not DiI-labeled, but were cultured for ~24 hours and then re-embedded in gelatin blocks and re-sliced at 50µm for Sema5B in situ hybridization (Fig 2.6D-G). Control shRNA-electroporated cortex showed no decrease in Sema5B expression in regions positive for GFP expression (bracketed areas, Fig 2.6D and E, respectively). In contrast, cortices electroporated with shRNA3 had demonstrable reductions in Sema5B RNA expression

Figure 2.7 Loss of Sema5B in organotypic slice culture causes DiI-labeled cortical fibers to misproject into the subventricular and ventricular zones of the pallium.



A) Ex vivo electroporation was used to create effective Sema5B "chimeras". Endogenous Sema5B expression is shown in fuschia, GFP indicating the electroporated region is green, DiI label is red, and the overlap of DiI and GFP is shown in yellow. Left: As per Polleux (Hand *et al.*, 2005), the positive electrode was placed laterally to direct shRNA constructs into the lateral aspect of the dorsal VZ near and/or including the corticostriatal boundary. Centre: A DiI crystal was placed on the slice in the cortical plate to label corticofugal axons. Far right: schematic of the cortical layers. GFP+ electroporated regions were examined for the presence of DiI-labeled fibers that should normally be coursing through the IZ, and are represented here as yellow fibers within the green VZ. B-G) E13.5 slices electroporated with control (B-D) or shRNA1 (E-G). B, E) Greyscale images of GFP fluorescence in the VZ. C, F) Greyscale images of DiI-labeled corticofugal axons. D, G) Merged

images of GFP and DiI – showing segregation or overlap of DiI labeled axons and electroporated VZ. A white line is shown in each of B-G, circumscribing the VZ/SVZ, and defining it from the IZ. Cortical axons in a control slice demonstrate normal pathfinding along the IZ (red arrow; D). Cortical axons in an shRNA knockdown slice show significant errors in direction of targeting and are found in the GFP+ VZ (red arrowheads; F). H, I) Low magnification confocal images of DiI-labeled cortical axon projections in control (H) or shRNA3 (I) plasmid electroporated cortex. A white line is shown to define the IZ from the VZ/SVZ. Control axons maintain their trajectory in the IZ (red arrows), whereas Sema5B knock down allows penetration of axons into the VZ (red arrowheads). J, K) Guidance errors were quantified as the average number of DiI-labeled axons that invade GFP-expressing VZ per slice, per condition (±SEM; J) or the percent of slices per condition that displayed guidance errors (±SEM; K). By either measure, both shRNA1 and shRNA3 produce a significant increase in the numbers of VZ-penetrating axons compared with control. Scale bar in B represents 250μm for B-G. Scale bar in H represents 100μm for H and I.

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within the GFP-positive region (bracketed areas, Fig 2.6F and G, respectively), while the remaining cortical ventricular zones expressed Sema5B as usual (red arrows, Fig 2.6F). Therefore, in addition to their effects on the recombinant form, our shRNA constructs were effective in the knock down of endogenous Sema5B.

After a 24 hour culture period DiI-labeled slices were examined for the presence of labeled axons amongst VZ cells expressing GFP, which indicates positive transfection. Control constructs expressed within the VZ (GFP expression – Fig 2.7B) had no effect on the pathfinding of cortical axons (DiI labeling – Fig 2.7C) which did not enter the region of GFP expression (merge – Fig 2.7D; confocal images – Fig 2.7H; quantification Fig 2.7J), but rather remained within their proper course in the IZ (red arrows in Fig 2.7C, H). In comparison, when the VZ was electroporated with shRNA1 (Fig 2.7E-G) or shRNA3 (Fig 2.7I), cortical axons were found traversing the normally well-maintained boundary between the IZ and the SVZ/VZ (demarcated by a white line) and were found within the regions of Sema5B knock down

(merge – Fig 2.7G; confocal images Fig 2.7I; red arrowheads in Fig 2.7F and I; quantification Fig 2.7J). Overall, knock down of Sema5B by shRNA electroporation caused a significant increase in the number of axons per slice that entered the VZ (15.2 ± 1.5 axons – shRNA1; 11.5 ± 1.3 – shRNA3) compared with control constructs (2.2 ± 0.7 axons; one-way ANOVA; main effect of plasmid type, $p<0.001$). Similarly, when analyzing Sema5B loss of function in broader terms, we found that shRNAs against Sema5B increased the percentage of slices exhibiting cortical axon guidance defects from 8.33 ± 0.7% in control electroporations to 88.5 ± 2.4% (shRNA1) and 75.0 ± 3.3% (shRNA3). This increase in the rate of guidance defects is significant between control and experimental conditions (Fig 2.7K; one-way ANOVA; main effect of plasmid type, $p<0.001$). By either measure, shRNA3 appears to be somewhat less effective in Sema5B knock down as shRNA1; however, both shRNAs produce guidance defects in descending cortical axons consistent with a loss of repulsion from the VZ/SVZ. This supports our hypothesis that Sema5B mediates repulsion of descending cortical axons from the germinal zones of the developing telencephalon.

2.4 Discussion

In this study I show that the class 5 semaphorin Sema5B is expressed in a spatial and temporal pattern consistent with a role in maintaining the trajectory of corticofugal axons towards subcortical structures such as the thalamus. I provide evidence showing that Sema5B is both sufficient and necessary to inhibit cortical axons from entering specific territories such as the ventricular zone during their guidance to subcortical targets.

2.4.1 Exogenous Sema5B is inhibitory to cortical axons in vitro

Sema5B is found in regions of the developing telencephalon that are specifically avoided by corticofugal axons. The strongest expression of Sema5B is within the ventricular and subventricular zones (VZ/SVZ), particularly in the dorsal pallium, but it is also expressed in moderate levels along the CSB, both of which are a pattern reminiscent of Pax6 (Jones *et al.*, 2002; Puelles et al., 2000). The CSB is an intermediate target for cortical axons as they navigate into the subcortical regions (Molnár and Cordery, 1999), so I postulated that dorsal cortical axons arriving earliest to the CSB might have a stronger response to Sema5B than lateral cortical axons. The pause of axon outgrowth at boundary regions is a common occurrence during nervous system development, also being found in thalamic axons and centrally-projecting sensory axons, and thus is likely an important developmental process although the underlying mechanism is not known. The CSB may provide inhibition that prevents or slows further outgrowth of cortical axons until this accumulation of cortical axons has occurred.

I found that both groups showed significant avoidance responses to Sema5B in vitro, including growth cone collapse; however, there was no difference in the degree of this repulsion. Thus, mechanisms other than degree of responsiveness to Sema5B likely account for the differential pause exhibited at the CSB *in vivo*. For example, it is possible that cortical axons only cross the CSB once there are sufficient homophilic interactions amongst corticothalamic axons to overcome narrow inhibitory barriers, and possibly to permit heterophilic interactions between the growth cones of cortical and thalamic axons and promote their subsequent fasciculation (Molnár, 2000). It has also been argued that no

interaction exists between thalamic and cortical axons; that they actually travel in mutually exclusive domains within the internal capsule (Bagnard *et al.*, 2001). Alternatively, this pause at a major histogenic boundary and subsequent accumulation of descending cortical fibers may be important for the continuity of topographical organization as they guide toward their final targets.

2.4.2 Sema5B is sufficient to inhibit cortical axons in situ

While I have shown that Sema5B is indeed capable of inhibiting cortical axons in vitro, this does not necessarily reflect the endogenous function, as the context of modifying factors found in extracellular spaces and on cell surfaces along an axon's trajectory, including proteoglycans, adhesion molecules, and other membrane-bound or secreted guidance cues, can influence the ultimate behavior elicited by a particular guidance cue. This has been demonstrated most importantly with Sema5A, which can act as either a permissive or inhibitory substrate depending upon local co-expression of heparin or chondroitin sulfate proteoglycans (Kantor *et al.*, 2004). It has been suggested that the same is true at least in vitro for Sema5B (Shipp and Hsieh-Wilson, 2007). Our gain of function organotypic cell overlay assay was employed to assess whether there exists such a modulation of the demonstrated in vitro inhibitory behavior of Sema5B. Although this may reflect a complete "overwhelming" of endogenous local cues, the creation of ectopias in brain slice culture are often used and are generally assumed to be acting in an endogenous fashion (Flames et al., 2004; López-Bendito *et al.*, 2006; Marín et al., 2001; Polleux and Ghosh, 2002). I chose to make our cell overlay on the presumptive internal capsule, a region of the brain along the pathway of descending

cortical axons that does not express Sema5B. By adding these ectopias I showed that the addition of Sema5B can block the entry of axons into a normally permissive zone. In some instances the ectopic Sema5B placement actually resulted in aberrant turning of axons into regions of endogenous Sema5B expression along the CSB (Fig 2.4L). This presumably reflects a guidance phenomenon where an axon challenged by multiple non-permissive substrates will opt for the less repulsive of the two (Bagnard *et al.*, 2000). The inhibition of cortical axons by a gain of Sema5B expression in the internal capsule demonstrates a sufficiency of inhibition by Sema5B, not only in vitro, but even in the physiologically relevant environment provided by the ex vivo slice culture system.

2.4.3 Thalamic axons are non-responsive to Sema5B

Much in the same manner as was performed to examine the descending cortical projection to the internal capsule, I also examined the reciprocal thalamic projection. This fibre set also encounters regions of Sema5B expression flanking its path through the subcortex and is also thought to pause at the CSB (López-Bendito and Molnár, 2003). I speculated that the developing nervous system would make repeated use of a cue that creates inhibitory domains flanking an equivalent, though reversed guidance path. In addition, it has also been shown that a permissive bridge of cells from the lateral ganglionic eminence migrates between the medial ganglionic eminence (MGE) and the globus pallidus, and is absolutely necessary in order to permit thalamic axons entry to the subcortex (López-Bendito *et al.*, 2006). The factor within the MGE and globus pallidus that is responsible for the inhibition of thalamic axons has not been identified, and I postulated that Sema5B might subserve that

role. It was therefore surprising to discover that this is not the case; dorsal thalamic axons were not inhibited by the presence of Sema5B expressed in islands of HEK293 cells in vitro, or by overlays of cells creating ectopic regions of Sema5B expression within the internal capsule.

What is most interesting is that not only are thalamic axons not inhibited by the same cue that inhibits cortical axons, but they also appear to guide normally and find their cortical targets in spite of an environment where cortical axon guidance has been disrupted. This contrasts with the argument that the interaction of descending and ascending projections near the CSB is a crucial event for the guidance of each fiber set (Vanderhaeghen and Polleux, 2004). In further support against the "handshake" hypothesis, thalamic and cortical axons have been shown to segregate from each other in culture, while Sema3A expressed within the internal capsule (Skaliora *et al.*, 1998) amplifies these homotypic fasciculations (Bagnard *et al.*, 2001; Bagnard *et al.*, 1998). In vivo, a disjunct between cortical and thalamic axon guidance has been demonstrated in the COUP-TFI mouse mutant, where thalamic projections are defective in their guidance to the cortex although cortical projections reach and pass the CSB normally (Zhou et al., 1999). Vice versa, thalamic axons find their way normally in a cortex-specific Pax6 mutant independent of deviations in cortical pathfinding (Piňon et al, 2008). If the integrity of both axon sets was necessary for the guidance of each as has been suggested (Hevner *et al.*, 2002), I would expect that the lack of cortical axons in the internal capsule of our slice culture assays would disable the guidance of the thalamic axons therein. Our results that thalamocortical fibres are not affected by Sema5B and grow properly into the cortex while corticofugal fibres are deflected by Sema5B would suggest that the thalamocortical

projection cannot rescue the cortical projection, supporting previous evidence that the two fibre sets are not necessarily co-dependent for their proper targeting.

2.4.4 Sema5B is a necessary inhibitory component of the descending cortical projection

In order to demonstrate that Sema5B was not only sufficient to inhibit the descending cortical projection but also functioned as an inhibitory factor along the corticofugal projection in situ, I employed ex vivo electroporation of shRNA expressing plasmids with subsequent slice culture. This allowed us to determine the degree of Sema5B loss of function guidance defects, if any, in the same type of system with which I analyzed the Sema5B gain of function.

In our gain of function experiments I created ectopic domains of Sema5B expression within the normally non-expressing presumptive striatum and internal capsule. In order to electroporate shRNAs from a ventricular injection, it was required that I examine the sema5B-expressing tissue that it was actually feasible to access, i.e. the VZ of the dorsal/lateral pallium, a region that strongely expresses Sema5B within the developing mouse nervous system (see Fig 2.2). Therefore, if Sema5B acts endogenously to create inhibitory domains for the descending cortical projection, I would expect that the loss of Sema5B at the appropriate stage of development would result in cortical axon guidance errors. I found that where shRNAs against Sema5B were expressed, cortical axons no longer maintained their trajectory within the intermediate zone (IZ) of the cortex, but could be found invading the VZ that they would normally avoid.

Inhibitory guidance cues other than Sema5B are also present in the developing cortical VZ, including slit1 and slit2. Slit1 is expressed in the cortical plate and the germinal zones of the LGE, MGE and diencephalon (Bagri *et al.*, 2002). Slit2 is present in a medial^{high} to lateral^{low} gradient, and has been shown to direct cortical axons away from midline structures (López-Bendito *et al.*, 2007). It is therefore important to note that although cortical axons displayed defective axon guidance in the vicinity of Sema5B-negative VZ, it appears that the slit2 gradient was maintained as cortical axons maintained their overall lateral trajectory. If the electroporation was fully penetrant, and more axons were seen to deviate into the ventricular zone, I would expect that eventually the tract would develop relatively normally due to the slit gradient. It is likely, however, that a full knock-out would have a more significant corticofugal pathfinding defect extending from the dorsal cortex into subcortical regions. It is purely speculative as to whether such a mutant would ultimately retain appropriate connectivity between the deep layer cortical neurons and its various subcortical targets.

Based upon in situ hybridizations showing Sema3A expression within the VZ, in conjunction with in vitro repulsion studies, it has been argued previously that Sema3A might be the inhibitory factor keeping the VZ free of cortical axons (Bagnard *et al.*, 1998). Subsequent studies however have demonstrated that the Sema3A protein is active within the cortical plate and although it does repel cortical axons, it is attractive to the dendrites of the same cortical neurons. Interestingly, Sema3A exogenously overexpressed at the VZ actually reverses the polarity of cortical neurons, such that their dendrites grew toward the source of Sema3A, while the axons were erroneously repelled toward the cortical plate (Polleux *et al.*,

2000). Furthermore, as the leading process of a migrating neuron is analogous to the apical dendrite, it is not surprising that Sema3A has also been shown to attract radially migrating neurons to the cortical plate (Chen et al., 2008). Together, these two findings exemplify that Sema3A is not likely present in the VZ to repel axons, therefore leaving the possibility open for another inhibitory guidance cue, such as Sema5B. In the absence of immunohistochemistry, it was crucial that I demonstrate the Sema5B expression in the VZ corresponded to a real activity. By knocking Sema5B down within the VZ, and showing the aberrant invasion of cortical axons into the GFP-positive, Sema5B-negative regions, I showed that Sema5B is normally active in the VZ where the transcript is found, and that this activity corresponds to the creation of domains inhibitory to corticofugal axons projecting toward the subcortex.

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3. The transmembrane Semaphorin 5B can function as a diffusible inhibitory guidance cue[2]

3.1 Introduction

The Semaphorin family contains 20 human isoforms with diverse functions. During embryogenesis, semaphorin function is critical for appropriate development of the nervous system (for a comprehensive review see Yazdani and Terman, 2006). The role of semaphorins as guidance cues has been extensively investigated and while typically described as repellants, many examples now demonstrate attractive semaphorin function (Bagnard et al., 1998; Wong et al., 1999; Pasterkamp et al., 2003; Kantor et al., 2004).

Intracellular signaling and extracellular factors modulate semaphorin function. Receptor combinations and intracellular cyclic nucleotide levels dictate growth cone response to semaphorins (Yazdani and Terman, 2006). Extracellularly, the presence of chondroitin and heparan sulphate proteoglycans also regulate responses to semaphorins, as well as many other guidance cues (Kantor et al., 2004; Shipp and Hsieh-Wilson, 2007). Semaphorin function in also regulated by proteolysis (Yazdani and Terman, 2006). For example, Sema3A, 3B, 3E, 3F, 3G are secreted repellants that require cleavage and subsequent dimerization to elicit activity; however additional cleavage can result in functional inactivation (Adams et al., 1997). The transmembrane Sema4D is also cleaved by MT1-MMP creating a secretable dimer that interacts with plexinB1 (Elhabazi et al., 2001), which is cleaved in turn (Artigiani et al.,

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2003). Identified enzymes that cleave semaphorins include matrix metalloproteinases (MMPs; McFarlane, 2003), and proprotein convertases (PCs; Seidah et al., 2008).

The transmembrane Sema5B has an important role in inhibiting descending cortical axons in the developing mouse brain (Chapter 2; Lett et al., 2009). Class 5 semaphorins are distinguished by a stretch of seven extracellular thrombospondin repeats (TSRs), in addition to the conserved semaphorin (sema) domain. Based on previous observations using cell island assays (Chapter 2; Fig 2.3, p84), collapse assays (To et al., 2007) and immunohistochemical analysis of adult hippocampus (O'Connor et al., 2009), I hypothesized that in the developing brain Sema5B may be proteolyzed into distinct secreted fragments to act as diffusible guidance cues.

I demonstrate here that endogenous Sema5B is found as multiple fragments with distinct though overlapping distributions in the developing brain. I show that recombinant full-length Sema5B is processed in HEK293 cells to release a diffusible fragment that can collapse cortical growth cones. In addition, Sema5B-expressing cell aggregates in collagen create inhibitory gradients for axons derived from cortical explants. Finally, I provide preliminary evidence that Sema5B may function via an interaction with a receptor complex containing TAG-1.

3.2 Methods

3.2.1 Animals: Timed pregnant CD1 mice were lethally anesthetized by chlorohydrate injection (0.25mg/kg, i.p.; Fisher). Embryos were harvested at E14. Experiments were performed in accordance with the Canadian Council for Animal Care and UBC Animal Care Committee.

3.2.2 Generation of Sema5B polyclonal antibodies: Two regions of Sema5B were chosen for antibody production based on their low homology to mouse Sema5A. These included an N-terminal region with part of the semaphorin domain (aa 20-133) and a C-terminal region (aa 994-1093). Recombinant peptides were produced using the glutathione-S-transferase (GST) fusion system (Amersham Pharmacia Biotech) and were purified with a glutathione agarose affinity column (Sigma). Female New Zealand white rabbits were immunized for antibody generation. For the first injection, 0.5 ml of 1mg/ml recombinant peptide was mixed with 0.5ml Freund's complete adjuvant. For boosts, 0.1mg of recombinant peptide in 0.5ml was mixed with 0.5 ml of incomplete Freund's adjuvant. Adjuvant and protein were emulsified before injection. Boost injections were given every two weeks and antibody generation was monitored with test bleeds and western analysis against the fusion peptide. Typically, terminal bleeds were collected after three boosts. The polyclonal antibodies were immunoaffinity purified with AminoLink columns (Pierce).

3.2.3 Sema5B peptide array blotting protocol: A peptide array of the first 200 and last 200 amino acids of mouse Sema5B was produced by the Peptide Array/Synthesis Facility at the

UBC Brain Research Centre. Each dot contained a peptide of 15 amino acids and the sequence of each sequential dot overlapped with the previous dot by thirteen amino acids (Winkler and McGeer, 2008). Primary antibodies used were our rabbit anti-Sema5B-N-term (5B-N; 1:200) or our rabbit anti-Sema5B-C-term (5B-C; 1:400). Secondary antibody used was peroxidase-conjugated donkey anti-rabbit (1:2500; Jackson Immunoresearch).

3.2.4 Brain immunohistrochemistry: E13.5-14.0 mice were perfused overnight with 4% paraformaldehyde and cryoprotected in 30% sucrose. For in situ hybridization, tissue processed as previously described (Chapter 2, p72-3). For immunohistochemistry, free floating sections were processed for immunohistochemistry with 5B-N or 5B-C and developed with donkey anti-rabbit-peroxidase (1:500; Chemicon) and diaminobenzidine (DAB, 0.3%; Sigma) as per Alcántara et al (1996). Alternatively, for fluorescence Sema5B-IHC secondary antibodies used was goat anti-rabbit Alexa 488. Anti-Nestin (1:350; AbCAM) or anti-Pax6 (1:200; DSHB) were detected with goat anti-mouse Alexa 586.

3.2.5 Cell lines, media and western blotting: Preparation of Sema5B-HA in pDisplay has been previously described (Chapter 2, p72). A secretable protein of Sema5B ectodomain fused to alkaline phosphatase (m655 in pBK-CMV), was kindly provided by A.W. Püschel (Münster, Germany). HEK293 cells were transiently transfected with m655, Sema5B, Sema3A or empty vector (pDisplay) with Lipofectamine 2000 (Invitrogen). Cells were maintained in OptiMEM media with low serum and L-glutamine for two days and prepared for collagen co-culture or media analysis. Media was concentrated with Amicon Ultra 30K MWCO centrifugal filter

devices (Millipore) and quantified by BCA protein assay (Pierce). To inhibit matrix metalloproteinase and verify the Sema5B-HA cleavage, 2.5x10^5 cells were plated into wells of a 24-well cell culture plate. After 4-5hrs, the cell culture medium was replaced (500uL) and GM6001 (Calbiochem) was added to final concentrations of 25uM, 20uM, 15uM and 10uM. Equivalent volumes of DMSO were added to additional wells as controls. The cells were treated overnight at 37°C in a CO2 incubator, and then the media was harvested and loaded onto an 8% SDS-PAGE gel. The following primary antibodies were used for western blot: rabbit 5B-N (1:400), rabbit 5B-C (1:200), mouse anti-HA (Clone HA-7, Sigma H9658), goat anti-mouse and anti-rabbit HRP-conjugated secondary antibodies (Jackson Immunoresearch). Membranes were developed with Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare).

3.2.6 Collapse assays and antibody blockade: Primary E13.5 neuron cultures were prepared and maintained as per (Mingorance-Le Meur et al., 2007). Neurons were cultured for 3 days, until most had generated an identifiable axon. For collapse, the following amounts were added to neuron cultures: Sema5B-HA = 0.532μg/μl; m655 = 0.270μg/μl; Sema3A = 0.360μg/μl. Cultures were fixed at t=0, 5, 15, 30, and 45 minutes after incubation with control, wildtype Sema5B, the secreted Sema5B ectodomain-AP fusion (m655) or, Sema3A conditioned media (see To et al., 2007) and stained with phalloidin-Alexa 488 (Molecular Probes). Three antibodies were tested for blockade of wildtype Sema5B media-induced collapse for a one hour period; 5B-N (1:100), a control rabbit polyclonal antibody against HRP (horseradish peroxidase; 1:250; Jackson Immunoresearch), and 4D7 against TAG-1 (mAb IgM;

1:125; DSHB). Neurons were double-labeled with phalloidin-Alexa-488 and anti-Tuj1 (Covance). Tuj1 was visualized with goat anti-mouse Alexa-568. In both assays collapse was quantified as the percent of neurons per sample whose axon had a collapsed growth cone. Six to ten samples were taken from each condition at each time point (each containing n=5-15 neurons). ANOVA was used to assess statistical significance (GraphPad InStat 3).

3.2.7 Three dimensional collagen co-cultures: Collagen gels were prepared and quantified as previously described (Lumsden and Davies, 1983). Briefly, the neocortex was dissected into cubes of ~300um and allowed to recover for one hour at 37^{o}C in the culture medium (neurobasal plus L-glutamine, D-glucose, pen/strep, 5% serum, B27, HCO3-). Explants were cultured with control, Sema5B-HA or m655-expressing cell aggregates for 2-3 days, then fixed with 4% PFA and processed by DAB IHC tuj1 (used 1:4000), with biotinylated goat-anti-mouse secondary antibody and streptavidin/peroxidase (1:400 and1:500, Jackson). TIFF images were analyzed in Adobe Photoshop (CS3.0). An area of 122x586 pixels was selected approximately 60 pixels from the proximal or distal side of the explant. Axons entering proximal areas were converted to a proportion of the distal outgrowth. Assay was performed in triplicate, with each experiment of n = 20-35, except m655, where n=10-12. The final number is the average of the three experimental averages, with SEM calculated across experiments. ANOVA was used to assess statistical significance.

3.3 Results

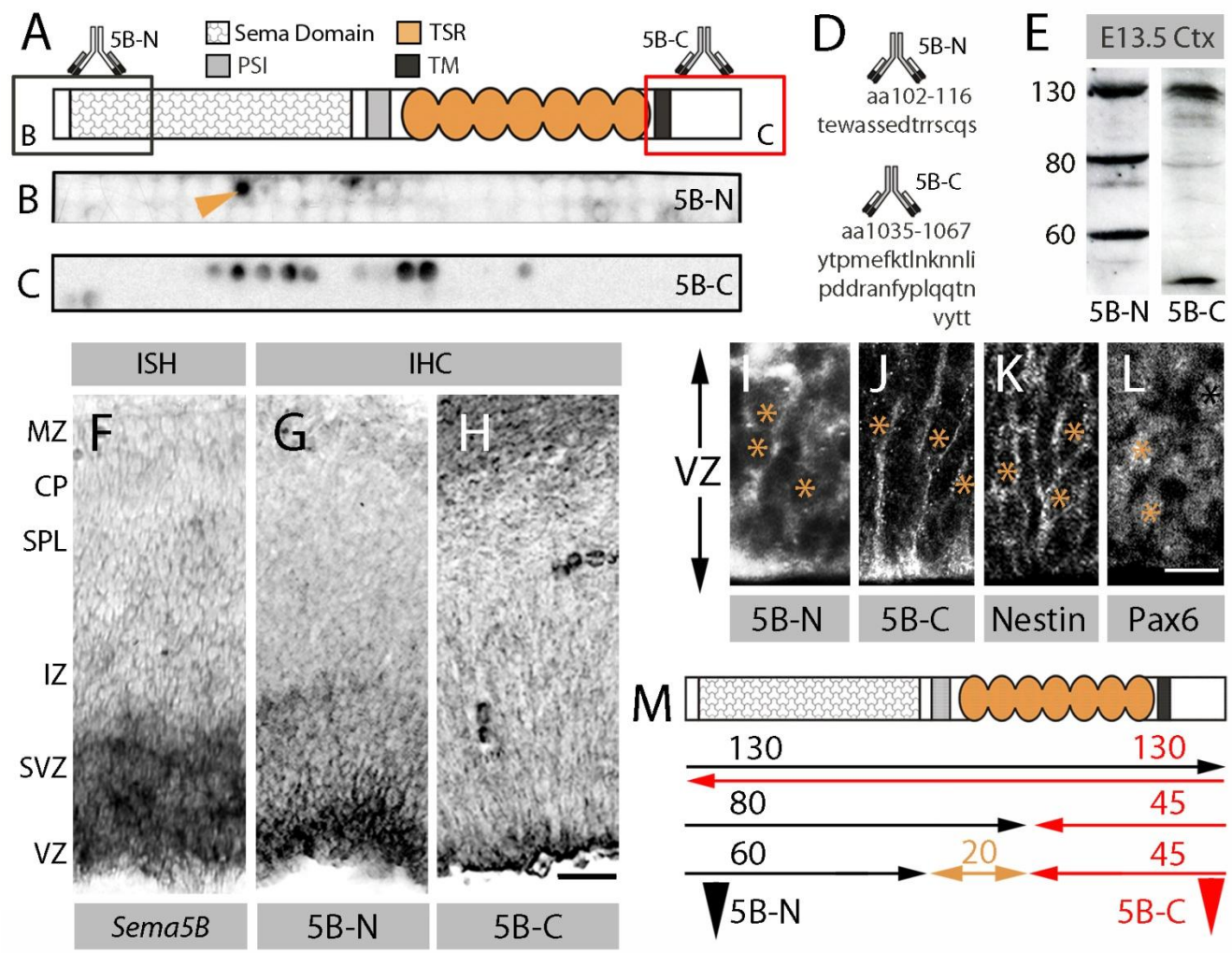
3.3.1 Endogenous expression of Sema5B

To examine Sema5B distribution, two polyclonal antibodies recognizing the extracellular N-terminus and the intracellular C-terminus (Fig 3.1A; 5B-N and 5B-C) were previously generated. To confirm that the antibodies recognized mouse Sema5B, arrays of the first 200 N-terminal and last 200 C-terminal amino acids were probed with 5B-N and 5B-C. 5B-N recognized aa102-116 (arrowhead; Fig 3.1B; TEWASSEDTRRSCQS; Fig 3.1D). 5B-C had a broader range of recognition, but was restricted to the expected region of binding (Fig 3.1C) corresponding to aa1035-1067 (YTPMEFKTLNKNNLIPDDRANFYPLQQTNVYTT; Fig 3.1E).

Sema5B is highly expressed in the E14 mouse brain (Chapter 2, Fig 2.1 and 2.2, pp78-82) and has a predicted molecular weight of approximately 123kDa. While previous studies have identified a single Sema5B transcript from mouse brain RNA (Adams et al., 1996), western analysis of E14 brain lysates with 5B-N and 5B-C revealed numerous bands (Fig 3.1D, F). Antibody 5B-N recognized 3 prominent bands, corresponding to approximately130kDa, 80kDa, 60kDa while antibody 5B-C recognized a similar band at 130kDa and a second prominent band at 45kDa. Based on the molecular weights of the fragments recognized by the two antibodies it appears there are three versions of Sema5B (Fig 3.1M). First, there is a presumptive full-length Sema5B of 130kDa. Secondly, an 80kDa N-terminal fragment that could pair with the 45kDa C-terminal fragment, and thirdly a 60kDa N-terminal fragment that could pair with the C-terminal fragment of 45kDa with an additional proteolytic fragment not recognized by either antibody (orange arrow; Fig 3.1M).

I next examined 5B-N and 5B-C labeling in the developing brain to see whether the different Sema5B peptides were differentially distributed. Antibody 5B-N labeling was very similar to the mRNA distribution, primarily surrounding radial glial somas in the ventricular zone, with decreased labeling in the subventricular zone (Fig 3.1G, I). The C-terminal antibody also labeled radial glia, however, it was not concentrated at the cell bodies (Fig 3.1H), but labeled radial glial end-feet and the marginal zone in a pattern similar to nestin (Fig 3.1K). Pax6 was used to label radial glial cell nuclei (Fig 3.1L). Therefore, N-terminal and C-terminal fragments appear differentially distributed, with N-terminal fragments found around radial glia cell bodies and C-terminal fragments localized along their processes.

Figure 3.1 Sema5B expression in the embryonic mouse neocortex.
A) Schematic of Sema5B. SEMA: semaphorin domain; PSI: plexin-semaphorin-integrin domain; TSR: thrombospondin repeats; TM: transmembrane domain. Antibodies 5B-N and 5B-C are depicted in the regions to which they were raised. B, C) Peptide arrays of Sema5B. B) N-terminal 200aa probed with 5B-N. C) C-terminal 200aa probed with 5B-C. D) Amino acid sequences recognized by 5B-N and 5B-C. E) Western blot of cortical lysate probed with 5B-N and 5B-C. F) Sema5B ISH. G) 5B-N labeling. H) 5B-C labeling. I-L) High magnification of VZ labeled for 5B-N (I), 5B-C (J), Nestin (K), or Pax6 (L). Asterisks indicate labeled cells. M) Possible pairing of Sema5B fragments. Arrowheads denote epitopes. ISH: In situ hybridization; IHC: immunohistochemistry; MZ: marginal zone; CP: cortical plate; SPL: subplate layer; IZ: intermediate zone; SVZ: subventricular zone; VZ: ventricular zone. Scale bar represents 50µm in H, 10µm in L.



3.3.2 Recombinant Sema5B is secreted and collapses cortical neuron growth cones

Previously I showed that islands of Sema5B-expressing HEK293 cells are inhibitory for cortical axons (Chapter 2, Fig 2.3, p84). However, I did not examine whether this was due to a transmembrane or proteolytically released form of Sema5B. To determine whether Sema5B is processed in HEK293 cells, I transiently transfected cells with an HA-tagged recombinant Sema5B (Sema5B-HA), or m655, which is a secreted version bearing the Sema5B ectodomain (~107 kDa) fused at the C-terminus to human placental alkaline phosphatase (~55kDa). Conditioned media from the Sema5B-HA expressing cells contained a band around 100kDa that was detectable by both the HA and N-terminal antibodies (Fig 3.2A, lanes 2 and 3; schematic of cleavage product in G). This fragment became depleated from the media in a dose-dependent fashion in the presence of a braod-spectrum inhibitor of MMPs, GM6001 (Fig 3.2H). 5B-N detected 60kDa and 50kDa bands in m655 media (Fig 3.2A, lane 1; schematic of cleavage product in I), similar to the 60kDa fragment found in the mouse brain lysate (Fig 3.1E). Therefore, m655 also appears to be cleaved, releasing an N-terminal fragment that does not contain thrombospondin repeats.

To test whether Sema5B secreted fragments are inhibitory, I cultured cortical neurons in conditioned media from Sema5B-HA and m655 expressing cells and compared their activity to Sema3A collapsing activity. In comparison to control cultures (collapse = 31.33% ± 3.76 SEM), the m655 and Sema5B-HA media significantly increased growth cone collapse (Fig 3.2C, 80.61% collapse ± 3.52 SEM; Fig 3.2D, 72.08 collapse ±2.25% SEM) and both media were as

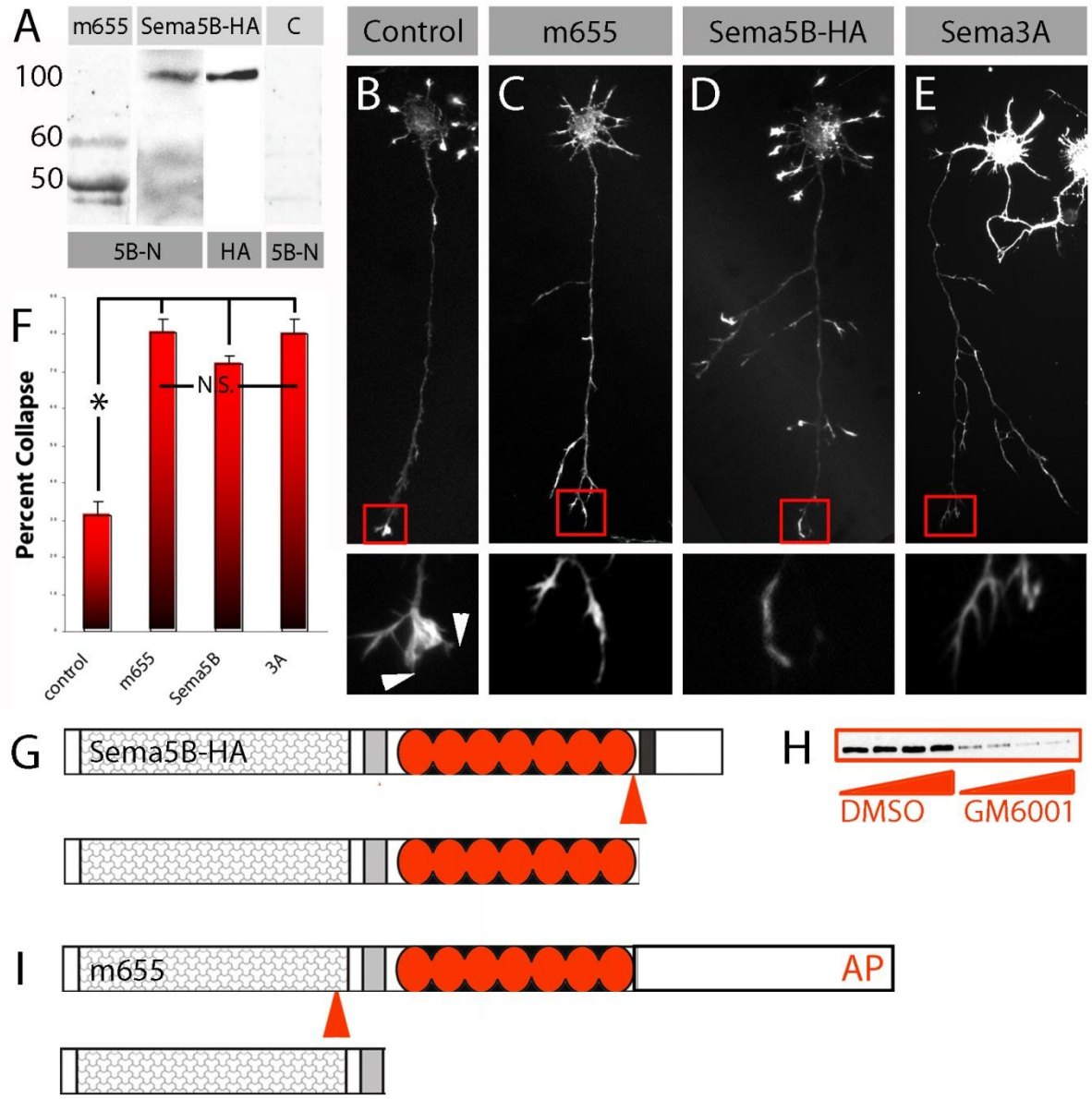


Figure 3.2 Recombinant Sema5B is secreted and collapses cortical neurons.
A) Western blot of media collected from HEK293 cells. Lanes from left to right, media collected from cells expressing: 1 –m655 (5B-N); 2 –Sema5B (5B-N); 3 –Sema5B-HA (αHA); 4 – empty plasmid (5B-N). Dissociated E13.5 cortical neurons were incubated with conditioned cell media. B) Control, C) m655, D) Sema5B-HA, E) Sema3A. B-E) Phalloidin labeling is shown in monochrome. Images were taken 15 minutes after addition of culture media. Boxed areas are shown in higher magnification. Arrowheads in B indicate filopodia of intact growth cone. Quantification of t=15 minutes shown in F. All non-control media were significant collapsing agents (ANOVA; $p<0.0001$). G) Schematic illustrating the cleavages and resultant secreted fragments from Sema5B-HA-expressing cells. H) the Sema5B-HA cleavage by HEK293 cells is mediated by a matrix metalloproteinase as shown by a dose-dependent reduction in the amount of secreted fragment with increasing amounts of the wide-spectrum MMP inhibitor GM6001. I) Schematic of m655 (Sema5B ectodomain fused to alkaline phosphatase), and the putative cleavageresulting in the smaller-than-expected secreted fragment.

effective as Sema3A conditioned media (Fig 3.2E, 80.13% ± 3.97 SEM collapse; Fig 3.2F; ANOVA, Sema5B-HA vs m655 vs Sema3A = N.S.; ANOVA, control vs Sema5B-HA, m655 or, Sema3A, $p<0.0001$).

3.3.3 Recombinant Sema5B creates an inhibitory gradient

Collagen gel assays are extensively utilized to determine *chemotrophic* versus *chemotropic* activities of candidatesecreted guidance cues (Lumsden and Davies, 1983). When culturing cortical explants with Sema5B-HA or m655 expressing cells, I observed significant reductions in the proximal-to-distal ratio of neurite outgrowth towards the aggregates (0.34 ± 0.02 for Sema5B-HA, 0.46 ± 0.06 for m655, normalized to 1.0 ± 0.04 for control cells; Fig 3.3B, C) compared to control (Fig 3.3A). Compared to Sema3A, which produces repulsion detectable from a distance of at least one explant width and induces repulsive turning from a gradient source (Messersmith et al., 1995), both m655 and Sema5B-HA had a significantly shorter range of activity that nonetheless resulted in the creation of an inhibitory gradient detected and responded to by growing cortical neurite with a stalling of neurite extension (Fig 3.3).

3.3.4 Anti TAG-1 antibodies block the collapsing response of Sema5B

The N-terminal region of semaphorins is critical for conferring function (Koppel et al, 1997). I examined whether the N-terminal antibody would inhibit Sema5B-mediated growth cone collapse. As controls, I used a polyclonal antibody to HRP with no known rodent ligard (Jan and Jan, 1982) and the 4D7 monoclonal antibody against TAG-1 (Karagogeos et al., 1991).

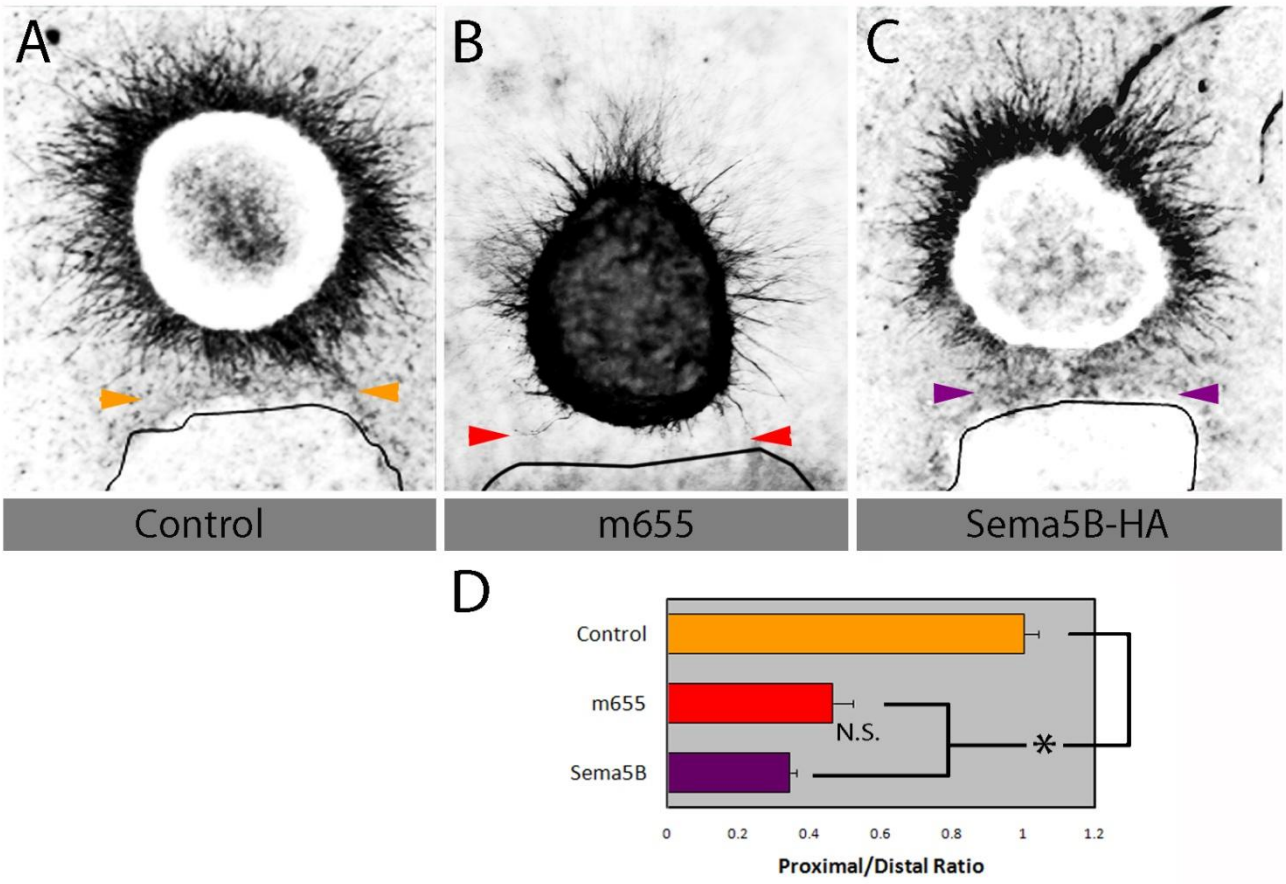
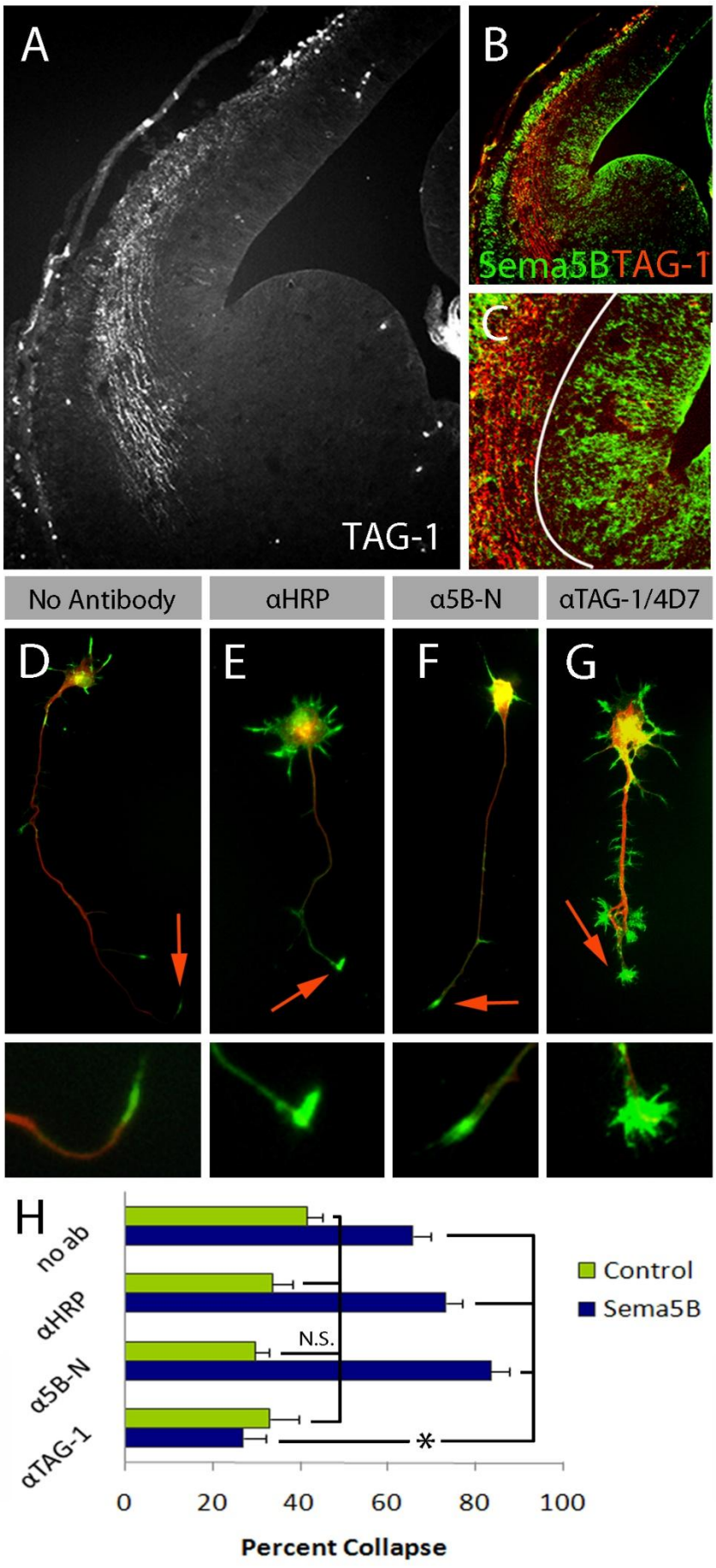


Figure 3.3 Sema5B creates inhibitory gradients in a 3D collagen matrix.
E13.5 dorsal cortical explants (DAB-IHC processed for Tuj1) apposed to aggregates of (A) control HEK293 cells (B) m655-expressing cells (C) or Sema5B-HA-expressing cells. Results quantified in D as the average ratio of proximal to distal axons. Arrowheads indicate axons proximal to cell aggregates. B) Fewer axons grow toward aggregates expressing m655 compared to control. C) Sema5B-HA also inhibits proximal axons. Controls displayed significantly more proximal outgrowth compared to Sema5B-HA and m655 (ANOVA; $p < 0.0001$). Assay was performed in triplicate, with each experiment of n = 20-35, except m655, where n=10-12.

The monoclonal antibody 4D7 was chosen because TAG-1 is expressed on a number of neuronal populations that respond to Sema5B (Chapter 2; Appendix B) while TAG-1-negative neurons we have tested are not responsive to Sema5B (Chapter 2; data not shown). Evidence has also shown TAG-1 as the obligate co-receptor for an unknown repellant in the spinal cord where Sema5B is expressed (Püschel et al., 1996; Law et al., 2008; Lett and O'Connor,

Figure 3.4 Antibodies to TAG-1 attenuate the collapsing effects of Sema5B-HA

A-C) E13.5 coronal section incubated with 4D7 against TAG-1, and 5B-N against the N-terminal of Sema5B. A) TAG-1 in single channel monochrome. B) The same section shown merged with TAG-1 in red, and Sema5B in green. C) High magnification of B, highlighting the exclusion of TAG-1 fibers from the Sema5B positive germinal zones. . Three DIV dissociated cortical neurons were incubated with Sema5B-HA media and one of the following: D) no antibody; E) αHRP (1:250), F) 5B-N (1:100), G) α-TAG-1/4D7; (1:125). Images are merges of green phalloidin and red Tuj1. Results are quantified in H. 4D7 was the only antibody effectively reducing Sema5B-induced collapse levels to baseline ($p<0.0001$). N.S. = not significant.



unpublished observations) and the 4D7 antibody can block this function (Masuda et al., 2003; Law et al., 2008).

Neither the 5B-N nor HRP antibodies reduced Sema5B-mediated collapse compared to control cultures (Fig 3.4). In contrast, the anti-TAG-1 monoclonal antibody 4D7 reduced the collapse response of cortical axons to Sema5B-HA to control levels (Fig 3.4D, E). Although, 4D7 incubation in cultures exposed to control media did not alter growth cone collapse (Fig 3.4E, $p = 0.365$). These results support a role for TAG-1 in mediating the inhibitory response to the secreted fragment of Sema5B in cortical axons.

3.4 Discussion

The function of many guidance cues is altered or regulated by post-translational modification. One mechanism of regulation is proteolytic cleavage by proteases such as metalloproteinases (McFarlane, 2003) and proprotein convertases (Adams et al., 1997). I show here that the predicted transmembrane protein semaphorin 5B is processed both *in vivo* and *in vitro* for the generation of a diffusible inhibitor with a critical role in telencephalic development (Lett et al., 2009).

3.4.1 Transmembrane Sema5B is proteolytically processed in mouse cortex

Previous evidence from *in situ* hybridization studies have shown that sema5B mRNA is strongly expressed in ventricular zones (VZ) during development of the mouse brain, indicating expression by radial glial cells (Chapter 2; Fig. 2.2, p80; Lett et al., 2009). To further

characterize Sema5B, our lab developed antibodies to the N- and C-terminals of full-length Sema5B to determine tissue localization and protein size. Surprisingly, although Sema5B is predicted to be a single-pass transmembrane protein, both antibodies detect several bands each upon western analysis, with the largest band near the predicted size. Earlier studies have shown a single transcript for Sema5B in the mouse (Adams et al., 1997); therefore, these additional fragments appear to result from post-translational processing.

3.4.2 Sema5B N- and C-terminals have distinct yet overlapping localizations

N-terminal antibody tissue labeling was mostly confined to the VZ, with progressively decreasing expression extending into the SVZ. Specifically, the N-terminus appeared to be localized diffusely within interstitial spaces between VZ (radial glial) cells. Even in the SVZ where 5B-N labeling is weaker, labeling appears localized extracellularly. In contrast, rather than being restricted to the VZ, the C-terminal fragment was distributed along the length of radial glia in the E13.5 cortex, resembling the distribution of nestin, an intermediate filament specific to radial glia. This differential localization of Sema5B fragments is reminiscent of the distribution of F-spondin in the spinal cord floor plate (Zisman et al., 2007). F-spondin is a secreted cue that is cleaved and segregated into two portions, one acting as a short-range repellent from floor plate cells, while the other accumulates as an attractive substrate in the basement membrane underlying these cells. Together these F-spondin fragments direct commissural axons past the floor plate. Similary, while the N-terminal of Sema5B diffuses from the VZ to create an inhibitory gradient for corticofugal axons, the remaining TSR-containing

C-terminal portion could potential serve an adhesive function along radial glia for neurons migrating to the cortical plate.

3.4.3 Cells expressing Sema5B generate inhibitory gradients

Processing of Sema5B into a number of smaller fragments could result in several functional outcomes. To first approximate the functional significance of a secreted Sema5B fragment, we examined the collapsing properties of a secreted recombinant Sema5B (m655) on cortical neurons. Surprisingly, m655 was also proteolyzed to produce fragments containing the conserved sema domain and were similar to the smallest sema domain-containing fragments identified by western analysis of tissue. Contrary to being inactivated, proteolytically cleaved m655 was quite effective at growth cone collapse.

The secreted N-terminal fragment generated by proteolytic processing of Sema5B-HA was considerably larger than the m655 fragments and more closely resembled the higher molecular weight bands observed in tissue. This larger fragment also increased collapse frequency and over time was as effective as m655 and the well-characterized repellant Sema3A, though it took slightly longer (approximately 30 minutes) to reach maximal collapse. One possibility is that the TSRs counteract the inhibitory effect of the sema domain. In this regard m655 is possibly more effective for collapse due to a lack of TSRs, compared to the larger Sema5B-HA fragment likely containing some or all of the TSRs. Nonetheless, these results show Sema5B is processed into a several sema domain-containing fragments that collapse cortical growth cones.

As collapse assays only tests acute inhibition, collagen gel assays were used to assess whether Sema5B could create stable inhibitory gradients for axon guidance. Both secreted m655 and "full-length" Sema5B-HA were found to inhibit cortical axons at a distance from the cell aggregates. Axons seem to have "stalled" when directly faced with the Sema5B-HA gradient, where they did not appear to turn away, but grew straight and stopped prior to being able to contact the aggregates. I noted that axons in the m655 gradient appeared to display a slight repulsive turning response (see Fig 3.3), though this is a preliminary observation and was not quantified. One possibility is that the TSRs counteract the inhibitory effect of the sema domain and thus m655 is possibly more effective at establishing a repulsive gradient due to a lack of the TSRs, compared to the larger Sema5B-HA fragment which likely contains some or all of the TSRs.

The tissue lysate contains multiple N-terminal fragments that are smaller than the predicted full-length Sema5B. Similarly, the media of both Sema5B-HA and m655 expressing cells also present with smaller fragments that closely resemble molecular weights of the *in vivo* fragments. The observed differences in proteolytic fragments likely reflect a difference in proteolytic enzyme expression. We have previously shown that Sema5B does indeed function as an inhibitory guidance cue *in vivo* (Lett et al., 2009). Although I cannot rule out that some Sema5B is expressed as a full length molecule *in vivo*, I propose that the inhibitory activity is predominantly the function of secreted sema-domain containing fragments being released from Sema5B-expressing tissues.

The presence of numerous sema domain-containing fragments *in vivo* suggests complex posttranslational processing that could be regulated both spatially and temporally. It is possible that multiple proteases are involved in a multi-stage processing of Sema5B as is the case for L1-CAM which is processed by a furin-like protease and subsequently by an MMP (Kalus et al., 2003). As the complement of proteases changes over time, Sema5B could similarly be susceptible to changes in protease activity, creating an additional level of spatiotemporal regulation and a sensitive mechanism for regulating such a widely expressed inhibitory activity. During periods of axonogenesis, however, the presence of inhibitors is necessary to create strict boundaries and non-permissive territories for the avoidance of errors in guidance and pathfinding. It is plausible to suggest therefore that Sema5B may be more highly processed during these periods of axon guidance to separate the sema domain from the TSRs, and enhance the inhibitory nature of Sema5B-expressing domains.

3.4.4 TAG-1 is a likely part of a multimeric receptor complex for Sema5B

Presently, Sema5B interaction with chondroitin sulfate proteoglycans has been ascertained (Shipp and Hsieh-Wilson, 2007), yet a high-affinity receptor for Sema5B has not been identified. During pathfinding, Sema5B-responsive corticofugal axons express TAG-1 (Karagogeos et al., 1991), a GPI-linked cell adhesion molecule. Sema5B is expressed in ventral spinal cord (VSC) during afferent sensory axon guidance and is inhibitory to TAG-1-expressing DRG axons in vitro (E12. 5 mouse; Lett and O'Connor, unpublished observations). Similarly, TAG-1-expressing chick retinal ganglion axons are also responsive to Sema5B (Wood JL & O'Connor, unpublished observations). In contrast, thalamocortical axons do not express TAG-

1 (Brummendorf and Rathjen, 1995) and do not respond to Sema5B (Chapter 2; Fig 2.5, p21; Lett et al., 2009). TAG-1 binding modulates the interactions of other proteins (Kuhn et al., 1991; Malhotra et al., 1998) and was recently identified as specifically affecting Sema3A sensitivity of the L1/neuropilin receptor complex (Law et al., 2008). Importantly, this and other studies also suggest the existence of a non-Sema3A inhibition located in the VSC, possibly Sema5B, which mediates its effect through TAG-1 (Masuda et al., 2003). To examine whether TAG-1 may play a functional role in the inhibition of Sema5B on neuronal growth cones, I examined growth cone collapse of cortical neurons in the presence of TAG-1 and Sema5B antibodies. Remarkably, the anti TAG-1 antibodies significantly attenuated growth cone collapse, while the Sema5B N-terminal antibody did not. I speculate that TAG-1 may interact with plexins and/or L1 along the developing corticofugal projection as a part of a multi-component receptor complex.

3.4.5 Conclusions

Sema5B is a prime candidate as a multifunctional cue. It is inhibitory *in vitro* and *in vivo* to cortical axons, and may be processed into numerous forms. The major secreted fragment is detectable by antibodies recognizing the N-terminus (HA and 5B-N), therefore indicating that the Sema5B secreted portion contains the semaphorin domain conferring overall function (Koppel et al., 1997). Presently I do not know whether TSRs confer an alternative function, though my results suggest possible attenuation of collapse activity. Moreover, some C-terminal fragments are predicted to contain only TSRs, potentially providing a permissive substrate for neurite growth.

3.5 References

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Chapter 4: Summary and Discussion of Results

Nervous system development is orchestrated by a multitude of three- and four-dimensional expression patterns of guidance cues, be they traditional or otherwise; secreted to produce repulsive or attractive gradients, or laid down as substrates that enhance or inhibit axon outgrowth. Understanding the regulation of these guidance cues, the growth cone behaviors they elicit, and how these behaviors might be modulated, is a crucial aspect to understanding the establishment of the complex array of the mammalian neural network and provide clues as to mechanisms and etiology of neurodegenerative disorders or neurological dysfunction resulting from erroneous neurodevelopment.

4.1 Summary of findings

Based on the initial discovery and characterization of Sema5B in embryonic mouse and rat expression by Dr. A.W. Püschel's lab, my first hypothesis was that semaphorin 5B has an important role in providing inhibitory guidance to aid tract formation in the mouse telencephalon. The work described in Chapter 2 critically supports this hypothesis, demonstrating that Sema5B is an inhibitory component of corticofugal pathfinding by use of in vitro culturing assays and both gain and loss of function in organotypic slice culture. I found that neocortical ventricular zones lacking Sema5B displayed erroneous penetration by cortical axons that would normally bypass this region, thereby indicating the necessity for Sema5B inhibition in this axon guidance system. Because like many semaphorins Sema5B presents with multiple putative cleavage sites, I further hypothesized that Sema5B might be cleaved from the cell surface to permit formation of *in vivo* inhibitory gradients. I went on to

establish in chapter 3 by way of western blot analysis, in vitro collapse assays and collagen gel assays that this inhibition is likely mediated by a secreted proteolytic fragment of Sema5B containing the N-terminal semaphorin domain. Neocortical sections labeled with 5B-N and 5B-C antibodies revealed distinct yet overlapping domains for the N- and C-terminal fragments. This implies that the proteolytic cleavage of the Sema5B N-terminus also leaves behind a separate membrane-spanning thrombospondin repeat domain. Function blocking antibodies were also used to demonstrate a potential for TAG-1 to participate in a receptor complex mediating Sema5B-induced growth cone collapse and axon guidance. Altogether my thesis provides the first known function for Sema5B in the developing forebrain and demonstrates the existence of a hitherto unsuspected processing event for a transmembrane semaphorin in the nervous system.

4.2 Principles of inhibitory guidance

Inhibitory guidance cues can function in various ways, depending on whether they are secreted or membrane bound. It is of importance to distinguish a secreted from a membrane-tethered cue, for the modes of action can differ substantially. Transmembrane or membrane-associated cues create inhibitory boundaries and may provide the absolute restriction of certain domains for given axon populations based upon physical contact between a growth cone and a boundary substrate. Inhibition in this manner is rather an all-or-nothing type of phenomenon, where the growth cone is either inhibited or not based upon its complement of receptors and signaling molecules (Keynes and Cook, 1995). In addition, a distinction can

be made between membrane-bound and secreted cues based upon whether they can impart directional information.

The penetrability of an inhibitory barrier is a function of the arrangement of the expressing cells. Complete inhibitory domains are created with uniform distribution and high levels of inhibitor expression, as seen between some rhombomeres (distinct sections/divisions of the rhombencephalon) during hindbrain development (Guthrie and Lumsden, 1991; Swiatek and Gridley, 1993; Chen and Ruley, 1998; Dottori et al., 1998). In contrast, non-uniform distributions of membrane bound cues can be utilized in cases of topographical targeting of axons for functional synaptic contact formation. For example, multiple and overlapping "gradients" of membrane-tethered Ephs and ephrins are used in this manner for the retinotopic patterning of axon termination in the optic tectum or the lateral geniculate nucleus of the thalamus (Uziel et al., 2006; Bouvier et al., 2008).

A barrier that is mobile, however, such as one composed of migrating cells as is the case of the corticostriatal boundary (CSB)/lateral migratory stream (LMS), may create a temporary boundary (Molnár and Butler, 2002; Carney et al., 2006), but one which is spread out enough, and therefore weak enough, to be eventually overcome by the many accumulated corticofugal axons. Similarly, dorsal root ganglion axons projecting into the spinal cord are known to pause and accumulate over a 24 hour period at the dorsal root entry zone prior to penetrating the dorsal root grey matter. The boundary cap cells at the dorsal root entry zone express Sema6A which acts via plexin A1 to mediate inhibition, but are also migratory cells of

neural crest origin (Mauti et al., 2007). Boundary cap cells located at the ventral root exit permit motor axon passage but restrict motor neuron somas from also migrating out of the CNS (Bron et al., 2007). Therefore it is possible that these regions where a boundary is only temporarily inhibitory likely operate in the same manner as the CSB or boundary caps. Often it is stated that pioneer axons pause in their outgrowth to permit accumulation of their slower growing counterparts (Molnár and Butler, 2002; Price et al., 2006). It may be more appropriate to say that these axons are incapable of extending beyond these inhibitory barriers without the combined positive interactions of the significant homotypic adhesions provided by a unified front of axonal growth cones, perhaps aided by the combined attractive response to a secreted positive cue, beyond this boundary.

Based upon the mRNA expression, my first hypothesis was that Sema5B was positioned in regions avoided by cortical axons and was therefore a candidate inhibitory molecule preventing axon invasion into these regions. This was quickly confirmed *in vitro* by two dimensional cell island/explant co-culture assays (Fig 2.3), where cortical axons avoided contact with Sema5B-expressing cells and would collapse in their vicinity. Gain of function analysis with organotypic slice cultures further provided evidence that Sema5B not only could inhibit isolated cortical axons, but was also inhibitory in a more physiological setting. These two experiments supported a substrate-bound role for Sema5B, especially as I showed with confocal microscopy that the expressing cells had penetrated into the slices where they could be contacted by pathfinding cortical axons (Fig 2.4). I postulated that for descending cortical axons, Sema5B was creating a strict inhibitory boundary at the ventricular zone, aiding in the

creation of a transient and mobile barrier along the CSB, and creating domains of inhibition flanking the presumptive internal capsule for its appropriate formation and definition (Chapter 2, Fig 2.2).

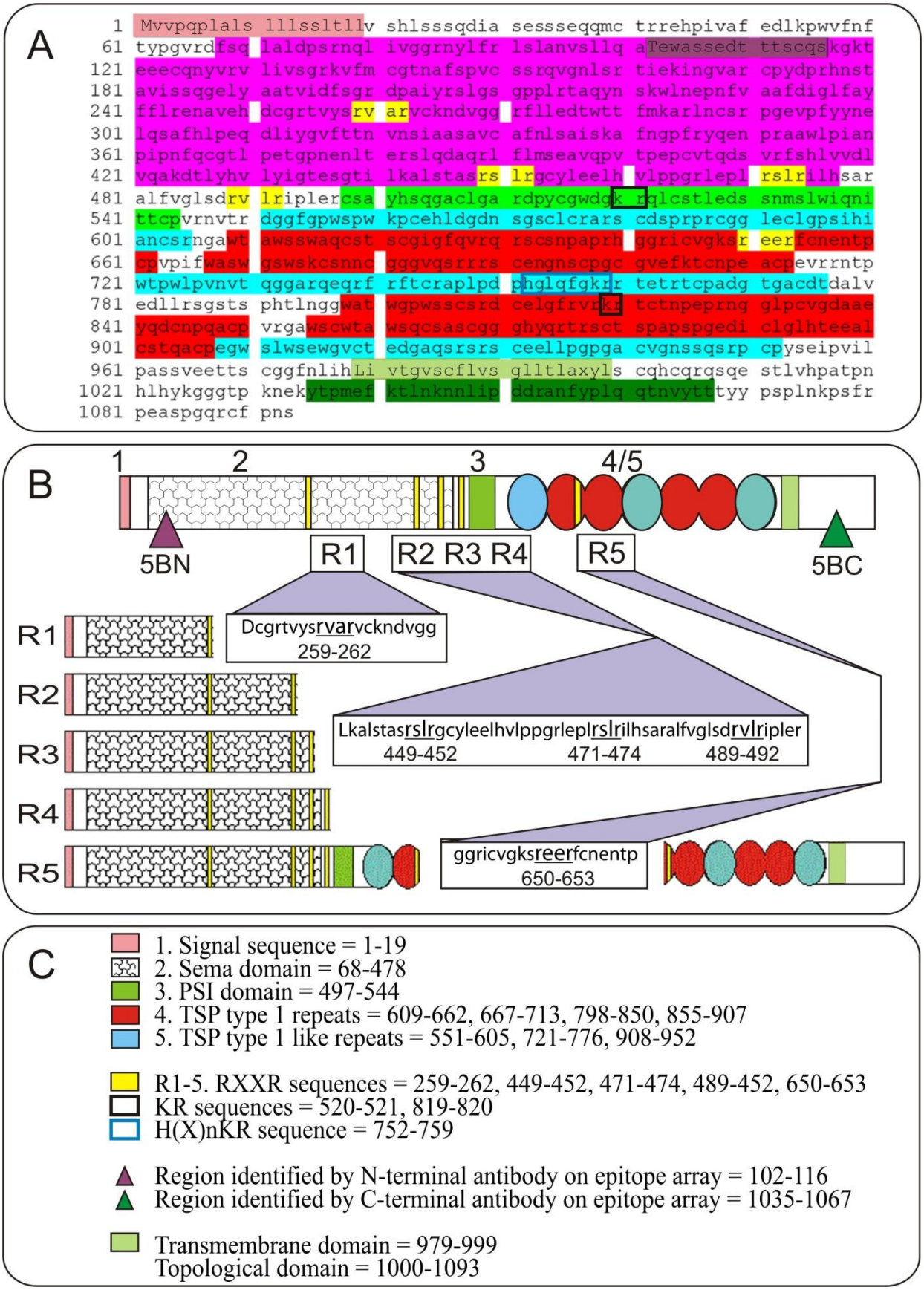
4.2.1 Transmembrane Sema5B is proteolyzed

Proteolysis is a common mechanism of regulating developmentally and functionally important molecules. Hormones are synthesized as prohormones, and activated by cleavage; the A-type ephrins are proteolyzed and released from cell membranes to permit endocytosis and signaling by their bound receptors (Hattori et al., 2000; Chen et al., 2007). Many semaphorins must also undergo proteolytic cleavage. The secreted classes of semaphorins are proteolyzed by furin and furin-like proprotein convertases (PCs) that cut proteins between basic residues (of a consensus sequence "$^{K}/_{R}$ Xn $^{K}/_{R}$", where X is any amino acid other than cys, and n=0, 2, 4, 6; Scamuffa et al., 2006). The repulsive activity of class 3 semaphorins is enhanced by a primary PC cleavage, and then substantially reduced in the event of a second cleavage (Adams et al., 1997; Klostermann et al., 1998; Koppel and Raper, 1998; Christensen et al., 2005; Varshavasky et al., 2008). The transmembrane Sema4D is cleaved by a transmembrane membrane matrix metalloproteinase (MMP) resulting in the release of the whole ectodomain (Kumanogoh and Kikutani, 2004). The soluble fragment dimerizes and can functionally repel or attract growth cones in the nervous system through a plexin B1-signaling mechanism also requiring a PC-mediated cleavage (Masuda et al., 2004; Ito et al., 2006).

Sema5B contains five "RXXR" PC cleavage recognition sites (Fig. 4.1), and three "KR" sites, leading to the hypothesis that Sema5B was a proteolytic target. When brain lysates were probed with the N- and C-terminal antibodies to Sema5B (referred to as 5BN and 5BC, respectively), several bands were detected in addition to the expected full length. Both Sema5B antibodies detected the full length protein; however, 5BN also identified bands of approximately 80 and 60kDa, while 5BC instead identified a band at about 45kDa Fig 3.1E). In the embryonic brain this implies that Sema5B, predicted to be a transmembrane cue, releases N-terminal proteolytic fragments. When I performed immunohistochemistry to determine the localizations of the N- and C-terminal fragments, I found they were indeed differentially localized (see Fig. 3.1). The N-terminal antibody stained predominantly within the VZ with progressively decreasing expression into the SVZ to the IZ. The staining was diffuse among the radial glia cell bodies and interstitial in appearance. The C-terminal antibody distribution had a radial appearance similar to that of nestin, where the label outlined the radial glia cell membranes from ventricle to pia mater. This supports the idea that Sema5B is processed and that this processing is regulated to separate the inhibitory semaphorin domain from the thrombospondin repeats.

Because we found that the endogenous Sema5B existed in multiple fragments differentially localized within the cortical layers, we wished to re-examine the HEK293 cells expressing recombinant HA-tagged Sema5B for similar proteolysis. We had available to us a secretable version of the Sema5B ectodomain, fused to alkaline phosphatase (m655), which was used as a positive control for Sema5B secretion. Recombinant Sema5B was found in conditioned

Figure 4.1 Sequence analysis of Sema5B supports the endogenous cleavage hypothesis. A) The full 1093 amino acid sequence of Sema5B. All identified domains are highlighted and listed in the legend under C. The sema domain is highlighted in bold pink. Potential proprotein convertase (PC) cleavage sites (RxxR) are highlighted in yellow within whatever domain they are found. B) Schematic view of Sema5B. Major domains are highlighted in the corresponding colours to those in A and C, except the sema domain which is cross-hatched. R1-R5 refer to the five putative PC recognition sites. These regions are expanded to show the specific amino acids surrounding the relevant domains. R2-R4 are within 50 amino acids of each other, and might be difficult to distinguish on a western blot. Without including potential glycosylations, cleavage at each site would produce fragments in the following expected sizes: R1 – 27kDa; R2 – 48kDa; R3 – 50kDa; R4 – 52kDa; R5 – 70kDa. There are 7 N-linked glycosylations in the sema domain and another 4 between the sema domain and R5, which could add considerable weight to the protein. Unpublished data (Wang & O'Connor) suggests that glycosylation of Sema5B's sema domain can add between 10 and 20kDa to the predicted molecular weight. Therefore, R2-4 could create fragments of around 60-70kDa, while R5 could create a fragment of about 80-90kDa, both of which are consistent with the endogenous Sema5N western analysis in chapter 3.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121 eeecqnyvrv livsgrkvfm cgtnafspvc ssrqvgnlsr tiekingvar cpydprhnst
181 avissqgely aatvidfsgr dpaiyrslgs gpplrtaqyn skwlnepnfv aafdiglfay
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B
1
2
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4/5
5BN
R1
R2 R3 R4
R5
5BC
R1
R2
R3
R4
R5
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449-452
471-474
489-492
ggricvgksreerfcnentp
650-653
C
1. Signal sequence = 1-19
2. Sema domain = 68-478
3. PSI domain = 497-544
4. TSP type 1 repeats = 609-662, 667-713, 798-850, 855-907
5. TSP type 1 like repeats = 551-605, 721-776, 908-952
R1-5. RXXR sequences = 259-262, 449-452, 471-474, 489-452, 650-653
KR sequences = 520-521, 819-820
H(X)nKR sequence = 752-759
Region identified by N-terminal antibody on epitope array = 102-116
Region identified by C-terminal antibody on epitope array = 1035-1067
Transmembrane domain = 979-999
Topological domain = 1000-1093

media (Fig 3.2A), detected by both antibodies to the HA tag (located at the N-terminal) and our N-terminal antibody (5BN), consistent with the release of the N-terminal (Fig 3.2A). The approximate size of the Sema5B-HA proteolytic fragment was consistent with the shedding of the entire ectodomain (~100kDa), implying cleavage near the cell surface, consistent with the activity of a transmembrane MMP or ADAM (A Disintegrin And Metalloproteinase; McFarlane, 2003). Indeed, when incubated with GM6001 – a wide-spectrum MMP inhibitor – the media fragment of Sema5B is rapidly depleted (Fig 3.2H). It was therefore concluded that an MMP is responsible for the proteolysis of Sema5B-HA in HEK293 cells.

The secretable m655 was not consistent with the expected molecular weight (a total of 155kDa for alkaline phophatase plus the Sema5B ectodomain). The 5BN antibody recognized only 50 and 60 kDa media fragments consistent with the size of the sema domain (see Figure 3.2I for the schematic). Notably, the fragment(s) identified by 5BN in the m655 conditioned media are similar in size to the smaller of the endogenous Sema5B fragments in brain tissue. Because there are seven glycosylation sites within the sema domain, the predicted 45kDa domain could easily migrate at 50-60kDa as seen in the tissue western analysis in Figure 3.1E. This similarity indicates that m655 may better approximate the conformation of the *in vivo* molecule, and as such is similarly processed. The next appropriate step would be to force expression of Sema5B-HA in the cells that express it endogenously (i.e. radial glia) to verify the endogenous proteolytic processing we see with 5BN with α-HA in western and immunohistochemical analysis.

4.2.2 Sema5B undergoes multiple cleavages to differentially regulate inhibition

Metalloproteases and proprotein convertases are two major types of proteases that serve relevant biological functions during development of the nervous system. These two groups of proteases often interact and undergo complex interdependent functional cross regulations (Scamuffa et al., 2006; Essalmani et al., 2008; Seidah et al., 2008). The spatiotemporal expression of proteases may have significant bearing on the relative levels of the various proteolytic Sema5B fragments; reflecting therefore an intricate and refined developmental regulation of the gross overall balance of repellant and inhibitory roles of the released Sema5B fragments *in vivo*.

There are nine mammalian secretory proprotein convertases (PCs; Seidah et al., 2008). Potential candidates expressed at the time of corticofugal development are PC5A (and possibly PACE4), and PCSK9/NARC-1. PC5A and PACE4 are highly regulated in their germinal zone expression; found at the cell membrane in complex with tissue inhibitors of metalloproteases (TIMPs) and heparan sulfate proteoglycans (Scamuffa et al., 2006; Essalmani et al., 2008; Seidah et al., 2008). PC5A is responsible for the cleavage of membrane-bound L1 in the CNS, at the RKHSKR motif (Kalus et al., 2003). In another membrane-tethered cue, integrin-α4, it cleaves at an HVISKR ST sequence, where the His in close proximity to KR is integral to the cleavage (Bergeron et al., 2003). Interestingly, Sema5B has a very similar sequence (Fig 4.1A) of H_{752}GLQFGKR R, which could potentially serve as a cleavage substrate for PC5A. The last PC with potential relevance to Sema5B processing is PCSK9 (proprotein convertase substilin-kexin-like-9), also known as NARC1 (neural apoptosis-regulated

convertase-1). Although the cleavage site has not yet been identified, PCSK9 is developmentally regulated in the developing neocortex, present at its highest between E12 and 15 in the mouse VZ (Seidah et al., 2003; Seidah et al., 2008). It is also overexpressed in a neuroepithelioma cell line, and plays a positive role in the stimulation of cortical neuron differentiation (Seidah et al., 2003; Poirier et al., 2006). Both PC5A and PCSK9 are embryonic lethal knock outs before E8 due to peripheral defects (Poirier et al., 2006; Essalmani et al., 2008), however they appear to be the most likely candidates for Sema5B cleavage. Mutational analysis would be required to fully elucidate which PC might mediate the mid-ectodomain cleavage observed *in vivo*.

Metalloproteases can be divided into two groups: the matrix metalloproteinases, which can be secreted (MMPs) or membrane tethered (MT-MMPs), and the ADAMs, which are primarily transmembrane molecules (McFarlane, 2003). Both groups are widely expressed in the CNS, and can mediate signaling by cleavage of guidance cues or their receptors (Galko and Tessier-Lavigne, 2000; Hattori et al., 2000; Schimmelpfeng et al., 2001). Evidence for specific activity of MMPs in the mouse cortex involves primarily secreted class 3 semaphorins in situations conferring attraction. Sema3A attracts migrating pyramidal neurons and dendrites (Polleux et al., 2000; Chen et al., 2008) via PKC mediated induction and activation of MMP2 (Gonthier et al., 2009). Meanwhile Sema3C attraction for cortical axons toward the IZ and SVZ requires an activation of MMP3 that is interfered with by inhibitory Sema3A signaling in these same axons (Gonthier et al., 2007). Though much information regarding embryonic expression is not available, according to the Allen Brain Atlas (http://mouse.brain-map.org), of the

approximately 29 members, the highest cortical expression in *adult* mouse brain is seen with the following MMPs (listed from lowest to highest signal strength): MMP7, MMP9, MT5-MMP, MT3-MMP, MMP13, MT4-MMP, and MT5-MMP. All of these have expression in the cortical plate, and could be expected to be expressed on migrating cortical growth cones. However, MT4-MMP, and MT5-MMP appear to have the most expression in the SVZ, near where the N-terminal of Sema5B is localized.

In heterologous cells, I have demonstrated that an MMP is responsible for cleavage of the Sema5B ectodomain (Chapter 3; Fig 3.2 G, H). To determine which type of metalloprotease is involved, there are four endogenous inhibitors of metalloproteases (TIMPs) to be tested (Bode et al., 1999). TIMP-1 inhibits soluble MMPs specifically, while TIMP-2 blocks all MMP activity, including both secreted and transmembrane, and TIMP-3 is an inhibitor for ADAMS (Brew et al., 2000). TIMP-4 is able to inhibit members of all metalloprotease families, including ADAMs, and is therefore not useful in the elimination process (Melendez-Zajgla et al., 2008). Once the general subtype is known, the specific MMP, MT-MMP or ADAM can be identified. Unfortunately, specific recognition sequences for MMPs are not conserved or are undetermined, making mutational analysis difficult.

Determining which MMP uses Sema5B as a cleavage substrate is relevant to the developmental changes in Sema5B described by O'Connor et al (2009) and my unpublished observations (Lett RLM). At earlier embryonic stages, starting around the initiation of neurogenesis in the cortex, the smaller 50-60kDa fragments predominate. Over time, the

smaller fragments found by western analysis become comparatively reduced, while the larger bands that more closely approximate the full ectodomain become progressively enriched (O'Connor et al., 2009). The changes in the relative amounts of the endogenous Sema5B proteolytic fragments may reflect increased levels of MMP expression with a concomitant reduction or inhibition of the relevant PC. Likely, endogenous Sema5B processing involves several proteases, as is the case for L1-CAM (Kalus et al., 2003); *in vivo*, Sema5B appears to undergo multiple cleavages in addition to that which releases the full ectodomain. One fragment appears to be generated by a cleavage separating the semaphorin domain from the thrombospondin repeats, as is the case for the m655 construct (see Fig 3.2 A, I).

Secreted cues can create gradients for "long distance" repulsion or inhibition. In terms of secreted gradients, it is the slope or steepness that determines the nature of the repulsion or inhibition (Isbister et al., 2003). A very steep gradient may produce an effect akin to a physical barrier, causing collapse and retraction; whereas a more gradual slope may have more subtle effects leading to slowed outgrowth and turning or branching. In vitro, the inhibitory nature of a secreted cue can be tested by a collapse assay – where dissociated neurons are incubated with the cue of interest and are later quantified in terms of numbers of collapsed growth cones. This only speaks towards identifying a cue as inhibitory, but does not qualify the nature of the inhibition, be it growth inhibition, or growth cone repulsion. Gradients of inhibitory cues are usually thought to be repellant, where in a purely repulsive gradient; axons sensing an increasing amount of an inhibitor will turn and grow away from the source,

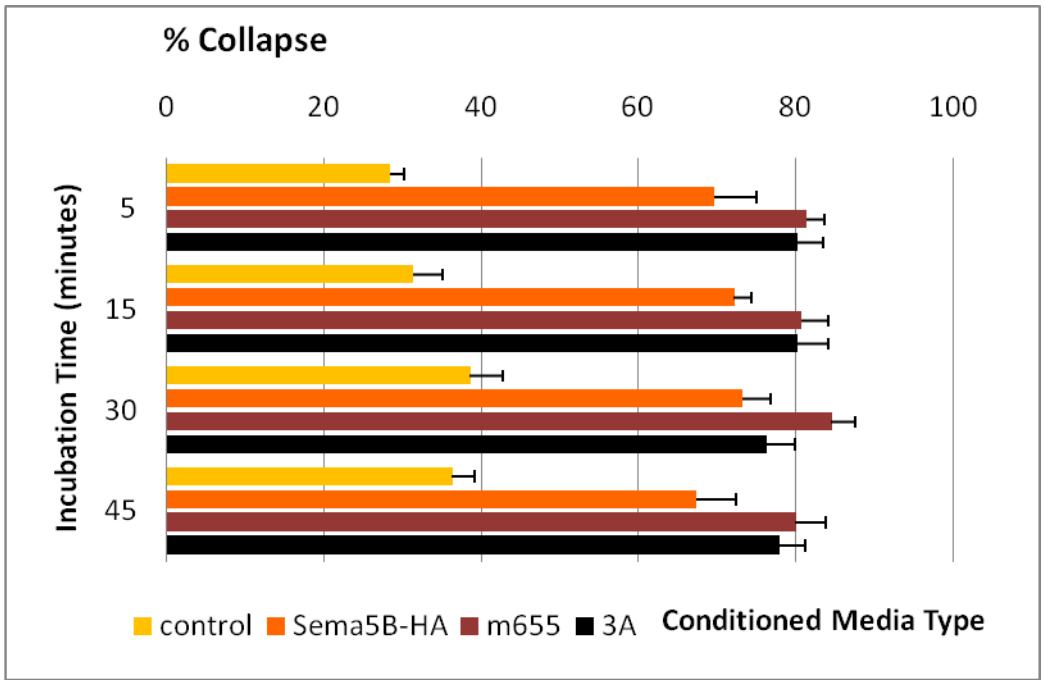
as has been elegantly shown with assays by Moo-Ming Poo (Lohof et al., 1992; Zheng et al., 1996).

As an alternative to repellant guidance, a secreted inhibitor may rather inhibit axon growth without providing directional information. The gradient retains an important aspect here, for increasing amounts of the inhibitor would serve to slow and eventually halt the forward progression of a growth cone. This is comparable to the effects of substrate-bound ephrin gradients used in Eph-expressing retinal axon targeting (von Philipsborn et al., 2006). I will refer to this particular form of inhibition as "growth cone stalling" – where the growth cone may or may not collapse, but the axon itself can no longer advance owing most likely to a balance in retrograde flow, actin assembly, and coupling of microtubules and the substrate with F-actin (Gallo and Letourneau, 2004). A recent study of DRG centrally projecting axons growing within the spinal cord has suggested that Sema3A not only repels axons, but also can inhibit growth cone advance through inhibition of the growth promoting NGF/TrkA signals (Ben-Zvi 2008). Other similar examples of secreted gradients for axon growth inhibition are found throughout the nervous system during development (Campbell and Holt, 2001, 2003; Sanford et al., 2008), and Sema5B is possibly one such protein; capable of growth inhibition, and repulsion, depending on circumstances.

When dissociated cortical neurons were incubated with conditioned media from Sema5B-HA cells, a significant proportion of axonal growth cones collapsed compared with media from control cells. This effect was similar to but slightly less effective than that of the renowned

collapsing agent Sema3A, and to the effect of m655 media, both of which reached a maximum collapse by five minutes. Sema5B-HA media reached a height of collapse at 30 minutes, but never collapsed cells to the same extent as m655 media. In the collagen gel assays of chapter 3, I demonstrate that recombinant Sema5B can diffuse from a cell aggregate to inhibit cortical axon growth from a distance. This does not however support a directive "repellant" role for the Sema5B ectodomain, although closer analysis of growth cones and turning angles in assays with Sema5B-HA vs m655-expressing cell aggregates may support a more directive role for the m655 secreted form (see Fig B.1 for additional examples).

Figure 4.2 Time course of semaphorin-induced cortical growth cone collapse.
4 DIV cortical neuron cultures were incubated with media from control cells (yellow bars), or cells expressing Sema5B-HA (orange bars), m655 (maroon bars), or Sema3A (black bars). Data from the 30 minute time-point was depicted in Fig. 3.2 of this thesis. Sema5B-HA-induced collapse was evident at 5 minutes, but reached a maximum of 73% at 30 minutes. At 45 minutes the amount of collapse was in decline. m655 was immediately as effective as Sema3A at producing collapse of around 80%, which was largely maintained for all examined time points. The percent of growth cones under Sema3A-induced collapse at 30 minutes was already in decline.



The proteolysis of endogenous Sema5B and apparent segregation of the N- and C-terminal fragments *in situ* caused a slightly different light to be shed on the gain of function experiments (Fig 3.1). Contact inhibition as seen in the cell island assay remained possible because based on western analysis not all of Sema5B was proteolyzed in heterologous cells. Alternatively, or perhaps additionally, a short range gradient from cell islands could mediate the avoidance and collapse I describe in chapter 2. I also demonstrated that transplanting Sema5B-expressing cells onto the normally non-Sema5B-expressing internal capsule was sufficient to induce corticofugal pathfinding errors. Usually these errors amounted to simple stalling of outgrowth near the border of ectopic Sema5B-expressing cell. Occasionally corticofugal axon interaction with Sema5B ectopias resulted in mistargetting back towards the cortex (Fig 2.4). Both stalling and inappropriate turning seem to be similar, if not slightly more pronounced when the organotypic cell overlay assay was performed with the secretable m655-expressing cells (Fig B.1C). Furthermore, the stalling behavior and occasional repulsive turning seen in the organotypic overlay with full-length Sema5B-HA expressing cells was highly reminiscent of the growth cone and axon outgrowth of cortical explants in the collagen gel assays I used to test for Sema5B inhibitory gradients from expressing HEK293 cell aggregates, though again there is a hint of increased repulsion, rather than mere axon inhibition (Fig 3.3; Fig B.1).

The absence of most or all of the thrombospondin repeats could underlie this proposed difference in growth cone responses (inhibitory versus repulsive) to the respective gradients of Sema5B-HA and m655 expressing cells. Work with the chicken version of Sema5B has

employed mutations lacking the thrombospondin repeats (δTSR), lacking the sema domain (δsema), and variants in-between (Wang, Lett & O'Connor, unpublished data). Cells expressing δTSR were substantially more repulsive to growing axons, whereas δsema cells became a preferred substrate. The wildtype Sema5B, similar to the mouse Sema5B-HA, was cleaved to release the ectodomain, and axons in the vicinity were likely to stall, rather than be repelled. It would seem as though the TSRs are auto-modulatory toward the sema domain. The regulation of the degree of Sema5B repulsion would therefore be regulated by the proteases. Because the *in vivo* fragments seem to represent both the Sema5B-HA and m655 fragments, I believe that *in vivo*, the Sema5B sema domain is repulsive, and that the different fragments are created from alternative cleavages that would mediate different aspects or levels of inhibition – growth inhibition (stalling) and repulsive guidance.

4.2.3 Redefining the corticofugal model – Sema5B as a secreted inhibitor

In order to determine the necessity for Sema5B in corticofugal pathfinding, I used electroporation of Sema5B-shRNAs to knock down Sema5B expression in the dorsal ventricular zone near the CSB. With either construct, Sema5B was reduced sufficiently to permit inappropriate descending cortical axon growth into the VZ (Fig 2.7). In the context of Sema5B as a secreted inhibitor, I propose the following model.

Sema5B is expressed by the radial glial progenitors, destined for the cell surface by an export signal sequence, and becomes processed along the way by a furin-like proproteinase convertase, at a site between the sema and TSR domains. Alternatively, Sema5B may be

expressed on the cell surface prior to cleavage by MMPs and surface expressed PCs, which are often MMP-dependent. Either way, the N-terminal fragments bearing the sema domain would be released by the radial glia near the somas, therefore concentrating in the ventricular zone, and diffusing into the SVZ. This gradient, based on immunohistochemistry, appears as relatively steep, with a high concentration in the VZ and a much lower concentration in the SVZ (Fig 3.1).

Axons descend from the cortical plate due to repulsion primarily by Sema3A, while being attracted toward the SVZ by an attractive semaphorin, Sema3C. As they arrive at the Sema5B gradient in the IZ just outside the SVZ they would be inhibited from entry into the germinal regions. The slit-2 gradient from medial cortical regions also inhibits the robo-expressing cortical axons. Therefore, cortical axons could be assumed to encounter two inhibitory gradients pushing from the ventricular and the medial toward the lateral domains, along the developing IZ toward the CSB. Repulsion is maintained from the cortical plate primarily by continuous Sema3A expression, and from Sema5B in the germinal zones.

There are two additional regions of Sema5B expression that are significant due to their locations, though these are yet to be functionally dissected and therefore may underlie other developmental processes. I believe that Sema5B expression from the ventrolateral regions at the terminal migration destination of the CSB/LMS also likely maintain and funnel the corticofugal trajectory toward the subcortex. In addition, and perhaps more importantly,

Sema5B expression along the corticostriatal boundary may also contribute to the temporary barrier to cortical axon progression at around E13.5 and for 24 hours thereafter.

Figure 4.3 Initial guidance of the corticofugal projection.
Subplate neurons, shown in dark orange, form the pioneer projection to subcortical regions. Axons are repelled by slit1 and Sema3A from cells of the cortical plate, and descend toward the subventricular zone (SVZ) attracted by a gradient of Sema3C. There is some indication that reelin, secreted by Cajal-Retzius cells, and crucial to the appropriate inside out development of the cortex, may also act as a repellant for cortical axons. Meanwhile, though more important for inhibiting cortical axons from crossing the diencephalic midline, a slit2 medially derived gradient also promotes lateral guidance of cortical axons. Finally, cortical axons maintain avoidance of the SVZ and VZ germinal layers due to Sema5B, produced by radial glia, cleaved, and secreted to create a sharp inhibitory gradient from ventrical to intermediate zone.

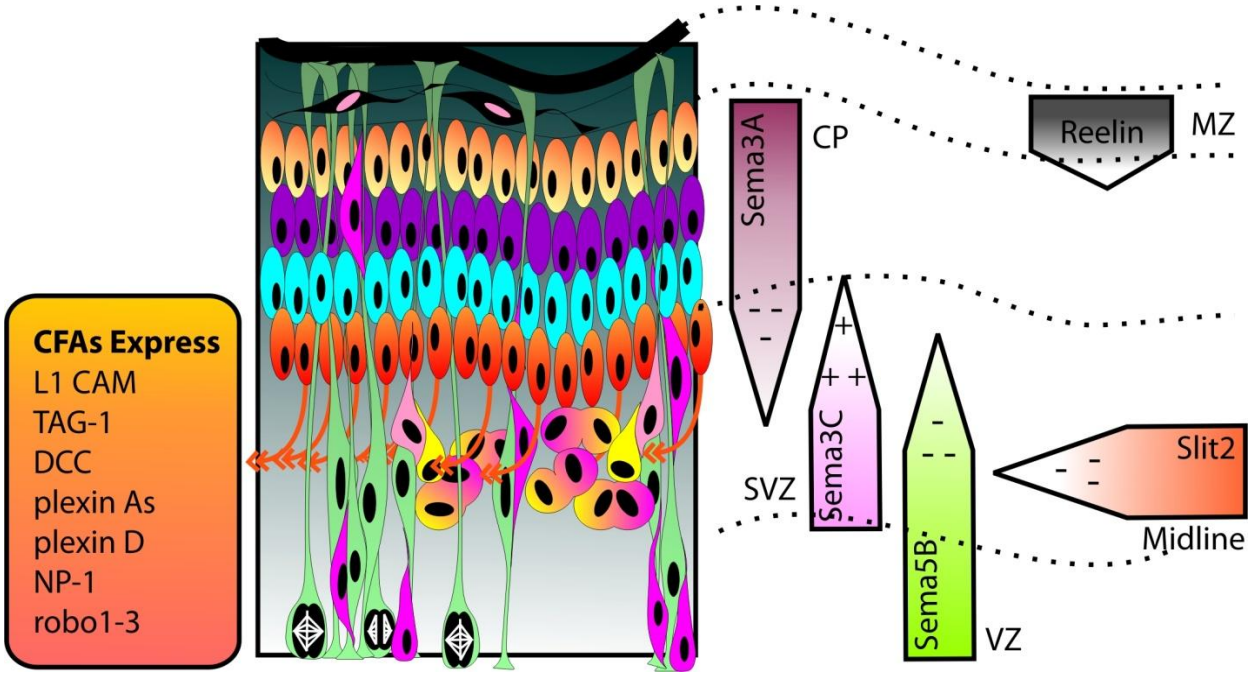
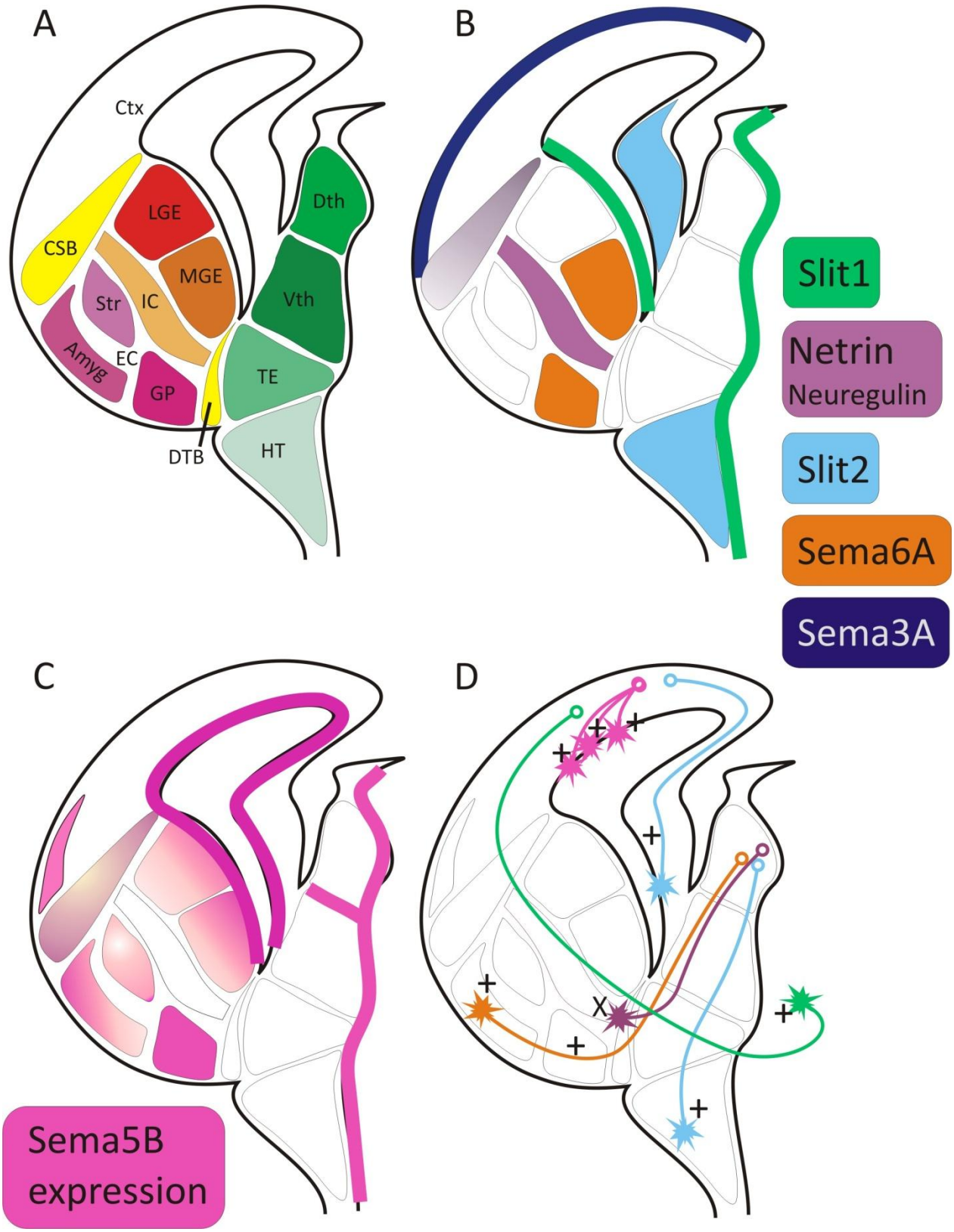


Figure 4.4 Mutant phenotypes of corticofugal and thalamocortical pathfinding.
A) Schematic of mid-late developing anterior CNS. Major regions are illustrated. Ctx: neocortex; CSB: Cortico-striatal boundary; Amyg: amygdala and related basal nuclei; Str: Striatum; GP: Globus pallidus; LGE: Lateral ganglionic eminence; MGE: Medial ganglionic eminence; IC: Internal capsule; EC: External capsule; DTB: Diencephalic-telencephalic boundary; Dth: Dorsal thalamus; Vth: Ventral thalamus; TE: Thalamic eminence; HT: Hypothalamus. Note the colours in A do not relate to the legend in B. B) Expression of other guidance cues relevant to cortical and thalamic pathfinding. C) Schematic of Sema5B expression, shown in pink. Expression flanks the cortical projection. D) Guidance errors made by cortical and thalamic axons in the context of various losses of function. 'X' denotes gain of inhibition, '+' denotes loss of inhibition, or novel permissive region. Colors of axons correspond to the color of the cue indicated in the legend. Pink corticofugal axons descend inappropriately into the germinal zones of the neocortex in the absence of Sema5B. Green cortical axons grow into ventomedial locations in the absence of Slit-Robo signaling in the cortical midline. Purple axons are prevented from penetrating beyond the DTB without the permissive corridor created by migrating LGE cells through the MGE (López-Bendito et al., 2006). Internal capsule cells in this corridor normally express netrin and neuregulin, which provide attraction to draw these axons through the subcortex, and without which, Dth axons do not seem to progress. Most recently, Sema6A has been found to provide inhibition in the MGE and GP for the maintenance of the initial thalamic subcortical projection. In the absence of Sema6A, Dth axons enter the subcortex too ventrally, and grow along that trajectory into amygdaloid nuclei. Although some of these axons do innervate the appropriate cortical regions eventually, these mutations help provide important information for the coordinated regulation of telencephalic tract formation.



4.2.4 Evidence against the handshake hypothesis.

The so-called "handshake hypothesis" states that the growth cones of corticofugal and thalamocortical axons must physically interact within the LGE. Subsequent to initial contact, these axons utilize each other as scaffolding on which to fasciculate in order to reach their respective reciprocal targets in the thalamus and cortex. This interaction is said to be necessary for correct pathfinding past the pioneering stage and eventual targeting. Around E14.5 in the mouse, the time corticofugal fibers are about to enter the LGE after their pause at the CSB, thalamocortical axons have nearly completed growing through the putative internal capsule (Molnár and Butler, 2002). Cortical axons are thought to be attracted to the internal capsule in the region of the LGE by netrin-1 expression (Fig. 4.2B), which, in addition to both secreted and substrate bound neuregulin, also creates attraction to the ascending thalamic axons (Métin et al., 1997; Braisted et al., 2000; Bagri et al., 2002; Dickson and Gilestro, 2006). It is just beyond the CSB where the growth cones of cortical and thalamic axons have been proposed to interact; an occurrence that is considered vital to their proper continued guidance by using the reciprocal pathway as physical scaffolding to arrive at their reciprocal targets (Vanderhaeghen and Polleux, 2004).

Though evidence exists both in support and refutation of this model, the work presented in Chapter 2 supports the idea of independence between corticofugal and thalamocortical tracts. Both sets of axons are inhibited by an unknown factor in the MGE (López-Bendito and Molnár, 2003; Molnár et al., 2003; López-Bendito et al., 2006), which I had originally hypothesized, albeit incorrectly, to be Sema5B (see expression pattern in Fig. 4.2). Not only are thalamic axons non-responsive to Sema5B, but their trajectory to the cortex remains

intact, in spite of errors and stalling of cortical axons attempting to form the reciprocal pathway (Fig 2.5, see also Fig 4.2). Therefore, thalamic axons do not utilize the Sema5B present in the MGE and GP, as I had suspected, but also my evidence points to thalamic axons not requiring interactions with cortical axons at or near the CSB in order to reach their final targets in the cortical plate.

Evidence now shows that Sema6A is responsible, at least in part, for inhibiting thalamic axons from entering the basal telencephalon and amygdaloid body (Little et al., 2009), while it appears to be Sema5B that performs this role for corticofugal axons (Chapter 2). Cortical and thalamic axons therefore do not express the same complement of receptors, particularly the plexin semaphorin receptors. In addition, although Pax6 is a necessary component for the proper development of the cortex and its lamination, the guidance errors of thalamocortical axons in a Pax6 mutant are significantly more severe. This may however reflect the change in adhesion and exclusion presented by the CSB due to the resultant down-regulation of R-cadherin along this boundary (Andrews and Mastick, 2003).

Further support for the independence of thalamocortical pathfinding can be found among mutants. In the *reeler* knock out cortex, thalamic projections meet their appropriate targets in what should be layer 4 of the cortical plate in spite of complete cortical disarray (Molnar et al., 1998; Little et al., 2009). Similarly, thalamic axons in Sema6A mutant cortices are misrouted through the ventral subcortex and amygdalae, missing the internal capsule and CSB; however, they are still eventually able to target the appropriate cells of the cortex (Little et al.,

2009). In fact, these thalamocortical axons are unlikely to have encountered corticofugal axons, as they miss the CSB target. Therefore thalamic axons do not rely on the cortical trajectory, but in addition, the arealization of the cortex is also not a result of the supposed handshake, for cortical identity is also maintained in the absence of this interaction.

4.2.5 A receptor for Sema5B?

Although corticofugal/corticothalamic and thalamocortical axons travel the same general pathway in the telencephalon (Coleman et al., 1997), there are expression differences that dictate differential responsiveness along these respective and mutual guidance pathways (López-Bendito and Molnár, 2003). One major difference is that only the corticofugal projection responds to Sema5B, even though it is ideally positioned to guide both (Chapter 2; Lett et al., 2009). Another significant difference is that although both tracts express L1-CAM, only corticofugal axons express axonin-1, also known as transient axonal guidance-1 (TAG-1). TAG-1 is responsible for modulating the functions and interactions of many other proteins, including cell adhesion molecules such as L1 and nrCAM and integrins, as well as guidance cue receptors (Felsenfeld et al., 1994; Malhotra et al., 1998; Wolman et al., 2008).

TAG-1 is expressed along several major tracts in the developing central nervous system (Karagogeos et al., 1991; Vaughn et al., 1992). The entire corticofugal tract expresses TAG-1 until around E17-18; around the time that layer VI axons enter the dorsal thalamus (Fujimori et al., 2000) and the follower corticospinal axons from layer V decussate at the junction of the brainstem and spinal cord (Wolfer et al., 1998). The loss of TAG-1 at this stage is thought to be

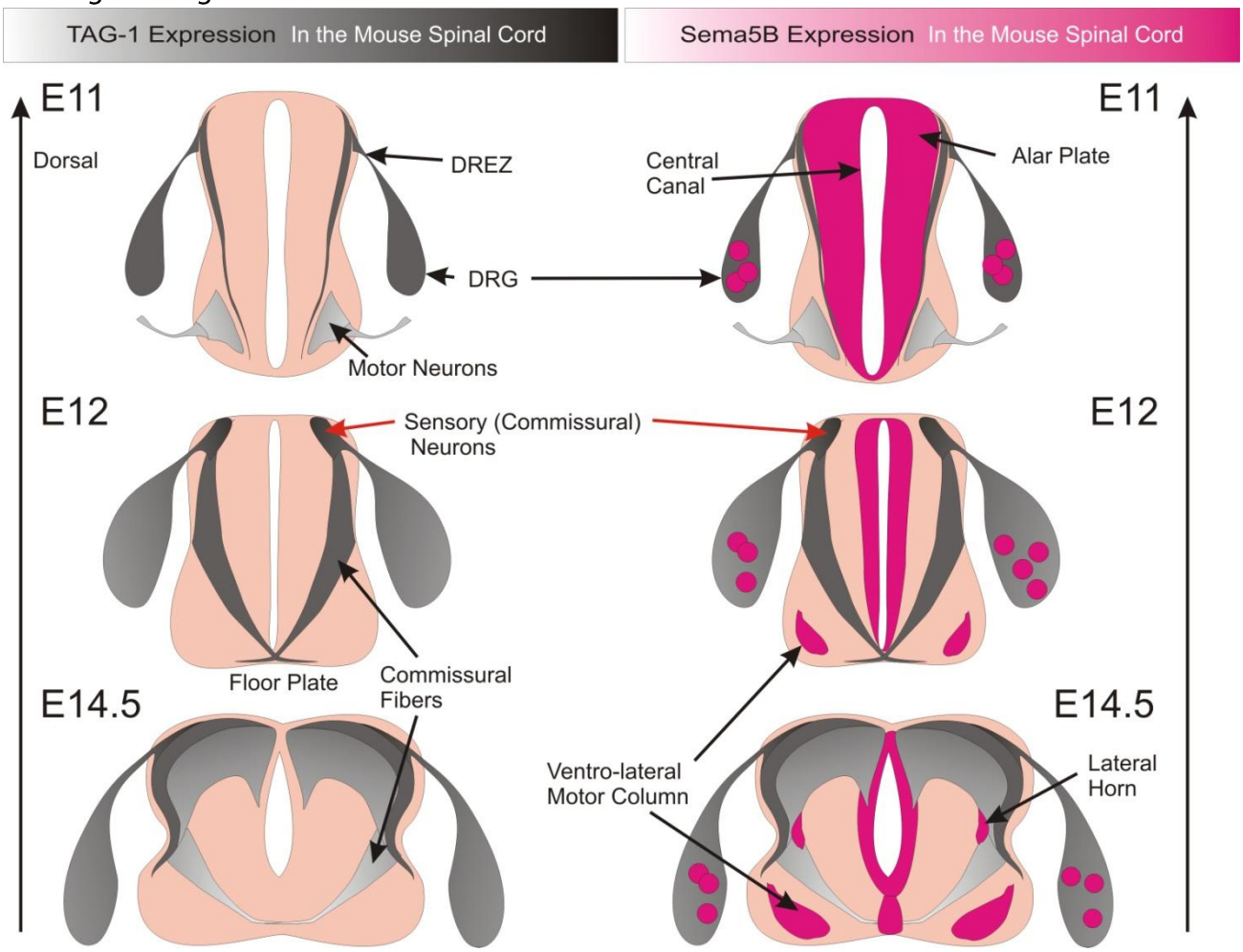
commensurate with an altered sensitivity to certain guidance cues, in particular those expressed along the midline (Fujimori et al., 2000). Chapter 2 illustrates that the TAG-1 expressing corticofugal axons show a repulsive response to Sema5B (see also the expression of TAG-1 in the cortex overlaid with Sema5B expression, Fig 3.4 A-C).

Recent studies have provided evidence for TAG-1 as a necessary component of a receptor complex mediating repulsion of sensory fibers descending into the ventral spinal cord (Masuda et al., 2003). Some of this TAG-1-mediated repulsion is induced by Sema3A via the neuropilin-L1 complex while some of this repulsion is from an unknown source in the ventral spinal cord (Law et al., 2008). I believe that one possible constituent of this repulsion is Sema5B as it is strongly expressed in the ventral spinal cord.

Sema5B is a potential candidate inhibitor for axon projections entering the ventral spinal cord. The spatiotemporal characteristics of pathfinding by dorsal root ganglion (DRG) axons and dorsal spinal sensory axons (commissural fibers) expressing TAG-1 during spinal cord development (Fig4.3; Vaughn et al., 1992) appear to coordinate with Sema5B expression at the corresponding appropriate ages (Appendix B; Fig B.2; Fig 4.3). At early stages, mouse Sema5B is expressed in the ventricular zones, as well as the secondary generative zone of the alar plate in the dorsal SC, which are exactly avoided by TAG-1 commissural fibers (Fig. 4.3). Later as DRG axons must enter the spinal cord, Sema5B expression becomes restricted to the VZ and the ventrolateral motor column and the early lateral pre-ganglionic autonomic motor

Figure 4.5 TAG-1 and Sema5B expression in the developing mouse spinal cord .

Dorsal root central fibers are TAG-1-positive, and apparently do not lose this expression (Karagogeos et al., 1991). Motor neuron expression of TAG-1 is lost very early, while TAG-1 expression in the developing commissural fibers, which are from the secondary sensory (commissural) neurons in the dorsal horn, is highly dynamic. Expression is high until these axons have passed the floor plate, around which point TAG-1 is downregulated. The left-hand panel shows TAG-1 expression alone. The strength of TAG-1 expression is depicted by the grey gradient. Dark greys indicate higher expression. Light greys indicate low expression. Adapted from primary data in Vaughn et al, (1992). The right-hand panel shows the same TAG-1 expression, but includes Sema5B expression, depicted in magenta. Early in development, Sema5B is expressed in the secondary differentiating field of the alar plate in the dorsal spinal cord. As neurogenesis in the dorsal spinal cord ends, Sema5B expression is down-regulated to the VZ of the central canal. Expression begins to show in the ventrolateral motor columns of the ventral spinal cord. At E14.5, Sema5B in the spinal cord is low, except at the VZ, the floor plate (to a certain extent), and the emergent lateral horn consisting of preganglionic sympathetic neurons. Expression in the ventrolateral motor pool remains robust. Throughout these stages, a subset of cells in the DRG also appears to express Sema5B, though the significance of this is unclear.



horn (Fig B.2 G, H). Sema5B-expressing territories are strictly avoided by TAG-1 fibers in the developing spinal cord.

Retinal ganglion axons also must enter the optic tectum without prematurely penetrating the neuronal layers (Chatzopoulou et al., 2008). The layers of the optic tectum express Sema5B, and the TAG-1 positive retinal axons also avoid Sema5B in co-culture with expressing cells (unpublished observations, Wood JL and O'Connor TP). Therefore, TAG-1 expression in axons can be correlated to sensitivity to the inhibitory effects of Sema5B. In further substantiation of these observations, I also show in chapter 3 that 4D7, a monoclonal IgM antibody with a proven blocking function against TAG-1 blocks Sema5B-induced cortical growth cone collapse, while our 5B-N antibody binds but does not block this action (Fig 3.4). Further investigation will reveal whether this is a direct interaction between Sema5B and TAG-1, or as for Sema3A the TAG-1 requirement for a repulsive growth cone response is mediated through a complex of receptor molecules. My hypothesis that TAG-1 mediates Sema5B-induced inhibition could be lent further support if I were to assess Sema5B-knockdown from the cortical VZ in a TAG-1 knockout background, demonstrating a lack of misguidance. Additional co-cultures with TAG-1-/- cortical explants and Sema5B-expressing cells would confirm a loss of response to Sema5B due to lack of TAG-1 activity. I could also knock down both Sema3A and Sema5B in the spinal cord, and examine whether the dual loss mimics the errors in commissural axon guidance as seen in with the loss of TAG-1 by Law and colleagues (2008).

4.3 Not just another semaphorin repellant…

4.3.1 The benefits of a two-in-one protein

One of the interesting aspects of this protein is the implication that, in addition to being relatively unique as a secreted molecule released from a transmembrane molecule, Sema5B may very well be a "two-in-one" protein. Many active soluble inhibitors are synthesized as a proprotein with an initial inactive or less potent state, and are then cleaved to remove an autoinhibitory domain, or some additional sequence that reduces activity. This case is found with many semaphorins, other guidance cues, and a large number of growth factors and morphogens (Essalmani et al., 2008; Seidah et al., 2008). By contrast, Sema5B is created as a transmembrane protein that is cleaved not to release a single active peptide, but rather into two portions, both of which have demonstrably important functions. This would categorize it along the lines of the reported two-in-one F-spondin protein that is cleaved in half and provides substrate adhesion and repulsion for commissural axons traversing the floor plate of the ventral spinal cord (Tzarfati-Major et al., 2001; Zisman et al., 2007).

I believe that the proportion and number of the thrombospondin repeats that would be retained by the transmembrane portion subsequent to cleavage is suggestive of a potentially significant biological activity. As described earlier, Sema5B may undergo different proteolytic events (either as a function of the temporal or spatial regulation of protease expression) and that these cleavages may alter the overall ability of Sema5B to induce inhibitory or repulsive actions based on differential retention of thrombospondin repeats (TSRs) within the secreted N-terminal fragment. During the height of neurogenesis and corticogenesis, the predominant

in vivo N-terminal fragments are 80 and 60 kDa. The relative proportions of these fragments have been found to change as development proceeds, such that the 60kDa fragment becomes less prominent in favor of the 80kDa fragment (O'Connor et al., 2009).

The cleavage of Sema5B has multiple implications. For one, as I have previously discussed, the N-terminal portion bearing the sema domain would be secreted in vivo to create inhibitory gradients from its expression locale. I have argued that this is the case for the ventricular zone in the dorsal cortex. If the sema domain is released from the membranes, as our data suggests, then what becomes of the remaining portion? Based upon the sizes observed with western analysis and the distribution of N- versus the C-terminal fragments of Sema5B (as discussed in Chapter 3 and earlier in this chapter), I have concluded that during corticogenesis these two portions are segregated and that the N-terminal consists of the sema domain, whereas the remaining membrane-tethered C-terminal portion bears the TSRs. The N-terminal fragments are found in the interstitial spaces between the radial glia cells bodies and are localized in a gradient that is high in the ventricular zone and graded toward the intermediate zone. The C-terminal antibody labels very differently from the N-terminal and, like radial glial cytoplasmic proteins such as nestin or plasma membrane-tethered proteins such as N-CAM, clearly outlines the fibers of the radial glia and not the interstitial spaces (Fig 3.1). An analysis of cortical tissue lysate with the C-terminal antibody to Sema5B on a western blot indicates that after cleavage enough of the Sema5B protein is left behind to contain a majority of the seven thrombospondin repeats. I propose that the TSRs are therefore expressed along the length of the radial glial fibers.

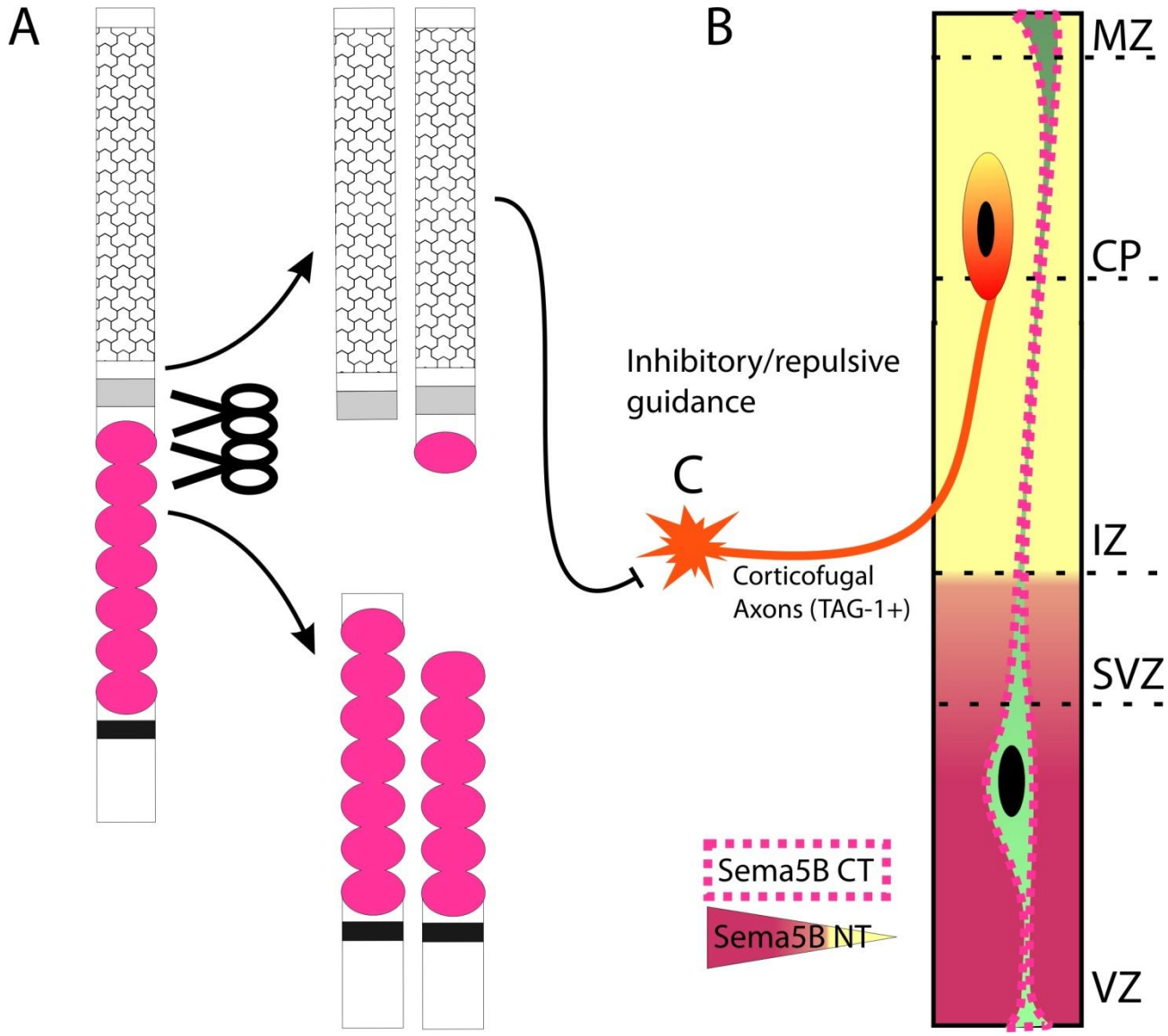


Figure 4.6 Divergently localized fragments of proteolyzed Sema5B in the cortical layers. A) Schematic of Sema5B and the major cleavages that occur in the cortex in vivo to produce N-terminal fragment(s) bearing the semaphorin domain (hatched) responsible for the inhibitory guidance of corticofugal axons. The C-terminal portion(s) remain tethered to the plasma membrane and bear most if not all the TSRs (pink). B) The N-terminal fragments are depicted as existing extracellularly in a gradient that is high within the VZ and graded in the SVZ toward the IZ where its presence is negligible. This gradient repels the TAG-1+ corticofugal axons (orange), shown in ©, before they enter the germinal zones, and restricts them to the IZ. The membrane-bound C-terminal portions of Sema5B appear to diffuse along the entire length of radial glial fibers, and are depicted as pink dots along the radial glial cell (green).

4.3.2 Left behind: Implications for the function of the isolated membrane-bound TSRs

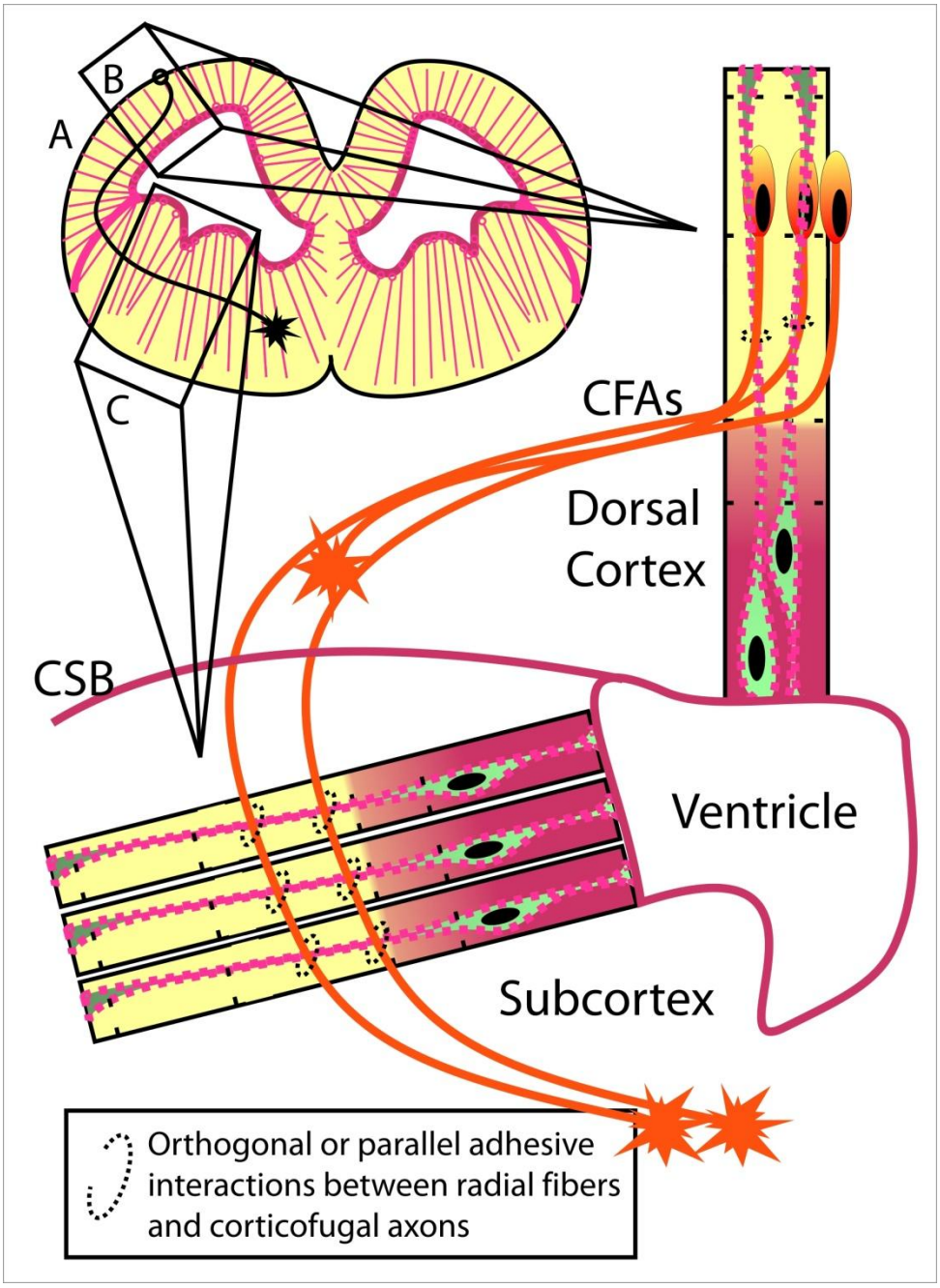
The ramifications for the remaining Sema5B-TSRs on radial glia are numerous. Thrombospondin repeats have a multitude of important functions in the nervous system, particularly during development (Adams and Tucker, 2000): 1) they may provide a permissive substrate for neurite outgrowth (Hamel et al., 2008); 2) the prototypic molecules bearing thrombospondin repeats (TSP-1 and -2) are known to promote synaptogenesis in the dorsal neocortex and hippocampus (Christopherson et al., 2005); 3) they can mediate interactions with heparan and chondroitin sulfate proteoglycans (Yamada et al., 1997; Hamel et al., 2005), and; 4) they are capable binding and activating latent TGF-β1 into its active form (Schultz-Cherry and Murphy-Ullrich, 1993).

One potential function for the Sema5B-TSRs is to mediate permissive interactions with migrating neurons and growing axons. The distribution of the C-terminal fragment of Sema5B indicates that it is localized along the radial fibers of the primary neural progenitors. In unpublished experiments, it has been shown that the Sema5B-TSRs in chick are highly permissive to growing DRG axons that normally are repulsed by wildtype Sema5B, such that they prefer Sema5B-TSR-expressing cells compared to control cells (Wang, W., Lett, R.L.M., and O'Connor, T.P). In a similar fashion, though untested, I hypothesize that Sema5B-TSRs are highly permissive to cortical axons as well, and may therefore aid in their outgrowth toward the IZ along radial fibers, and subsequently across these fibers as they pathfind through the IZ toward the CSB. This type of positive substrate may be necessary to permit continual out-growth of the axons (Jay, 2000; Cooper, 2002; Bard et al., 2008), which cannot advance

without the appropriate signals shifting the balance toward growth cone protrusion (Suter and Forscher, 2000; Wolman et al., 2008; Lowery and Van Vactor, 2009).

In the closest relative to Sema5B, Sema5A, the thrombospondin repeats are crucial to its overall function by mediating interactions with heparan and chondroitin sulfate proteoglycans (Kantor et al., 2004). These interactions presumably effect the conformation of the molecule, altering its binding capabilities with presently unknown axon guidance receptors to elicit either repulsion or attraction, depending on the proteoglycans present. An immunological receptor (plexin-B3) was determined and validated in heterologous cells (Artigiani et al., 2004; Sadanandam et al., 2007), but was disputed when the CNS axon system shown to respond to Sema5A did not rely upon Plexin-B3 signaling (Kantor et al., 2004).

Figure 4.7 Sema5B-TSRs as a permissive substrate for corticofugal axon extension.



A) Cross section of the mouse cortex at E14 demonstrating the organization of radial glial fibers. B) In the dorsal cortex, CFAs may interact with Sema5B-TSRs expressed along radial fibers, both in parallel as they descend and orthogonally as they turn laterally within the IZ. C) Similarly in subcortical regions, CFAs may interact with radial fibers as they grow across them via the putative internal capsule. Sema5B-TSRs are shown as pink dots along radial fibers. The N-terminal gradient is shown in dark pink and CFAs are in orange. CFA: corticofugal axon; CSB: corticostriatal boundary.

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Appendix A: Testing guidance cues: common functional assays

A.1 Three-dimensional repulsion assays for secreted guidance cues

Typically, the actions of secreted molecules have been examined by co-culturing cell aggregates that express guidance cues with neuronal explants in 3-dimensional matrices (Lumsden and Davies, 1983, 1986), composed of polymerized collagen, matrigel, or similar support. In these matrices, a repellant, an attractant, or a trophic factor can be determined by monitoring the differences in neurite behavior on the side of the explant proximal to the aggregate compared with the distal neurites. A ratio greater than 1.0 indicates attractive tropic or trophic activity, whereas a ratio less than 1.0 is indicative of an inhibitory or repulsive factor. This assay can also determine whether or not a protein is secreted and capable of generating gradients.

A.2 Two-dimensional assays for substrate bound guidance cues

Contact-dependent assays are performed in two dimensions (Walter et al., 1987). A commonly utilized assay is the membrane stripe assay, where explants are seeded on orthogonally oriented lanes of cell membranes prepared from expressing or non-expressing cells. Neurites extending from the explant therefore make a choice of which substrate they prefer to extend upon (Mann et al., 1998; Skutella et al., 1999). Inhibitory substrates will contain less overall outgrowth than controls, which would alternatively contain less overall outgrowth with respect to a permissive substrate (Walter et al., 1987; Castellani, 1997). A simpler and more accessible system is the "cell island" assay (Heron et al., 2007; Lett et al., 2009). Rather than using cell membranes, which are generated from disrupted cells, a contact-response assay is

generated by seeding low densities of cells expressing guidance molecules on coverslips in co-culture with explants.

A.3 Organotypic slice cultures provide a physiologically relevant ex vivo model

Two- and three-dimensional explant co-cultures evaluate the activity of an isolated factor in development during processes such as migration or axon guidance; however, this method is unable to examine a factor in the context of any other cues that would normally be present. A 300um thickness of the telencephalon allows for the maintenance of a certain amount of 3-dimensionality to allow for examination of cues in a pseudo-physiological environment including endogenous distribution of other cues (in addition to the one being studied) within an arrangement of cytoarchitechture that is within physiologically normalcy of the tissue. Within this system, both axon guidance and cell migration may be studied under both loss and gain of function paradigms, if certain caveats regarding developmental distortions after long term culture are taken are into account and controlled for (Roberts et al., 1993; Humphreys et al., 1996).

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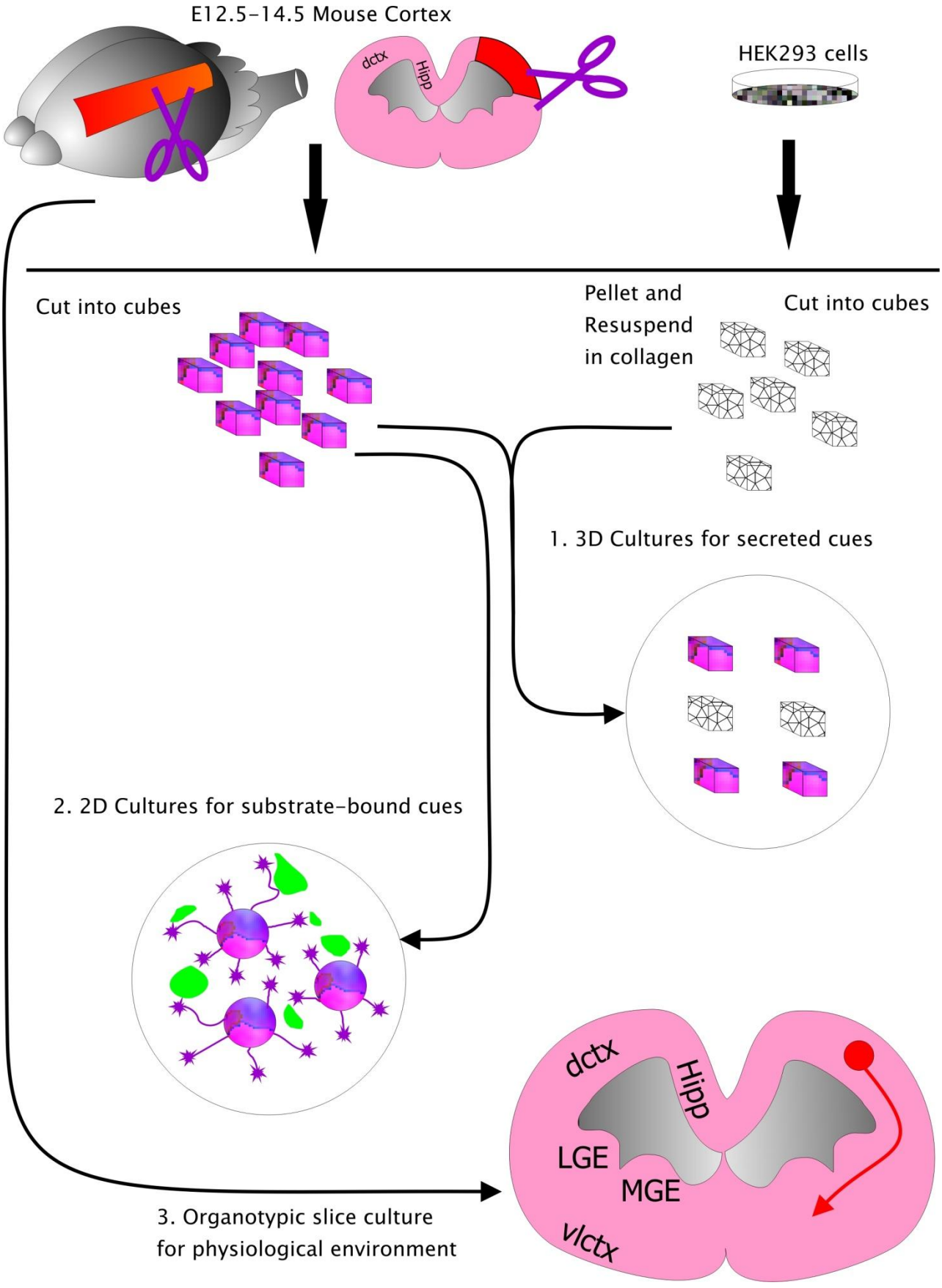
Figure A.1 Assays for the evaluation of guidance cue function.
The best age to create explants and slice cultures to test cortical projection axons in their responses to a guidance cue is between E12.5 and E14.5 when subplate neurons and deep layer neurons are established and undergoing axonogenesis and pathfinding. For assays 1 and 2, the dorsal neocortex is isolated (shown in red on whole brain or coronal slice view), and then diced into small cubes. For assay 1, HEK293 cells expressing a guidance cue are grown to confluence, then spun down into a pellet and resuspending in a collagen mixture. Once the collagen is solid, the cell aggregates are also cut into cubes. Brain explants and cell aggregates are placed as shown in (1), and cultured to determine secreted capabilities. For assay 2, brain explants are plated on coverslips that are also seeded with below confluence amounts of guidance cue-expressing HEK293 cells, shown as green "islands" surrounding explants in (2). Assay 2 is a contact-mediated axon guidance assay. More intricate and physiologically "normal" assessments of guidance cue function can be performed in slice cultures, or assay 3. Whole brains are embedded and coronally on a vibratome. They can be manipulated by electroporation, or by the addition of HEK293 cells directly to their surface. DiI is often used to label axons, or migrating cells, and can be used to follow these as they react to knock downs or novel ectopias of expessing cells.


E12.5-14.5 Mouse Cortex
dctx
Hipp
HEK293 cells
Cut into cubes
Pellet and
Resuspend
in collagen
Cut into cubes
1. 3D Cultures for secreted cues
2. 2D Cultures for substrate-bound cues
dctx
Hipp
LGE
MGE
vlctx
3. Organotypic slice culture
for physiological environment

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Appendix B: Supplemental data for chapters 3 and 4.

B.1 The secreted version of Sema5B and Sema5B-HA have similar properties *in situ*

This thesis is composed of two manuscripts, of which one is published and the second under review at the time I am writing. Some of the work for the support of arguments made in chapter 3 is still relatively preliminary and cannot be included in the manuscript in such time as the appropriate replications and quantifications can be completed. In Figure B.1 I show that the m655 protein, that is engineered to be secreted, produces phenotypically similar results in the organotypic gain-of-function assay (Fig B.1 C), and as shown under several conditions in Chapter 3 is inhibitory, and possibly repulsive (see discussion, pp136-139), for cortical axons when secreted into the collagen gel assay (Fig B.1 D). I have shown in Chapter 2 that Sema5B-HA produces cortical axon misguidance in gain-of–function assays, and in Chapter 3 that Sema5B-HA is cleaved for the creation of inhibitory gradients for cortical axons in collagen gel assays. The intentionally secreted version of Sema5B (m655) was used as a positive control to demonstrate and support the evidence that the wild type, HA-tagged Sema5B was also acting as a secreted inhibitor.

B.2 Additional evidence that Sema5B is an inhibitor for TAG-1 expressing axons

Chapter 3 also provides evidence of the cell adhesion molecule, TAG-1, as likely to be a necessary component of a multimeric receptor complex mediating the inhibitory effects of Sema5B. I came to this hypothesis by combination of evidence in the literature (Furley et al., 1990; Karagogeos et al., 1991; Vaughn et al., 1992 ; Felsenfeld et al., 1994; Milev et al., 1996;

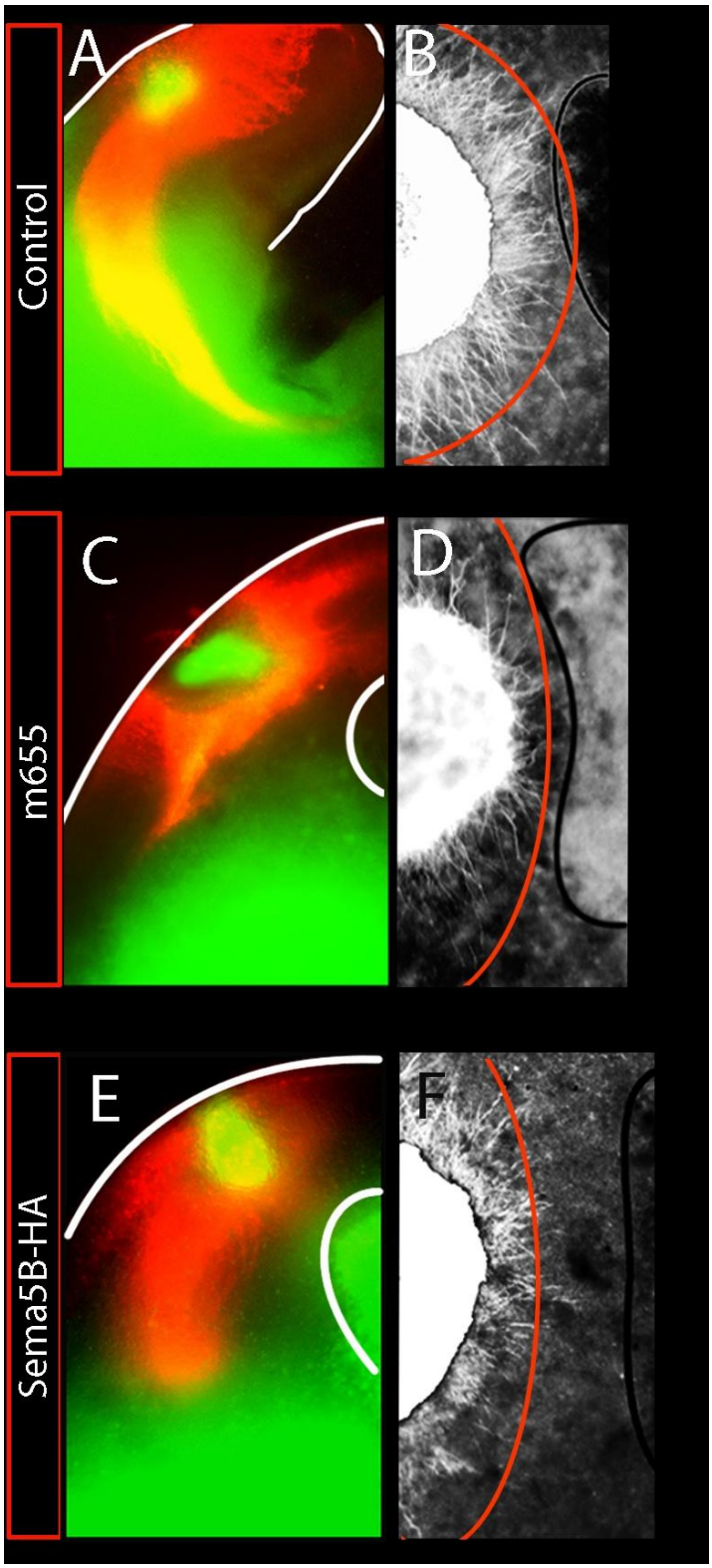

Figure B.1 Cells expressing the secreted ectodomain of Sema5B (m655) cause corticofugal misguidance.
A, C, E) E14 slice cultures labeled in the cortex with DiI and overlaid with HEK293 cells expressing (A) control plasmid, (B) the m655 construct, or (C) the Sema5B-HA construct. (A and E are originally shown as a part of Fig 2.4, and are here for comparison to (C)). M655 cells produc the same type of aberrant inhibition of descending cortical axons as was seen with the wildtype Sema5B-HA. The sample number was very small (n=6), where all but one slice displayed aberrant descending cortical projections. B, D, F) Explants from E13.5 cortex cultured in collagen 3-D matrix with aggregates expressing (B) control plasmid, (D) the m655 contruct, or (F) the Sema5B-HA construct. The aggregates are outlined in black. The red outline borders the edge of axon outgrowth. In controls,

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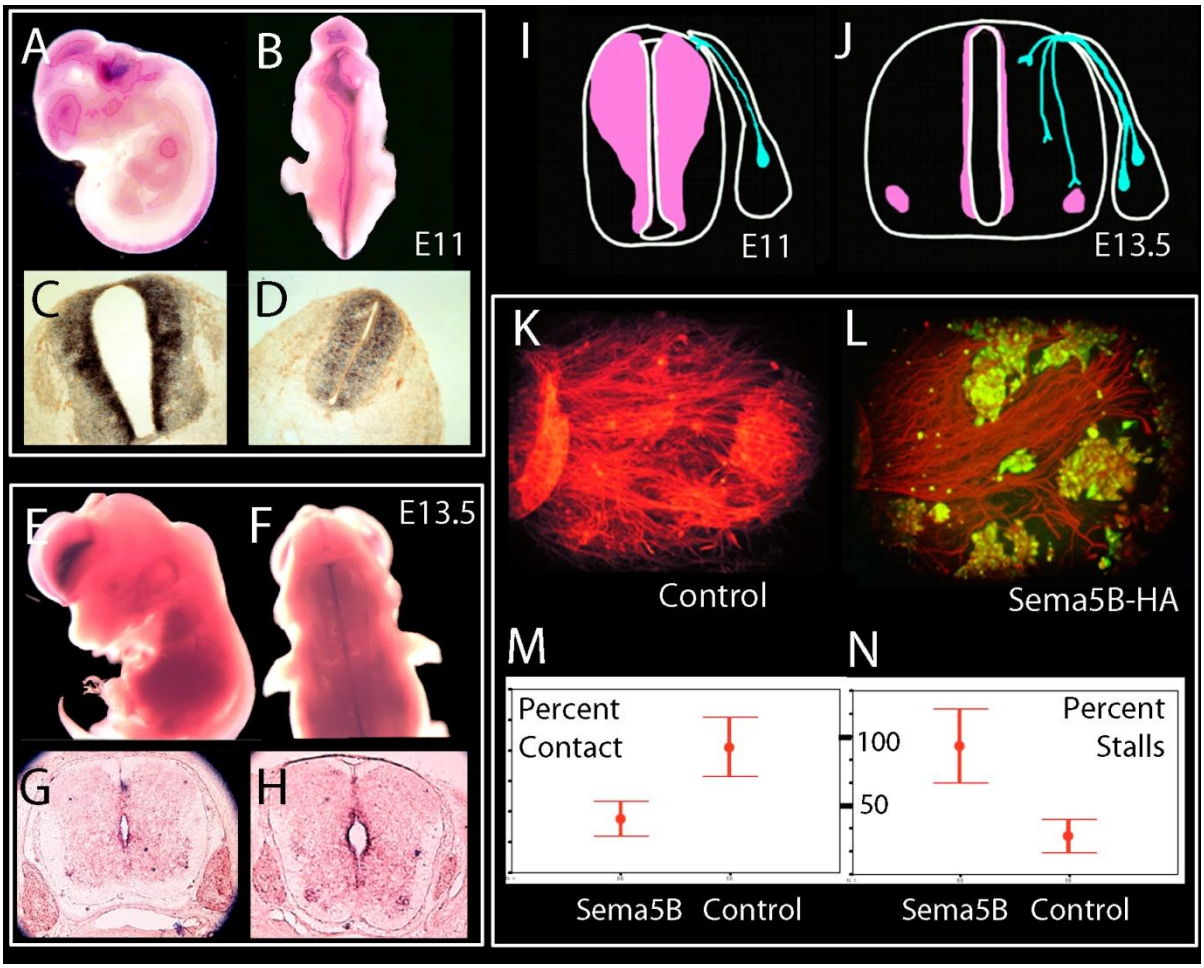
Fujimori et al., 2000; Denaxa et al., 2001; Chatzopoulou et al., 2008; Wolman et al., 2008), particularly the recent work regarding inhibitory signaling in the ventral spinal cord of vertebrates (Masuda et al., 2003; Law et al., 2008), and my own observations of that all tested TAG-1-expressing axons are responsive to Sema5B. There is clearly much work to be done in terms of fully elucidating this

interaction. I have new evidence to suggest that, as I have suspected, TAG-1 does not interact directly with Sema5B. Pull-down assays are currently underway using axons responsive to Sema5B in order to extract the full receptor complex and for the subsequent identification of TAG-1 as part of that complex.

For now my evidence is based upon the repeated claim of an "unknown inhibitor" present in the ventral spinal cord at times which I know Sema5B to be expressed (Fig B.2 A-H). Sema5B is expressed in the dorsal differentiating fields early on, when dorsal root ganglion (DRG) axons are not ready to enter the spinal cord. At the time of DRG axon penetration of the spinal cord, Sema5B is notably absent from these dorsal regions and is only found along the ventricular zones, as is the case of the entire neuraxis, and in neuron pools in the ventrolateral and lateral domains, corresponding to subpopulations of motor neurons, and preganglionic sympathetic neurons (Fig B.2 I, J). Sema5B is significantly inhibitory to mouse DRG axons (Fig B.2L compared to control, K; quantified in M, $p<0.001$), both NT3 and NGF-dependent (not shown separately here), which are known to consistently and stably express TAG-1, even in long-term culture (Karagogeos et al., 1991; Vaughn et al., 1992). In addition, any axons that do contract cell islands collapse and stall, compared to a very low stalling rate against control cells (Fig B.2 N, $p<0.001$). It would be intriguing to determine if the loss of Sema5B and Sema3A would be adequate to phenocopy the disruption seen by interfering with TAG-1 alone.

Figure B.2 Sema5B in the spinal cord reflects regions avoided by TAG-1 fibers
A-H) in situ hybridization for Sema5B. A-D) E10.5-11 mouse. A) Whole mount, sagittal view. B) Whole mount, posterior view. C) Transverse section of E11 spinal cord at rostral level (around upper thoracic). D) Transverse section of E11 spinal cord at caudal level (around lower lumbar – note the less advanced development at this posterior level). E-H) E13-13.5 mouse. E) Whole mount, sagittal view. F) Whole mount, posterior view. G) Transverse section of cervical spinal cord. Note expression in the ventrolateral motor columns and in ventricular zone (surround central canal). H) Transverse section of thoracic spinal cord. Expression is similar to G, with the addition of expression in the developing lateral horns corresponding to pre-ganglionic sympathetic neurons. I, J) Schematic of Sema5B expression at E11 and E13.5, with corresponding DRG innervation of the spinal cord. K-L) Island assays performed with E13 mouse DRGs in co-culture with control HEK293 cells (K) or Sema5B-HA-expressing cells (L; labeled for HA in green). Axons were labeled for neurofilament (shown in red). M) Quantification of the percent contact between DRG axons and cells. DRG axons were significantly inhibited from contacting Sema5B-HA expressing cells (student's t-test, $p<0.001$). N) Axon stalling, as described earlier in the text, was significantly increased in proximity to Sema5B-HA cells, compared with control ($p<0.001$). This represents an additional TAG-1 expressing population of axons that are repelled or inhibited by recombinant Sema5B.



B.3 References

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Appendix C: UBC Research Ethics Board

The University of British Columbia

ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: **A99-0227**

INVESTIGATOR OR COURSE DIRECTOR: **Tetzlaff, W.G.**

DEPARTMENT: **Zoology**

PROJECT OR COURSE TITLE: **Expression of Semaphorins and Their Receptors in Axotomized Facial and Brain to Spinal Cord Projection Neurons**

ANIMALS: **Rats 276 Mice 182**

START DATE: **99-08-01** APPROVAL DATE: **2002-01-28**

FUNDING AGENCY: **Christopher Reeve Paralysis Foundation**

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

[redacted]

Approval of the UBC Committee on Animal Care by one of:
Dr. W.K. Milsom, Chair
Dr. J. Love, Director, Animal Care Centre
Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
323-2194 Health Sciences Mall, Vancouver, V6T 1Z3
Phone: 604-822-8155 FAX: 604-822-5093

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-1448

Investigator or Course Director: Timothy P. O'Connor

Department: Cellular & Physiological Sc.

Animals: Chicken 250
Invertebrates 1000
Mice 182

Start Date: **April 1, 2004** **Approval Date:** **November 3, 2006**

Funding Sources:

Funding Agency:	Canadian Institutes of Health Research
Funding Title:	Regulation of semaphorin guidance activity in the developing nervous system
Funding Agency:	Canadian Institutes of Health Research
Funding Title:	Development of a high throughput screen for bioactive compounds that stimulate neural regeneration

Funding Agency:	Natural Science Engineering Research Council
Unfunded title:	N/A
Funding Title:	Signalling mechanisms of neuronal growth cone motility

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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