Expression of Chick Semaphorin 5B During Neuronal Development

by

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Abstract

During development, the formation of a functional nervous system requires precise pathfinding of axons to their targets. Growth cones at the leading edge of these axons use the information supplied by a variety of cues in the environment to navigate their course. The semaphorins, comprising a large family of neuronal guidance cues, were first identified in the grasshopper limb bud and were shown to be important for proper Ti1 axon pathfinding. Most of the studies from invertebrates and vertebrates have demonstrated an inhibitory role but more recent studies have shown them to have a bi-functional nature, also acting as attractive cues. The transmembrane semaphorin, chick semaphorin 5B (cSEMA5B), is unique from other semaphorin in that it contains both an inhibitory SEMA domain and a region of thrombospondin type-1-like repeats that have been associated with neuronal outgrowth. To determine whether this semaphorin plays a role in axon guidance in the developing chick nervous system, its expression was analyzed using a variety of techniques including *in situ* hybridization, RT-PCR, and immmunocytochemistry. Expression of cSEMA5B is first clearly identified at E5 in the spinal cord, DRGs, retina, and in a variety of neuroepithelia associated with the tectum, ventricular regions, and the olfactory system. Expression within the spinal cord is dynamic being first broadly expressed in both dorsal and ventral regions at E5 with the ventral expression persisting through E11. At this later stage the expression is associated with large-diameter cells in the lateral motor column. Expression within the retina occurs along the retinal ganglion cell layer and is relatively uniform in distribution and is maintained from E5 through E10, while the expression in the tectum appears to occur in a gradient with highest levels in the anterior region. Results from these studies, along with

some important *in vitro* studies performed by others in our lab, have suggested that cSEMA5B is important for the neuronal pathfinding within the spinal cord, retina, and developing tectum.

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List of Abbreviations

bp	basepairs
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
CNS	central nervous system
Cy3	indocarbocyanine
CUB	complement-binding
DH	dorsal horn
DRG	dorsal root ganglion
ECM	extracellular matrix
EN	external nuclear layer
EP	ependymal layer
DEPC	diethyl pyrocarbonate
FITC	fluorescein isothiocyanate
GCL	retinal ganglion cell layer
GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
IN	inner nuclear layer
kb	kilobase
kD	kilodalton
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MICAL	flavoprotein monooxy-genase

ml	milliliter
mM	millimolar
NBT	4-nitro blue tetrazolium chloride
NGF	nerve growth factor
Npn	neuropilin
NT	neurotrophin
OFL	optic fiber layer
ON	optic nerve
ONH	optic nerve head
OTK	off-track
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLMC	presumptive lateral motor column
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
RGC	retinal ganglion cell
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SGC	stratum griseum centrale
SO	stratum opticum
μg	microgram
μl	microliter
μM	micromolar

VH	ventral horn
WM	white matter

Acknowledgements

I would firstly like to thank Dr. Timothy P. O'Connor for allowing me to conduct my research in his lab, for his excellent guidance throughout the entire duration of this degree, his passion for science, and for his friendship in and out of the lab. I would like to thank my committee members, Dr. W. Tetzlaff and Dr. C. Roskelley for their suggestions and scientific advise. I would like to thank Dr. Jennifer Bonner for her scientific guidance, her friendship while sharing an office. I would like to thank my fellow graduate students in Anatomy as they made this whole process a lot of fun. I would like to thank my parents who have supported me in whatever endeavors I have gone through. Finally, I would like to thank my Lord Jesus Christ who gives me strength and guidance always.

Introduction:

Axon guidance:

The generation of a functional nervous system requires precise pathfinding of axons to their appropriate targets. How neurons accomplish this feat while making relatively few errors is unclear, however, the precise temporal and spatial distribution of guidance factors is a requirement. Guidance cues evoke stereotyped responses from neurons including attraction or repulsion, and can act locally if bound to cell membranes and the ECM, or at a distance if they are secreted. The growth cone, a highly dynamic, actin-rich sensory structure found at the leading edge of the axon, is responsible for interpreting these signals and translating them into a number of growth cone behaviors including outgrowth, turning, fasciculation, retraction, and stalling (Suter and Forscher 2000). When first describing a growth cone over a century ago, Ramon y Cajal proposed that this structure responds to guidance factors to direct axonal growth. This has proven to be true and in the subsequent century, numerous guidance cues have been identified, and their effects on growth cone behaviors have been characterized. Even though a number of families of guidance cues have been identified, the precise mechanisms underlying growth cone behavior and response to these guidance cues are not resolved.

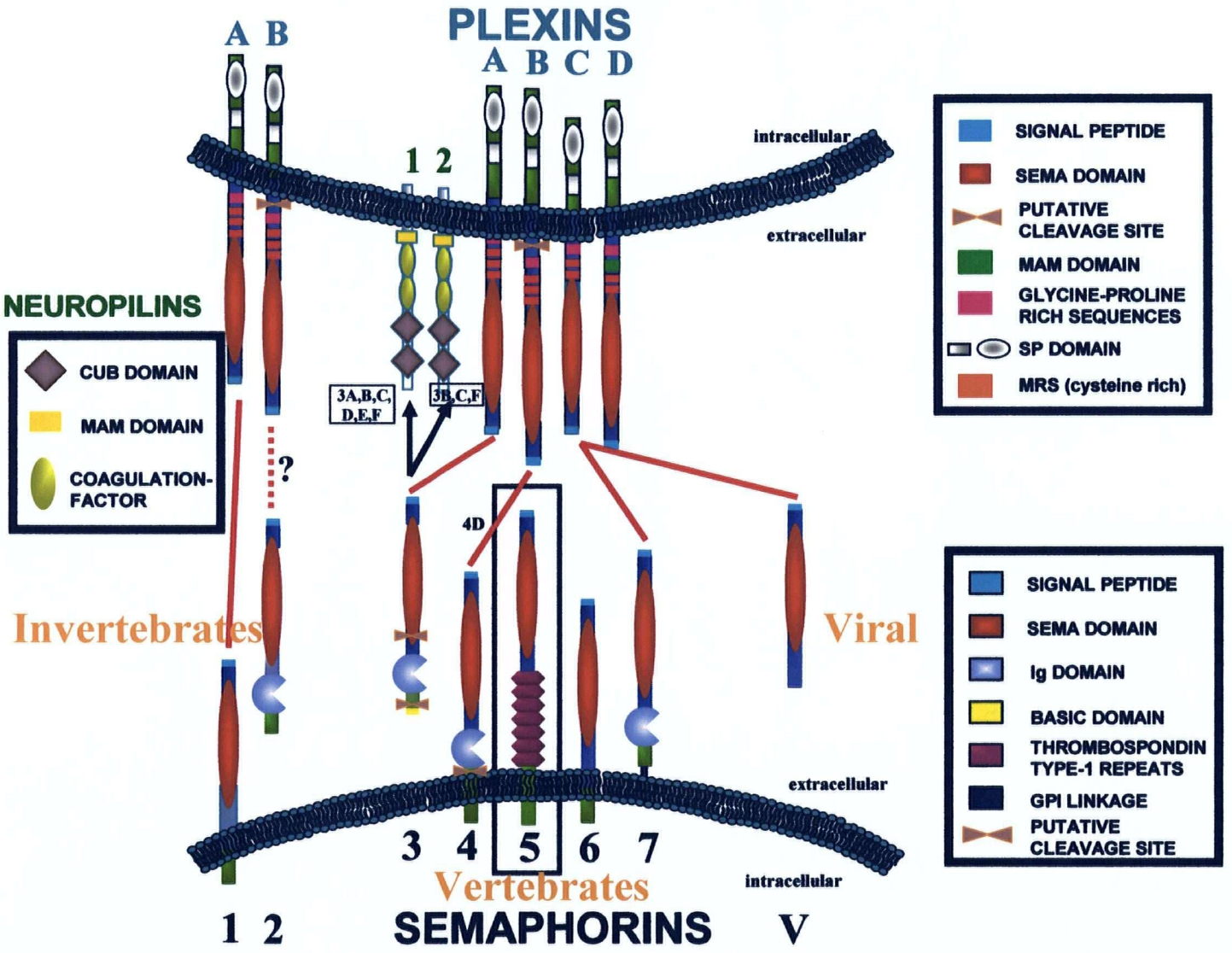
Semaphorins:

To date the major families of guidance proteins identified include the Ephrins, Slits, Netrins, and Semaphorins (Grunwald and Klein, 2002). These guidance cues can localize to the membrane, as is the case with ephrins and some members of the semaphorin family, or they may be secreted proteins like netrins, slits, as well as some of the semaphorins. Different families are typically associated with a particular response,

either attractive or inhibitory, but many exhibit bi-functional guidance properties. Of these guidance cues, semaphorins comprise the largest family. With more than 20 different members identified to date, the semaphorins are subdivided into eight different classes based on domain structure and species of origin (figure 1; Semaphorin Nomenclature Committee 1999). Semaphorins comprise a diverse family of secreted and membrane associated proteins characterized by the presence of a conserved semaphorin (Sema) domain, 500 amino acids in size. The semaphorins may be bound to the membrane through either a transmembrane domain (classes 1, 4, 5, 6) or through a glycophosphatidyl inositol linkage (class 7), or they may be secreted as is the case with classes 2, 3, and V (figure 1). The first identified semaphorin, grasshopper Sema-1a (previously named fasciclin IV), was initially implicated in axon guidance using antibody perturbation experiments (Kolodkin et al., 1992, 1993). Subsequent purification of a secreted semaphorin, chick Collapsin-1 (Sema3A), demonstrated the chemorepulsive nature of this semaphorin as acute addition led to rapid actin depolymerization and growth cone collapse of DRG neurons in vitro (Luo et al., 1993). Following this initial characterization, a number of studies went on to demonstrate the repulsive nature of semaphorins for a variety of neuronal populations (Matthes et al., 1995; Tanelian et al., 1997; Bagnard et al., 1998; Chedotal et al., 1998; Shoji et al., 1998; Winberg et al., 1998b; Yu et al., 1998; de Castro et al., 1999; Miyazaki et al., 1999; Rabacchi et al., 1999; Roos et al., 1999, Steup et al., 1999; Yu et al., 2000; Tamamaki et al., 2003). In addition to functioning as repulsive guidance cues, semaphorins have also been found to function as attractive cues (Wong et al., 1997; Bagnard et al., 1998; Song et al., 1998; de Castro et al., 1999; Wong et al., 1999; Bagnard et al., 2000; Fujioka et al., 2003). For

Figure 1: Semaphorins and Semaphorin Receptors

Genes encoding semaphorins are highly conserved from invertebrates to humans. There are at least 20 known semaphorins that can be divided into 8 subclasses based on structural similarities. Classes 1, 2, and 5 are invertebrate semaphorins, classes 3 through 7 are vertebrate semaphorins, and class V are viral associated semaphorins. Common to all semaphorins is a 500-amino acid length semaphorin domain at their amino termini (orange), which is highly conserved between invertebrates and vertebrates. The carboxyl regions of the semaphorins are more variable. Classes 2 through 4, 7, and a subset of V contain an Ig domain (blue/white), while class 5 members contain a thrombospondin type-1 repeat domain (purple). The semaphorins are further distinguished biochemically as being secreted, membrane glycosylphosphatidylinositol (GPI)-anchored, or transmembrane molecules. A schematic representing the two classes of semaphorin receptors, the neuropilins and plexins, depicts the various domain structures associated with each. There are two classes of neuropilins, both of which have a very small intracellular domain, which interact with the secreted class 3 semaphorins and the plexins, and are only found in vertebrates. The plexin family has four classes (A-D) in vertebrates, with known interactions with semaphorins from classes 3, 4, and 7. There are two classes (A&B) in invertebrates that are known to interact with semaphorins from class 1. Known interactions between the receptors and semaphorins are shown with lines (red).


PLEXINS
A B
A B C D
1 2
intracellular
extracellular
NEUROPILINS
CUB DOMAIN
MAM DOMAIN
COAGULATION-FACTOR
?
3A,B,C,D,E,F
3B,C,F
4D
Invertebrates
Viral
extracellular
3 4 5 6 7
Vertebrates
intracellular
1 2
SEMAPHORINS
V
SIGNAL PEPTIDE
SEMA DOMAIN
PUTATIVE CLEAVAGE SITE
MAM DOMAIN
GLYCINE-PROLINE RICH SEQUENCES
SP DOMAIN
MRS (cysteine rich)
SIGNAL PEPTIDE
SEMA DOMAIN
Ig DOMAIN
BASIC DOMAIN
THROMBOSPONDIN TYPE-1 REPEATS
GPI LINKAGE
PUTATIVE CLEAVAGE SITE

example, while Sema3A is repulsive for cortical axons, another semaphorin, Sema3C acts as an attractive guidance cue for these same neurons (Bagnard et al., 1998). Similarly, in the developing grasshopper limb, the Ti1 neurons turn away from their normal pathway to contact cells ectopically expressing Sema-1a, suggesting an attractive response (Wong et al., 1999). In addition, the activation of the cGMP pathway in Xenopus spinal neurons can convert Sema3A repulsion to attraction (Song et al., 1998). Additional examples of both repulsive and attractive roles for these proteins *in vitro* have been demonstrated (Raper, 2000).

Genetic Analysis of Semaphorins:

Along with many *in vitro* studies describing the function of semaphorins, a number of studies have described a role for semaphorins *in vivo*. Observations on a number of mutants from classes 1 and 2 in invertebrates, and from classes 3 and 6 in vertebrates, have led to many conclusions about the *in vivo* function of these semaphorins (Behar et al., 1996; Taniguchi et al., 1997; Shoji et al., 1998; Catalano et al., 1998; Winberg et al., 1998; Yu et al., 1998, 2000; Roy et al., 2000; White et al., 2000; Bahri et al., 2001; Leighton et al., 2001; Ginzburg et al., 2002). There are three semaphorins (*Ce-sema-1a* or *smp-1*, *Ce-sema-1b* or smp-2, *Ce-sema-2a* or *mab-20*) in *C. elegans* and mutants for each have been described (Roy et al., 2000; Ginzburg et al., 2002). While the majority of defects associated with *Ce-sema-2a* include errors in morphogenesis and epithelial cell-to-cell contacts, these embryos also display errors in axon guidance and cell migration (Roy et al., 2000). Mutants exhibit a number of fasciculation and pioneer guidance defects in at least one of the DA and DB motor neurons, as well as errors in neuroblast cell migration (Roy et al., 2000). A mutation in either the *Ce-sema-1a* or *Ce-*

sema-1b also results in a number of defects in epidermal cell morphogenesis and cell-to-cell contacts (Ginzburg et al., 2002). In addition to errors in morphogenesis, *Ce-sema-1a* and *Ce-sema-1b* mutants exhibit mild defects in the guidance of PLML and PLMR axons, including premature stopping and unusual branching (Ginzburg et al., 2002). In *Drosophila*, null mutations in *D-Sema-1a* result in premature stalling of motor axons and failure of these axons to defasciculate (Yu et al., 1998). In addition, ectopic expression of D-Sema-1a in muscle cells results in motor axons avoiding these targets (Yu et al., 1998). Expression of a truncated form of D-Sema-1a in these flies is able to rescue this phenotype, suggesting that the cytoplasmic domain of this transmembrane semaphorin is not required for this function (Raper, 2000). Recent studies on *D-Sema-1a* mutants have demonstrated that this semaphorin is also required for proper axon guidance and synapse formation in the adult Giant Fibre system (Godenschwege et al., 2002). Rescue experiments in these mutants further demonstrated that D-Sema-1a is required both pre- and post-synaptically (Godenschwege et al., 2002). Ectopic expression of D-Sema-2a in muscles prevents motoraxons from correctly pathfinding to these targets (Winberg et al., 1998). The converse is true in *D-Sema-2a* mutants, where many motorneurons are observed to incorrectly invade muscle fibers that normally express D-Sema-2a (Winberg et al., 1998).

In addition to genetic analysis of semaphorins in invertebrates, vertebrate mutants have also been described. Mice lacking Sema3A exhibit a number of defects in sensory and motor axon patterning in the developing spinal cord (Behar et al., 1996; Taniguchi et al., 1997), however, the overall morphology of the CNS in these mice is relatively normal (Catalano et al., 1998, Ulupinar et al., 1999). This may be due to functional redundancy

of different semaphorins. For example, sympathetic neurons are repulsed by multiple semaphorins from the class 3 family *in vitro* (Adams et al., 1997; Takahashi et al., 1998). Homozygous mutant mice lacking Sema6A, the mouse homologue of invertebrate D-Sema-1a, display a number of aberrant thalamocortical projections (Leighton et al., 2001). These neurons normally express Sema6A, which suggests that this guidance defect is acting cell-autonomously and that this semaphorin serves as a guidance receptor in these neurons (Leighton et al., 2001).

Thus, while the studies on the Sema3A knock-out mouse are not conclusive, other *in vivo* studies from invertebrates and mice support the *in vitro* findings and demonstrate that a number of different semaphorin classes normally function in axon guidance during development.

Semaphorin receptors:

Two families of transmembrane proteins, the neuropilins (Npn) and the plexins have been identified as receptors for semaphorins. The first receptors discovered belong to the neuropilin family of proteins, Npn-1 and Npn-2. They were first identified as semaphorin receptors in an expression screen which exploited their high affinity for semaphorin-3A (Kolodkin et al., 1997; He and Tessier-Lavigne, 1997; Fujisawa and Kitsukawa, 1998; Kolodkin 1998). Subsequent analysis demonstrated that this interaction was conserved for other class three members, however, different class members were shown to interact with different combinations of neuropilins for signaling (Nakamura et al., 2000). Specifically, Sema3A was shown to signal through a receptor complex consisting of Npn-1, while Sema3F signals through a complex consisting of Npn-2, and

Sema3C signals through a hetero-dimeric complex consisting of both Npn-1 and Npn-2 (Nakamura et al., 2000).

Npn-1 was originally characterized as a cell surface molecule with a restricted expression pattern in the optic tectum of Xenopus (Takagi et al., 1991). When over expressed in embryonic mice, Npn-1 leads to axon defasciculation and sprouting (Kitsukawa et al., 1995). The Npns are structurally characterized by the presence of two extracellular complement-binding (CUB) domains, followed by the presence of two coagulation factor V/VIII homology domains, a MAM domain, and a small cytoplasmic region (figure 1; Chen et al., 1997). The CUB domain was shown to be the interacting domain for Sema3A from analysis of various Npn-1 deletion mutants (Nakamura et al., 1998).

Many observations apart from the biochemical interaction studies have demonstrated that Npn-1 is required for functional Sema3A receptors. Antibodies to Npn-1 block Sema3A induced collapse of neurons *in vitro* (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). In addition, DRG neurons isolated form Npn-1 null mice do not undergo growth cone collapse in the presence of semaphorin 3A (Kitsukawa et al., 1997). The small cytoplasmic tail of Npn-1 interacts with a SEMCAP protein, neuropilin interacting protein (NIP) which is thought to induce clustering, however, it is the extracellular region of Npn-1 which is thought to mediate its function in semaphorin binding (figure 2C; Cai and Reed, 1999). Based on its structure and some key observations, it appears as though at least one additional transmembrane protein is required for Sema3A activity (Nakamura et al., 1998). Presently, an invertebrate equivalent for neuropilin has not been found.

Another family of transmembrane proteins, the plexins bind specifically to semaphorins either alone, or in combination with the neuropilins (Comeau et al., 1998; Winberg et al., 1998). There are nine known plexins, which subdivide into four families based on structure; plexin-A1-4, B1-3, C1, and D1 (Tamagnone et al., 1999). Plexins are transmembrane proteins characterized by the presence of an extracellular semaphorin domain and cysteine-rich region, as well as a conserved plexin-specific cytoplasmic domain (Tamagnone et al., 1999). There are 3 known classes of semaphorins which bind directly to plexins. In *Drosophila*, sema-1a and sema-1b bind plexin-A1 directly, while in vertebrates Sema4B binds plexin-B1, and Sema7A binds plexin-C1 (figure 1; Winberg et al., 1998; Lange et al., 1998; Xu et al., 1998).

Neuropilins and plexins interact to form a receptor complex for the secreted class 3 semaphorins. Initial results demonstrated that plexins form stable complexes with Npn-1 and Npn-2 when ectopically expressed in COS-7 cells (Takahashi et al., 1999). When co-expressed in COS-7 cells, neuropilins and plexins induce collapse in the presence of Sema3A, while expression of either one alone does not (Takahashi et al., 1999). Over-expression of a truncated plexin-A1 lacking the intracellular domain inhibits Sema3A induced collapse in cultured sensory neurons (Takahashi et al., 1999).

In addition to the Npns and the plexins, another family of receptor molecules shown to contribute to semaphorin receptor complexes includes L1, a member of the immunoglobulin superfamily of adhesion molecules. Analysis from L1 -deficient mice demonstrated that cortical neurons in these mice were unresponsive to Sema3A but not to Sema3B or Sema3E (Castellani et al., 2000). Further analysis demonstrated that the extracellular domains of L1 and Npn-1 directly associate to form a receptor for Sema-3A

(Castellani et al., 2000). Interestingly, a soluble form of L1, with an attached Fc domain, can convert Sema3A-induced axonal repulsion into attraction *in vitro* (Castellani et al., 2000, 2002).

Studies on plexin associated proteins from *Drosophila* have implicated the re-named Dtrk receptor *off-track* (OTK), as a binding partner required for Plexin A1 signaling (Winberg et al., 2001). This was supported from experiments which demonstrated that *otk* mutants displayed similar phenotypes to loss-of-function mutations of either *Sema1a* or *PlexA* (Winberg et al., 2001). Additionally, *otk* loss-of-function mutations interact genetically with Sema1a and PlexA, and reduced levels of *otk* suppress Sema-1a gain-of-function phenotypes (Winberg et al., 2001).

Finally, recent work by Giordano et al. 2002 has demonstrated that Plexin B1 (the Sema4D Receptor) and Met (the Scatter Factor 1/ Hepatocyte Growth Factor Receptor) associate in a complex when co-expressed in MLP29 epithelial cells. Binding of Sema4D to Plexin B1 stimulates the tyrosine kinase activity of Met, resulting in tyrosine phosphorylation of both receptors (Giordano et al., 2002). Cells that lack Met expression do not respond to Sema4D (Giordano et al., 2002). This same cells will respond to Sema4D is they are then forced to express Met (Giordano et al., 2002). These results suggest that the Met receptor may also contribute to a receptor complex for various Sema4D.

Genetic Analysis of Semaphorin Receptors:

A number of mutants have been described for both the neuropilin and the plexin families of receptors. There are two plexins (*plx-1, plx-2*), in *C. elegans*, but no obvious neuropilin genes (Fujii et al., 2002). PlexinA (*plx-1)* null-mutants exhibit a number of

morphological defects including the displacement of ray1, one of nine male-specific genital sensilla (simple sense organ) in the tale, and a partial loss of seam cells which lead to a discontinuous outer alae, a cuticular structure running longitudinally along the lateral surface of the body wall (Fujii et al., 2002). Interestingly, suppression of Ce-Sema-1a and Ce-Sema-1b displayed a similar phenotype to *plx-1*, whereas *Ce-Sema-2a* mutants exhibit a distinct phenotype (Fujii et al., 2002). In *Drosophila*, *PlexA* loss-of-function mutant phenotypes phenocopy those of *Sema1a* including a failure of ISNb growth cones to defasciculate from one another at any or all three of the ISNb choice points (Winberg et al., 1998). Also, *PlexA* and *Sema1a* loss-of-function mutations interact genetically, while *PlexA* loss-of-function suppresses *Sema1a* gain-of-function phenotypes (Winberg et al., 1998). Interestingly, over-expression of Plex A in all neurons leads to axon guidance defects in all parts of the motor projection and within the CNS (Winberg et al., 1998). Mutations in *PlexB* also result in axon guidance phenotypes (Hu et al., 2001). The ISNb neurons in flies overexpressing *PlexB* fail to innervate their normal muscle targets (Hu et al., 2001).

In mice lacking neuropilin-1, the phenotype is similar to that of Sema3A but it is much more severe, and mice arrest developmentally (Kitsukawa et al., 1997). These animals display severe abnormalities in cranial and spinal neurons, along with aberrant efferent projections within the PNS including abnormal limb innervation (Kitsukawa et al., 1997). Mice with mutations in neuropilins-2 also display a number of neuronal defects within the CNS and PNS, including severe defasciculation of oculomotor neurons (Giger et al., 2000, Marin et al., 2001). Mice that lack plexin-A3 have a number of axon guidance defects including an inability of the ophthalmic branches of the trigeminal nerve

to fasciculate (Cheng et al, 2001). These mice also display a number of guidance defects associated with the hippocampus including aberrant dentate granule cell and mossy fibre projections (Cheng et al., 2001).

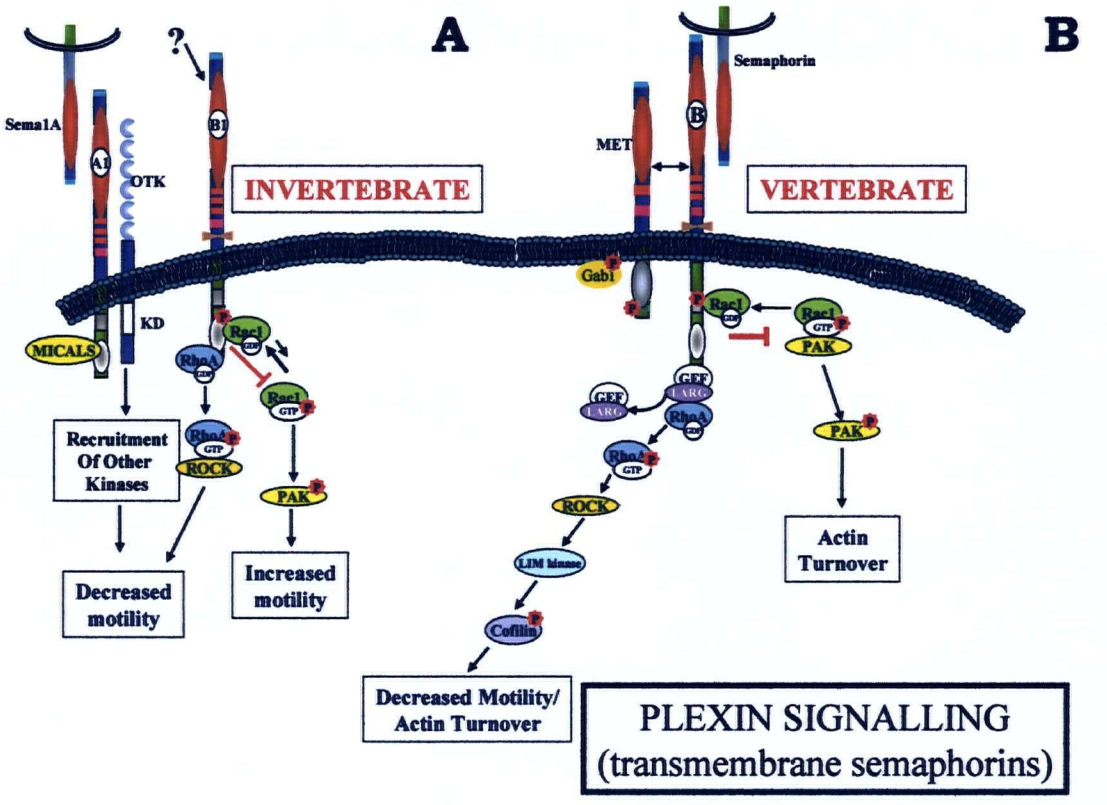
Semaphorin Signaling:

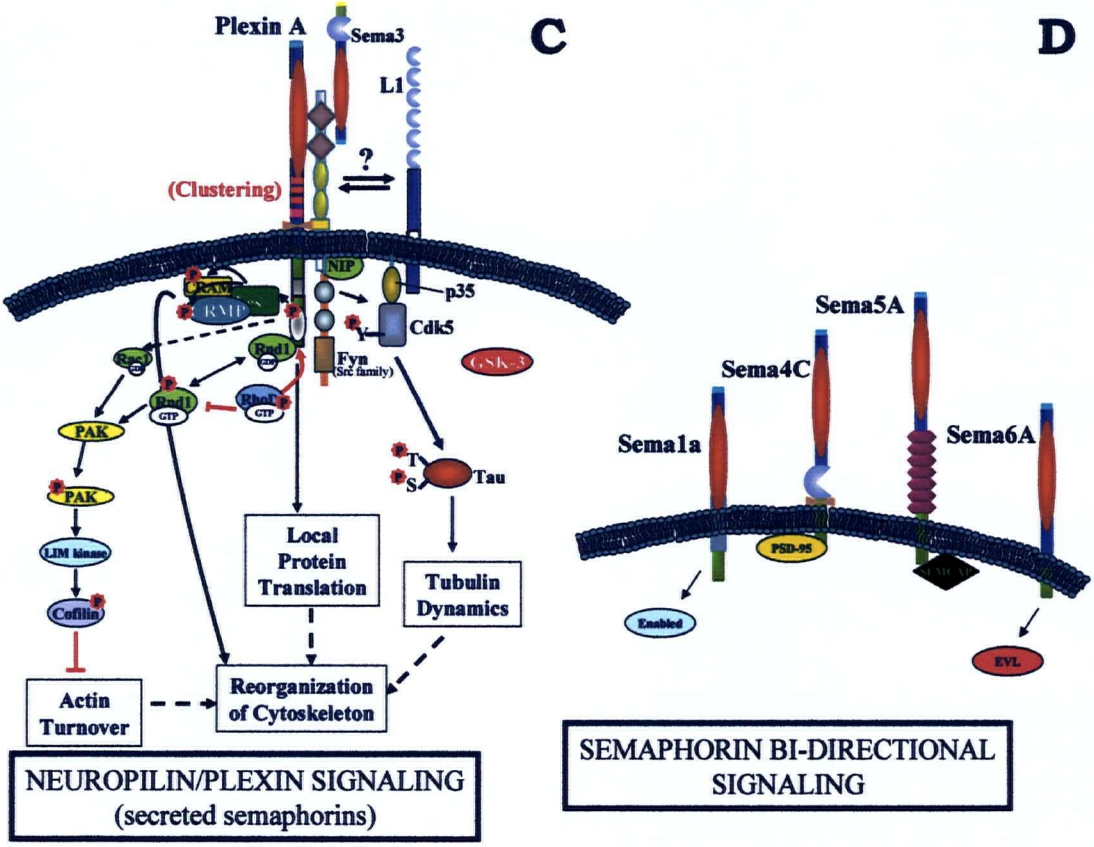
Recent studies have begun to identify the downstream signaling targets involved in transmitting the semaphorin signal (reviewed in Castellani and Rougon, 2002). A number of these studies have implemented the Rho GTPases in semaphorin signaling. Dominant negative Rac1 was shown to inhibit Sema3A-induced collapse of DRG neurons, while a constitutively active Rac1 partially mimicked the Sema3A growth cone collapse (Jin and Strittmatter, 1997). Both plexins and neuropilin signaling cascades result in the activation or inhibition of specific Rho GTPases including RhoA, Rac1, Rnd1, and RhoD (figure 2).

In *Droshophila*, PlexB was shown to bind activated Rac1, sequestering it from p21 activated kinase (PAK) its downstream effector (figure 2A; Driessens et al., 2001, Hu et al., 2001). In addition to an interaction with activated Rac1, PlexB also binds RhoA creating a scenario were signaling through PlexB leads to collapse by simultaneously inhibiting the Rac1 pathway, while leading to activation of the RhoA pathway (figure 2A; Driessens et al., 2001, Hu et al., 2001). This model was also supported from *in vitro* studies on interactions between Sema4D and plexin B1, which demonstrated that clustering of this plexin leads to an interaction with active Rac-1, that the collapse induced by Sema4D is characteristic of Rho activation, and that there was again an inhibition of PAK activity (figure 2B; Vikis et al., 2000; Rohm et al., 2000; Driessens et al., 2000). Again, Rac1 and RhoA activities are regulated through distinct

Figure 2: Semaphorin Signaling

A series of schematic representations of signal transduction pathways involved in semaphorin activity is shown. In (A), the invertebrate Sema1A interacts with Plexin A1 and OTK to recruit kinases including enabled. In (B), the vertebrate class 4 transmembrane semaphorins signal through the class B plexins associated with MET. This activation leads to a recruitment of the Rho family of small GTPases that signal to either inhibit, or induce collapse. The guanine exchange factor LARG interacts with plexin B1 and activates the RhoA pathway. In (C), the secreted semaphorins, signal through a clustered neuropilin/plexin complex to recruit Rnd1, which activates PAK leading to collapse. Recruitment of RhoD to the same binding region inhibits this Rnd1 activity. Signaling through plexin A1 also leads to activation of a number of other pathways involving CRMPs, Fyn, Cdk5, and a pool of GSK-3. Receptor interactions with L1 are thought to modulate semaphorin signaling through changes in cGMP levels. In (D) bi-directional signaling of transmembrane semaphorins may occur through a number of possible candidates.


A
B
Sema1A
A1
OTK
?
B1
INVERTEBRATE
KD
MICALS
Rac1
Rho4
Rac1
Recruitment Of Other Kinases
Rho4
ROCK
PAK
Decreased motility
Increased motility
Semaphorin
MET
B
VERTEBRATE
Gabi
Rac1
Rac1
PAK
GEF
LARG
GEF
LARG
Rho4
Rho4
ROCK
LIM kinase
Cofilin
PAK
Actin Turnover
Decreased Motility/ Actin Turnover
PLEXIN SIGNALLING
(transmembrane semaphorins)


Plexin A
Sema3
C
L1
?
(Clustering)
NIP
p35
Cdk5
Fyn
(Src family)
GSK-3
Rac1
Rnd1
Rnd1
Rho1
PAK
PAK
LIM kinase
Cofilin
T
S
Tau
Local Protein Translation
Tubulin Dynamics
Actin Turnover
Reorganization of Cytoskeleton
NEUROPILIN/PLEXIN SIGNALING
(secreted semaphorins)
D
Sema5A
Sema4C
Sema1a
Sema6A
PSD-95
Enabled
EVL
SEMAPHORIN BI-DIRECTIONAL SIGNALING

binding sites where Plexin B1 directly interacts with activated Rac1 (figure 2B; Hu et al., 2001; Vikis et al., 2002). Recent work by Aurandt et al. (2002) has implicated the activation of Rho by a Rho-specific nucleotide exchange factor LARG, in response to stimulation by Sema4D (figure 2B). The LARG protein was shown to interact directly with the C-terminal region of Plexin B1 through its PDZ binding domain (Aurandt et al., 2002; Perrot et al., 2002).

Sema3A signals through clustering of a neuropilin/plexin A1 complex (figure 2C). There appear to be differences in the downstream effectors of Plexin A1 compared to plexin B1 (figure 2B, C). While Rac1 activity is important for signaling through plexin A1, it does not appear to interact with this plexin (figure 2C; Kuhn et al., 1999; Rohm et al., 2000). While Rac1 does not interact with plexin A1, both the Rho-like GTPases Rnd1 and RhoD were shown to bind to the intracellular region of plexin A1, and actually compete for the same binding region (figure 2C; Rohm et al., 2000; Zanata et al., 2002). Interestingly, while Rnd1 activation by plexin A1 results in cytoskeletal collapse, binding of RhoD has an antagonist effect, blocking Rnd1 activity (figure 2C; Zanata et al., 2002). Plexin and neuropilin receptor complexes also induce activation of LIM kinase and subsequent inhibition of cofilin, resulting in a reduction in actin turnover and retraction of the growth cone (figure 2C; Aizawa et al., 2001).

The Collapsin response mediator proteins (CRMPs) are another family of proteins which were first identified by their possible involvement in the Sema3A-induced collapse of DRG growth cones (Goshima et al., 1995). Further evidence came from a study demonstrating that an anti-CRMP antibody blocks collapse induced by Sema3A (Wang and Stittmatter, 1997). A recent study examining the role of CRMPs in Sema3A

signaling demonstrated that they are linked to Plexin A1 through activation of Fes/Fps (Fes) tyrosine kinase which then phosphorylates plexin A1 and CRMP in a Fes/CRMP/CRAM(CRMP-associated molecule) complex (figure 2C; Mitsui et al., 2002). The activation of Fes appears to be required for Sema3A-induced collapse of COS7 cells expressing PlexA1, Npn-1, and Fes (Matsui et al., 2001). Signaling through Sema3A also appears to activate a pool of glycogen synthetase kinase 3 (GSK3), a seronine/threonine kinase implicated in a variety of growth factor signaling cascades (figure 2C; Eickholt et al., 2002). This activation occurs at the leading edge of the growth cone (Eickholt et al., 2002). Other downstream effectors of semaphorin signaling include the flavoprotein monooxy-genases (MICALS), which have been shown to interact directly with PlexA in *Drosophila* (figure 2A; Terman et al., 2002). The MICAL family of proteins have multiple domains which are known to be important for interactions with actin, intermediate filaments, and cytoskeletal-associated adaptor proteins, and may thus mediate the cytoskeletal alterations characteristic of semaphorin signaling (Terman et al., 2002). An additional kinase dependent pathway in Sema3A signaling involves the src kinase Fyn and the Thr/Ser kinase cyclin-dependent kinase 5 (Cdk5: figure 2C; Sasaki et al., 2002; Pasterkamp and Kolodkin, 2003). Evidence from *Xenopus* neurons suggests that Sema3A signaling also requires local protein synthesis in the growth cone, as the inhibition of translation in the axon prevents Sema3A induced collapse and turning (figure 2C; Campbell and Holt, 2001).

In addition to functioning as ligands, transmembrane semaphorins also contain intracellular regions which have been shown to interact with a variety of targets that may be involved in various signaling cascades. In *Drosophila*, Sema-1a is involved in

synapse formation and may signal bi-directionally via Enabled (figure 2D; Godenschwege et al., 2002). Other possible interacting proteins include the synapse associated protein PSD-95, SEMCAP-1, and EVL (ena vasp regulator) protein (figure 2D; Inagaki et al., 2001; Wang et al., 1999; Klostermann et al., 2000). While the list of possible candidates involved in semaphorin signaling is constantly increasing, a clear picture as to how the semaphorins are able to pass on guidance information to the cytoskeleton is unclear. Even more unclear is whether the transmembrane semaphorins are able to signaling bi-directionally, and whether this is crucial for their role in axon guidance. The studies on bi-directional Sema-1a signaling in *Drosophila* would suggest that the answer to the last two questions is yes (Godenschwege et al., 2002).

Transmembrane Semaphorins:

The majority of research on semaphorins in vertebrates has focused on characterizing the role of the class 3 secreted semaphorins during neuronal development. Recently, more work has begun to focus on the role of transmembrane semaphorins in development, however little is known about their function. Most of the studies on vertebrate transmembrane semaphorins have looked at the role of the class four semaphorin Sema4D (CD100), a semaphorin important in the immune system (Hall et al., 1996; reviewed in Suzuki et al., 2003). Recent experiments on the vertebrate semaphorin Sema6A, a transmembrane semaphorin homologous to the insect semaphorin class 1, have demonstrated its ability to repel sympathetic and DRG neurons *in vitro* (Xu et al., 2000). Analyses from mice lacking Sema6A transcript suggest that it is also important for proper development of the thalamocortical projection *in vivo* (Leighton et al., 2001). Based on research showing that a transmembrane semaphorin, semaphorin 1a has the

potential to be attractive for Ti1 neurons in the developing grasshopper limb, our lab focused on determining whether transmembrane semaphorins might play a similar attractive role in vertebrates (Wong et al., 1999). Using a series of degenerate primers to the grasshopper semaphorin 1a semaphorin domain, an embryonic chick cDNA library was screened to determine if other semaphorins with a conserved semaphorin domain might convey a similar function. One of the candidate genes that was identified corresponded to a class 5 transmembrane semaphorin, chick Semaphorin 5B (cSEMA5B).

Class 5 Semaphorins:

The class 5 family members are distinguished from other semaphorins by the presence of a unique protein domain containing seven thrombospondin type-1 repeats on their extracellular region, located 3' of the semaphorin domain. This domain is of particular interest as previous work has show that thrombospondin type-1 repeats can induce outgrowth of rat cortical neurons in culture, and is responsible for the attachment of various cell types, both neuronal and non-neuronal, when grown on a substrate containing thrombospondin (Neugebauer et al., 1991; O'Shea et al., 1991 Osterhout et al., 1992; Adams and Tucker, 2000). This class of semaphorins was first described in mice, and contains two known family members in vertebrates, Sema5A and Sema5B (Adams et al., 1996). These two family members are 58% identical and 72% similar to each other, with Sema 5A spanning 1077 amino acids, and Sema5B spanning 1093 amino acids (figure 4; Adams et al., 1996). The semaphorin domains in these mice are 64% identical to one another and are most similar to invertebrate semaphorin 1 domains (~60-66% similar). In mice, Sema5A and Sema5B are differentially expressed in embryonic and

adult tissues. Though expressed in similar regions, Sema5A is expressed primarily in mesodermal cells, while Sema5B is exclusively in the neuroepithelium (Adams et al., 1996). In addition, northern blot analysis on mouse tissue shows that the highest expression levels of Sema5B were found in brain tissue expressed as a single transcript, 5.9kb in size (Adams et al., 1996).

The expression of semaphorin 5B has also been examined in the forebrain of rats over a development period ranging from E15-P7 (Skaliora et al., 1998). SEMA5B is broadly expressed in all neuroepithelia throughout the brain at each of the ages examined. In particular, epithelial regions, such as the ventricular zone, which normally corresponds to high areas of neuronal proliferation, show strong expression (Skaliora et al., 1998). SEMA5B is distributed uniformly and is strongly expressed in cortical ventricular and subventricular zones at postnatal day 0 (Skaliora et al., 1998). This wide spread epithelial expression suggests that SEMA5B might play a general role in these proliferative tissues, possibly with cell migration (Skaliora et al., 1998). Interestingly in the rat, SEMA5B is expressed in discrete patches within the striatum and may be involved in the correct patterning of cells within this region of the brain. At P7, SEMA5B is highly expressed in the rat visual cortex (Skaliora et al., 1998).

In *Drosophila*, only one member of this class of semaphorins exists (Khare et al., 2000; Bahri et al., 2001). It is most similar to Sema5B but is not a homologue of either vertebrate class 5 members, and was thus given the designation of Semaphorin 5C, (DSema 5C; Khare et al., 2000; Bahri et al., 2001). In flies, DSema 5C is found at segment boundaries in regions giving rise to muscle attachments (Bahri et al., 2001). DSema 5C exists as two different protein isoforms that share a similar expression pattern.

The DSema 5C mutant flies are homozygous viable and display no obvious embryonic phenotypes (Bahri et al., 2001). As no detailed analysis was performed on the nervous system, a clear role for this semaphorin in *Drosophila* has not been established.

A possible role for class V semaphorins in axon guidance was suggested from studies examining the well known neurological disorder in humans, Cris-du-chat syndrome (Simmons et al., 1998). These studies reveal that the SEMA5A gene accounts for 10% of the deleted region associated with this disorder. In addition, recent studies on Sema5A in mouse have demonstrated its ability to repulse retinal axons *in vitro* (Oster et al., 2003). In these studies, Oster et al. 2003 examined the expression of this semaphorin in the developing retina and found it to be specifically expressed in the optic disk and along the optic nerve. They were able to demonstrate that the inhibitory response of RGC to Sema5A was maintained in the presence of L1, laminin, or netrin-1 signaling *in vitro* (Oster et al., 2003).

Hypothesis:

While the studies in both the mouse and rat have described the expression of Semaphorin 5B, they both have a number of limitations in that they have not examined the distribution of this semaphorin protein, they have only looked at particular regions or over a small developmental range, and they have not provided much insight as to the role of the class 5 semaphorins. In order to determine a functional role for this semaphorin, its expression was examined in the developing chick from E2 through until E17. The examination of cSEMA5B expression was completed using a series of *in situ* hybridization studies, and with antibodies raised against the N-terminal region of the protein. I hypothesize that cSEMA5B is expressed during in regions associated with

neuronal outgrowth and guidance, including the spinal cord, retina, and tectum. Based on its expression along with the results from functional *in vitro* studies performed by others in our lab, I propose that cSEMA5B is an inhibitory guidance cue that contributes to proper axon outgrowth and guidance within these structures. In addition, I hypothesize that cSEMA5B is expressed along many neuroepithelia and that it plays an inhibitory role in these highly proliferative regions, to facilitate there migration from these epithlial layers towards target tissue in the developing CNS.

Methods

RNA Work:

Probe sequence:

A cDNA sequence corresponding to the C-terminal cytoplasmic region of cSEMA5B was produced from the full-length cSEMA5B (obtained from an E10 chick cDNA library, Clontech) sequence cloned into the pLitmus29 vector. The following primers were generated to isolate this sequence from pLitmus29:

Primer1 (forward): $^{5'}$**AGCTGTCTGATCCCCATGAG**$^{3'}$

Primer 2 (reverse): $^{5'}$**GAAAAGAACATGCACAAACCC**$^{3'}$

The corresponding PCR product was subcloned into the vector bluescript29-KS, containing a T3 and T7 promoter sequence for the production of RNA.

Synthesis of DIG-labeled RNA probe:

To synthesize DIG-labeled antisense and sense RNA probes, the following reagents (Roche) were mixed in the following order at room temperature to produce a 20ul reaction mixture: 9.5μl of sterile DEPC treated dH_20, 4 µl of 5x transcription buffer, 2μl 0.1 M DTT, 2μl of nucleotide mix (pH 8.0), 1μl of Linearized bluescript-cSEMA5B plasmid (1 µg/µl), 0.5 µl Placental ribonuclease inhibitor (40 U/µl), 1μl of T7 or T3 RNA polymerase (10 - 20 U/µl). The resulting mixture was incubated at 37° C, for 2 hours. After the reaction was completed, a 1 µl aliquot was removed and separated on a 1.5% agarose/0.5XTBE gel containing 0.5 µg/ml EtBr in order to estimate the amount of probe synthesized. To remove any remaining plasmid 2 µl of ribonuclease-free DNase 1 (10 U/µl) was added to the reaction mixture and it was incubated at 37° C for 15 min. An RNA isolation column (GIBCO) was used to isolate the resulting RNA probe. As an

alternative method, RNA was isolated with the addition of a volume of 100 µl DEPC treated dH_20 and 10 µl of 4 M LiCl (DEPC-treated) to the reaction. The RNA was precipitated with the addition of 300 µl of ethanol followed by an incubation on ice for 30 minutes. The resulting mixture was spun in a microfuge for 20min. The pellet was washed with 70% ethanol, and spun down for another 2 minutes. All of the EtOH was removed with an aspirator and pellets were allowed to air-dry briefly for 2-5 minutes. The RNA pellet was re-dissolved in DEPC treated dH_20 at ~0.1 µg/µl (~100 µl) and stored at -80° C.

In situ hybridization Studies

Tissue Preparation:

Embryonic chicks isolated at various stages, were submerged in a solution containing 4% paraformaldehyde in DEPC-treated PBS, and were washed at 4°C for a period of 8 hrs. Chicks at E7 or older were first fixed via pericardial infusion, by inserting a needle into the heart and injecting PBS for 2 minutes to clear the blood followed by 10 minutes of Paraformaldehyde to ensure rapid fixation. These embryos were then sectioned into numerous parts before submersion in 4% paraformaldehyde solution at 4°C for 8 hrs. Following the fixation, the resulting embryos were submerged in a solution containing 30% sucrose in DEPC-treated PBS and were left on a rotator at 4°C overnight for cryo-protection. The embryos were submerged in O.C.T (optimal cutting temperature) compound (Tissue-Tek), and placed in a metal tray preteated overnight with 0.2N NaOH. Trays containing embryos submerged in O.C.T compound were frozen in a small vial of 2-methylbutane contained in a bath of liquid nitrogen. The

tissue was sectioned into 10um thick sections using a microtome (Bright Instruments) at –20°C. The sections were placed on slides and dried in an oven at 40°C overnight.

Prehybridization:

Slides were washed 2X for 5minutes in DEPC-treated PBS followed by two washes with DEPC-treated PBS containing 100mM glycine. Sections were treated with DEPC-treated PBS containing 0.3% Triton® X-100 and followed by two washes with DEPC-treated PBS. The sections were permeabilized for 20 minutes at 37°C with TE buffer (100mM Tris-HCL, 50mM EDTA, pH 8.0) containing 1μg/ml Proteinase K (RNase free). Following permeabilization, the sections were fixed with DEPC-treated PBS containing 4% paraformaldehyde at 4°C for 5 minutes and washed twice more with DEPC-treated PBS. Slides were then placed in RNase-free slide containers that contained 0.1M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Sigma), and the slides were allowed to shake for 10 minutes. Slides were washed again 2X 5min with PBS then the sections were incubated at 42°C for 1 hour in prehybridization buffer DIG Easy Hyb solution (Roche Molecular Biochemicals) containing 1mg/ml denatured and sheared salmon sperm and 1mg/ml yeast t-RNA.

In situ hybridization:

A wax pen (PAP) was used to encircle the sections to prevent leakage of hybridization buffer during the hybridization step. The slides were incubated in the presence of 200ul DIG Easy hybridization buffer (Roche) containing 50ng of DIG-labeled anti-sense or sense RNA probe. The sections were covered with a layer of parafilm to prevent evaporation and were placed in a humid chamber at 42°C for 24hrs or overnight.

Post Hybridization Procedure:

Following the hybridization step, the sections were put through a series of washes in a shaking water bath. The slides were washed twice for fifteen minutes in 2X SSC buffer at 37°C, followed by two washes with 1X SSC buffer for 30 minutes at 37°C, and twice more for 45 minutes in a 0.1 X SSC buffer at 37°C. Sections were washed on a shaking platform 2X for 10 minutes in buffer 1 (100mM Tris-HCl (pH 7.5), 150mM NaCl) at room temperature. Sections were covered for 30 minutes with blocking solution (buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum (Sigma)) at room temperature. The blocking solution was decanted and sections were incubated for 2 hrs in a humid chamber with buffer 1 containing 0.1% Triton X-100, 1% sheep serum, and a 1:500 (weight) dilution of sheep anti-DIG-alkaline phosphatase (Fab fragments; Roche) Sections were washed 2X 10 minutes in buffer 1 on a shaking platform at room temperature. Sections were incubated for 10 minutes with buffer 2 (100mM Tris-HCl (pH 9.5), 100mM NaCl, 50 mM $MgCl_2$), and were then covered with the color solution (10mL buffer 2 containing 200µl of nitroblue tetrazolium (NBT)/ 5-bromo-4-chloro-3-indolylphosphate (BCIP) stock solution (Boehringer Mannheim)) in a humid chamber for ~12-24 hrs until desired color was achieved. The color reaction was stopped with the addition of buffer 3 (10mM tris-HCl (pH 8.1), 1mM EDTA) followed by a brief wash with distilled H_2O. Sections were mounted with CytoSeal 280 (Richard-Allan) and overlaid by a coverslip.

Nissl Stained sections:

For sections prepared with a nissl-stain, 1 g cresyl violet acetate dissolved in 990 ml of ddH_2O and the mixture was heated to 35°C and stirred for several hours at 35°C.

The solution was filtered, allowed to cool at room temperature, and the pH was adjusted to 3.5 with glacial acetic acid. Sections prepared as outlined in tissue preparation above, were then submerged in this cresyl violet solution and stained for 5-15 minutes. The sections were washed with 10 dips in water, followed by 10 dips in 70% ethanol. Sections were dehydrated through ethanol by immersing the slides in 2 changes of 95% ethanol for 2 min each.

Northern Analysis

Total RNA from spinal cord, tectum, and gut tissues of various chicks ranging in age from E3 to E11 was TRIzol extracted (GibcoBRL). A total of 30μg of RNA, as measured by the absorbance at 280 nm, was electrophoresed in a 1% denaturing formaldehyde agarose gel for 3 hours at 70Volts. Electrophoresed RNA was then transferred to Hybond-N nylon membrane (Amersham) overnight in 10X SSC transfer buffer. The membrane was baked at 80°C for 2 hrs and incubated in prehybridization buffer (50% w/v deionized formamide, 5X Denhardt's solution, 1%SDS, denatured salmon testes DNA) at 42°C for 2-4 hrs. Membranes were then hybridized in the presence of anti-sense or sense RNA probes overnight at 42°C followed by a series of washes in SSC buffers of varying concentrations. The membranes were developed using a standard protocol from the DIG Northern Starter Kit (Roche).

RT-PCR studies

SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase was used to synthesize first strand cDNA for all RT-PCR studies (Invitrogen). Total RNA from spinal cord, tectum, and gut tissues of various chicks ranging in age from E3 to E17 was TRIzol extracted (GibcoBRL). A total of 1 ug of RNA was incubated in the presence of 50ng of random

primers and 1 μl of 10mM dNTP mix, to a total volume of 12ul with ddH_2O. The mixture was heated to 65°C for 5min followed by a quick chill on ice. A total of 4μl of 5X First-Strand Buffer, 2μl 0.1 M DTT, and 1ul of RNase inhibitor were added to the mixture. Following incubation at 25°C for 10 minutes, the mixture was incubated at 42°C for 2 minutes and 1 μl of SUPERSCRIPT II enzyme was added. The mixture was then incubated for 50 minutes at 42°C and the reaction was stopped by heating to 70°C for 15 min. A total of 2μl of first-strand cDNA mixture was used in the PCR reaction, and the primers used were the same as used to make the RNA probe. Following the PCR reaction, the product was subjected to separation on a 1% agarose gel in the presence of EtBr to confirm the size (figure 5B).

Affinity Purification of cSEMA5B Antibody

Purification of GST-fusion protein:

The pGEX plasmid containing cSEMA5B peptide corresponding to the region of antibody recognition and cloned downstream of GST, was supplied by Dr. Wenyan Wang. *E coli* DH5α cells transformed with pGEX vector containing either the cSEMA5B- glutathione-S-transferase fusion protein (GST) or GST protein alone were used to inoculate 25ml of LB supplemented with 50μg/μl of ampicillin (amp). These 25ml cultures were grown overnight in the orbitial shaker at 37°C. The resulting cultures were used to inoculate 500ml of LB supplemented with 50μg/μl of amp. This culture was grown for 1.5-2 hrs at 37°C until reaching an optical density(600) of 0.6-1.0. Protein expression was induced by the addition of IPTG (0.1mM final) followed by incubation at 37°C for 5 hours. The resulting bacteria were collected by centrifugation at 8000 rpm at 4°C for 10 minutes and the resulting pellet was re-suspended in 9ml of MT-PBS (150mM

NaCl, 16 mM Na_2HPO_4, 4mM NaH_2PO_4 (pH 7.3)). The suspension was sonicated six times at 30seconds each, with 30 seconds on ice between each sonication. Following sonication, 1ml of 10% triton-X-100 was added and the suspension was rocked at 4°C for 5 minutes. The resulting lysates were spun down at 8000 rpm for 10 minutes at 4°C and pellets were discarded. The supernatant was added to a 2ml slurry containing 160 mg (80mg/ml) of Glutathione beads (Sigma G4251) pre-incubated for 30 minutes in MT-PBS and washed 4X with 15ml of MT-PBS. The supernatant and beads were incubated at room temperature for 10 minutes. The mixture was centrifuged at 1000 rpm for 1 minute and the supernatant was removed. The beads were washed 5X in 15 ml MT-PBS with 1% triton-X-100 followed by 2 washes with MT-PBS (no Triton). The cSEMA5B-GST fusion proteins (or GST alone) were eluted by three washes with 2ml of elution buffer (50mM Tris HCl pH 8.0, 10mM Glutathione). Each elution wash was kept separate. 15µl of the resulting elutions were run on a 12% polyacrylamide gel to determine if elution was successful (figure 4). The OD_{280} for each elution fraction were measured to determine the peptide concentration (1OD ≃ 0.5mg/ml).

Affinity column preparation:

The column was prepared as outlined by the AminoLink® Kit (Pierce Biotechnology). A 500 µl solution of cSEMA5B peptide at 4mg/ml was diluted 1:3 in coupling buffer and loaded onto an equilibrated AminoLink® column. The resulting slurry was mixed at 4°C for 6hrs in the presence of AminoLink® Reductant solution to bind the cSEMA5B peptide to the column. The remaining active sites in the column were bound using a Tris-based quenching buffer in the presence of AminoLink® Reductant solution. Following a series of washes with 15mls of a 1M NaCl buffer (pH

7.5), the column was stored at 4°C in PBS buffer (pH 7.3) containing 0.05% sodium azide.

Antibody purification procedure:

Following equilibration of the column with 10ml of PBS (pH 7.3) containing 0.05% sodium azide, 2.0ml of sera was loaded onto the column and allowed to enter the gel bed. Sample was allowed to circulate though the column overnight at 4°C to allow for maximum binding of antibodies. The column was then washed with 30ml of AminoLink® wash solution. Antibody was eluted with Immunopure® IgG elution buffer and the elution fractions were monitored by absorbance at 280 nm. Fractions containing Ab were separated on a 12% polyacrylamide SDS gel and stained with coomassie blue to confirm the correct sizes for heavy and light chain regions (figure 12).

Antibody Staining

Embryo's were fixed in 3.7% paraformaldehyde via pericardial infusion (ie PBS in the heart for ~2-3minutes, followed by 20min of 3.7% para at room temperature). The fixed embryos were then imbedded in OCT compound and sectioned into 10um thick sections using a microtome at –20°C (Bright Instruments). Sections were mounted onto pre-cleaned Superfrost®D/Plus microsope slides (Fischer), and washed 3 X 5 minutes in PBS(A) containing 0.05% Triton X-100. Sections were then washed 2 X 10 minutes in PBS(B) containing 0.1% Triton X and 0.005% bovine serum albumin (BSA). To block sections, slides were incubated for 1 hour PBS(B) containing 10% goat serum and 1%BSA. Sections were then washed 2 X 10 minutes in PBS(A) followed by 2 X 10 minutes in PBS(B). Sections were incubated overnight at 4°C with primary antibody diluted appropriately in PBS(B) usually 1:1000 (10µl in 10ml). Sections were again

washed 2 X 10 minutes in PBS(A) followed by 2 X 10 minutes in PBS(B). Section were then incubated for 1 hour at room temperature with a Cy3 conjugated goat anti rabbit fluorescent secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted at 1:500 in PBS(B). Sections were washed 2 X 15 minutes in PBS(A). Sections were then covered in antifade (Molecular Probes) containing glycerol and PBS, covered with a coverslip and sealed using nail polish.

Antibody staining of dissociated cultures

Spinal Cords were isolated from E8 chick and subjected to trituration through a small bore pipette in the presence of DMEM-F12. The resulting suspensions were plated on poly-L-lysine and laminin (1ng/ml) coated coverslips in the presence of NGF (20ng/ml), and were allowed to grow for 12 hrs at 37°C in the presence of CO_2. After 12hrs cultures were fixed in 3.7% paraformaldehyde and stained as per protocol. Primary mouse anti-NeuN antibody was used at a dilution of 1:100, while anti-cSEMA5B rabbit antibody was used at a dilution of 1:1000.

Pre-absorption of antibody with fusion protein:

A total of 100μg of purified fusion protein was incubated with 10μl of affinity purified antibody, and 100μl of NeuN antibody in a final volume of 10ml of PBS. The next day, cells were stained as per protocol using the pre-absorbed affinity purified antibody and NeuN Antibody.

Westerns Blots

Approximately 100-200mg of various tissues including spinal cord, tectum, retina, and gut were isolated from chick at various stages in microcentrifuge tubes. These tissues were homogenized using a homogenizing pestle in the presence of 100μL of

RIPA buffer (150mM NaCl, 50mM Tris pH 7.4, 5mM EDTA, 5.0% NP-40, 1.0% sodium deoxycholate (DOC) and 0.1% sodium dodecyl sulphate (SDS), aprotinin, leupeptin, phenylmethyl-sulfonyl floride (PMSF)). The total volume was brought up to 500μl (200mg/ml) with the addition of RIPA buffer. The Cells were incubated in RIPA buffer on ice for 20 minutes. Supernatants were collected and assayed for protein concentrations according to the manufacturer's instructions (BioRad). A total of 40μg of protein was loaded and separated on a 12% SDS-poly acrylamide gel electrophoresis (PAGE) gels and then electro-transferred to Hybond ECL Nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were blocked with 3% milk protein in Tris buffered saline (TBS)-Tween-20 for 1 hour at room temperature. Membranes were probed with of rabbit anti-cSEMA5B antibody (1:1000) in TBS-Tween-20 overnight at 4°C. Blots were incubated with of anti-rabbit horse radish peroxidase (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA) in 1% milk in TBS-tween-20 for 1 hour at room temperature. Antibody was visualized by enhanced chemiluminescence (ECL) according to manufacturer's instructions (Amersham Pharmacia, Piscataway, NJ).

Cytoskeletal Extractions:

For cytoskeletal fractions, the tissue isolation was the same as above, however, homogenized tissue was incubated on ice with constant rotation for 20 min in 500μl of cytoskeletal buffer (50mM NaCl, 10mM Pipes pH 6.8, 3mM $MgCl_2$, 300 mM Sucrose, 0.5% triton-X 100, 1.2mM PMSF, 10μg/ml aprotinin, leupeptin; Hinck et al., 1994) to extract Triton insoluble proteins. The resulting lysates were spun down at 14,000 rpm for 10 minutes at 4°C and the insoluble pellet and supernatant were separated. A total of 100μl of SDS IP buffer (15mM Tris pH 7.5, 5mM EDTA, 2.5mM EGTA, 1% SDS) was

added to the pellets and pellets were boiled for 10-15 minutes at 100°C. The resulting suspension was brought up to 400μl with the addition of cytoskeletal buffer. Protein assays were performed for both the soluble and insoluble fractions according to the manufacturer's instructions (Biorad) to determine the amount of protein. A total of 25μg of protein from both fractions was loaded and separated on a 12% PAGE gel as described above.

Results

The structure of SEMA5B:

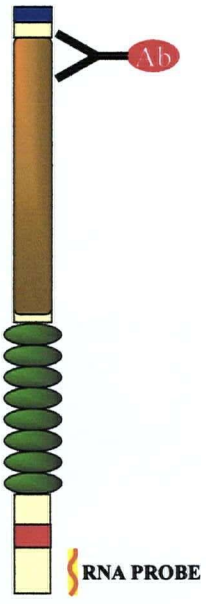
An analysis of cSEMA5B sequence shows that it is 1092 amino acids in length (figure 3). It is 77% identical and 87% similar to mouse Sema5B based on alignment using NTI Vector Suite 6.0 software. The structural domains of the protein include the semaphorin domain spanning approximately 500 amino acids, followed by seven thrombospondin repeats, a transmembrane region spanning 20 amino acids, and a short cytoplasmic region of 90 amino acids (figure 3A). Sequence analysis suggests that there may be a putative proprotein convertase cleavage sequence, RXR/KR, within the fifth thrombospondin repeat (figure 3B). A potential cleavage product is supported from westerns using an anti-cSEMA5B antibody which recognizes two distinct products (figure 11D, E). Precedence for the cleavage of semaphorins has been established for both the class 3 and 4 semaphorins (Adams et al., 1997; Elhabazi et al., 2001). Adams et al. 1997 demonstrated that furin-dependent proteolytic processing of Sema3a is required for an enhanced repulsive functionality. Similarly, Sema4D is cleaved by a metalloprotease-dependent process and exists as both a transmembrane and diffusible molecule in the immune system (Elhabazi et al., 2001).

Construction of an RNA Probe:

In mice, the cytoplasmic tails of the class 5 semaphorins consist of 80 (Sema5A) or 91 (Sema5B) amino acids. This region shows a lesser degree of similarity between 5A and 5B, than do other regions of the protein (Figure 4A). Indeed, an alignment of Sema5A and Sema5B nucleotide sequences demonstrated that the cytoplasmic region shows the greatest degree of divergence and thus provided an excellent sequence for the

Figure 3: Semaphorin 5B Structure and amino acid sequence

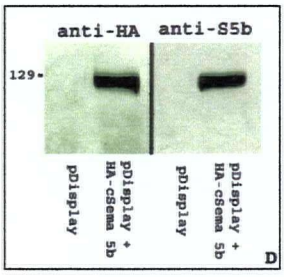
A schematic of cSEMA5B displaying the conserved regions between mice and chick. These include the signal sequence (blue), semaphorin domain (orange), thrombospondin repeats (green), and transmembrane domain (red) (A). Also present on the schematic is the representation of an affinity purified Ab used for the immuno studies, and a non-radioactive RNA probe used in the in situ and Northern studies. A western blot displays the specificity of the affinity purified Ab, as both a monoclonal HA antibody and the cSEMA5B antibody specifically recognize a recombinant cSEMA5B protein, containing an HA tag, which is over-expressed in HEK 293 cells. In addition, the amino acid sequence of cSEMA5B is displayed along with the conserved regions as indicated (B).



SEMA5B

A

-1092 AA IN LENGTH
-N-TERMINAL SIGNAL SEQUENCE
-500 AA SEMA DOMAIN
-SEVEN THROMBOSPONDIN REPEATS
-TRANS MEMBRANE REGION
-100 AA CYTOPLAMSIC REGION
-EXPRESSED IN THE BRAIN AND SPINAL CORD



The Amino Acid Sequence for cSEMA5B

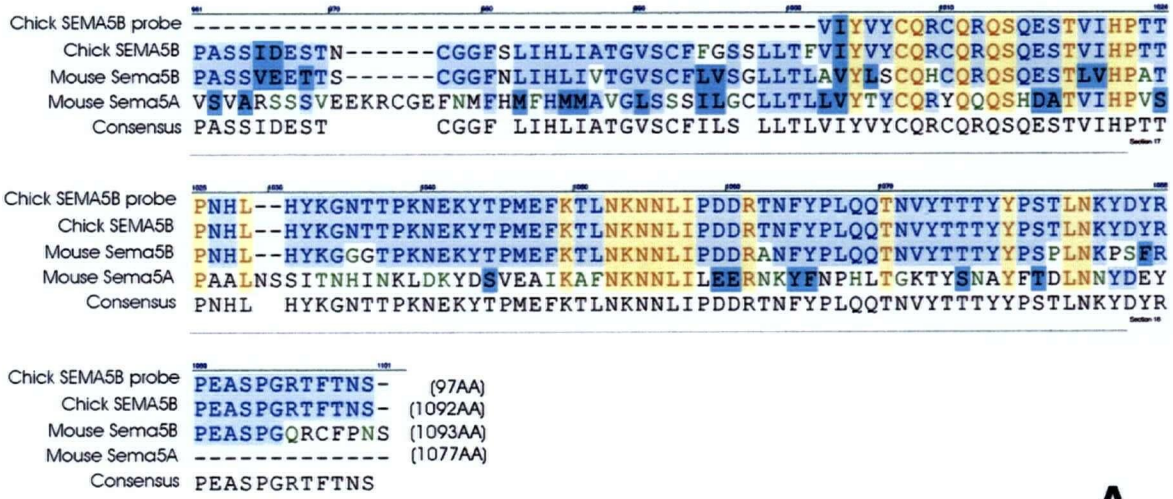
B

(1)MVVSRLKAISLSLPSLFLL{AFHLSASQNVPEYSEAEHQQCVRKEHPTIAF
EDLKPWVSNFTYPGVHDFSQLALDANRNQLIVGARNYLFRLSLHNVSL
IQATAWGSDEDTRRSCQSKGKTEEECQNYVRVLIV}YGKKVFTCGTNAFS
PVCSSRQVGNLSKIIDRINGVARCPYDPRHNSTAVITSRGELYAATVIDFSGRDPAIYR
SLGNVPPLRTAQYNSKWLNEPNFIAAYDIGLFTYFFFRENAVEHDCGKTVYSRVARV
CKNDIGGRFLLEDTWTTFMKARLNCSRAGEIPFYYNELQSTFYLPEQDLIYGVFTTN
VNSIAASAVCAFNLSAITQAFNGPFRYQENPRSAWLPTANPIPNFQCGTLSDDSPNEN
LTERVLQDAQRLFLMNDVVQPVTVDPYVTQDSIRFSKLVVDIVQGKDTLYHVMYIG
TEYGTILKALSTTNRSLRSCYLEEMQILPDGQREAIKSLQILHSDRSLFVGLNNGVLK
IPLERCSMYRTEGECLGARDPYCGWDNKQKRCTTIEDSSNMSLWTQNITECPVKNL
TTN*GRLGPWSPWQPCEHSDGDSTGSCMCRSRSCDSPRPRCGGRSCEGARI*EVANCSRN*GA*
*WTPWSSWALCSTSCGIGFQVRQRSCSNPAPRYGGRVCVGQSREERFCNENSPCP*LP*IFWSSWG*
*PWNKCSVNCGGGIHSRQRTCENGNTCPGCAVEYKTCNPESCP*EV*RRNTPWTPWMPVNITQN*
*GARQEQRFRYTCRAQLSDPHELQFGRKKTESRFCPNDGSAMCE*TDSLVDDLLKTGKTSARII
N*GGWSFWGAWSSCSRDCELGF**RIRKR**TCTNPEPKNGGLPCVGSAMEYQDCNPHPCP*VK*G*
*SWSCWTPWSQCSATCGGGHYQRTRTCTNPAPSSGEDICIGLHTEEALCNTHPC*E*GGWSEWSE*
*WSLCNEEGIQYRSRYCEVHSPDSSQCVGN*STQYQDCLYNEIPVILPASSIDESTNCGGFSLIH
LIATGVSCFFGSSLLTF(VIYVYCQRCQRQSQESTVIHPTTPNHLHYKGNTTPKNE
KYTPMEFKTLNKNNLIPDDRTNFYPLQQTNVYTTTYYPSTLNKYDYRPEASP
GRTFTNS) (1092)

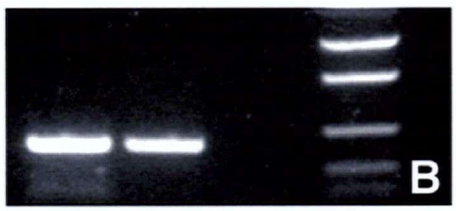
Signal Sequence ——— AAA	Tsp type-1-like repeat sequences — *AAA*
Antibody peptide-recognition sequence —— {AAA}	Transmembrane sequence —— AAA
Sema domain sequence — AAA	RNA probe sequence ——— (AAA)
	Putative cleavage sequence —— *AAA*

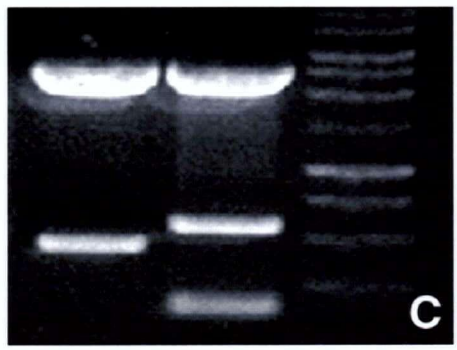
Figure 4: Analysis and alignment of cSEMA5B RNA Probe

An alignment of the cSEMA5B RNA probe sequence along with cSEMA5B, Sema5B, and Sema5A using Vector NTI suite 6.0 (A). Letters in yellow represent identical amino acids for all three sequences, while letters in light blue represents identical amino acids for cSEMA5B and Sema5B. A high divergence between Sema5A and Sema5B in mice over the probe region is indicated by a lack of blue or yellow lettering (A). The probe sequence was amplified from full-length cSEMA5B via PCR, and is 350bp in size when run on a 1% agarose gel at 90V (B). A double digest using NotI and SmaI releases the probe sequence from the bluescript29 KS vector in the first lane, while a PvuII digest confirms the correct orientation of the probe in the second lane (C). Both the antisense and sense RNA probes are ~350bp in size when run on a 1% agarose gel (D). The amount of probe is determined to be 100ng/μl when compared to a series of standards of known DIG-labeled RNAs concentrations, using the same color development process employed in the in situ hybridization procedure (E). The RNA isolated from tissue runs as two bands as most of the RNA present is rRNA which runs at ~4.8kb and ~ 2kb in size, corresponding to the 28S and 18S bands (F). The antisense probe recognizes dot blots of total RNA on a nylon membrane following the northern blot process (G). The sense probes also cross react with the total RNA, but to a much lesser degree (G).

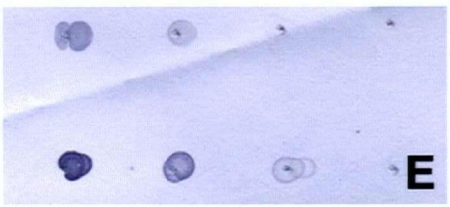

Chick SEMA5B probe
Chick SEMA5B
Mouse Sema5B
Mouse Sema5A
Consensus
PASSIDESTN------CGGFSLIHLIATGVSCFFGSSLLTFVIYVYCQRCQRQSQESTVIHPTT
PASSVEETTS------CGGFNLIHLIVTGVSCFLVSGLLTLAVYLSCQHCQRQSQESTLVHPAT
VSVARSSSVEEKRCGEFNMFHMFHMMAVGLSSSILGCLLTLLVYTYCQRYQQQSHDATVIHPVS
PASSIDEST CGGF LIHLIATGVSCFILS LLTLVIYVYCQRCQRQSQESTVIHPTT
PNHL--HYKGNTTPKNEKYTPMEFKTLNKNNLIPDDRTNFYPLQQTNVYTTTYYPSTLNKYDYR
PNHL--HYKGNTTPKNEKYTPMEFKTLNKNNLIPDDRTNFYPLQQTNVYTTTYYPSTLNKYDYR
PNHL--HYKGGGTPKNEKYTPMEFKTLNKNNLIPDDRANFYPLQQTNVYTTTYYPSPLNKPSFR
PAALNSSITNHINKLDKYDSVEAIKAFNKNNLILEERNKYFNPHLTGKTYSNAYFTDLNNYDEY
PNHL HYKGNTTPKNEKYTPMEFKTLNKNNLIPDDRTNFYPLQQTNVYTTTYYPSTLNKYDYR
PEASPGRTFTNS- (97AA)
PEASPGRTFTNS- (1092AA)
PEASPGQRCFPNS (1093AA)
---------- (1077AA)
PEASPGRTFTNS

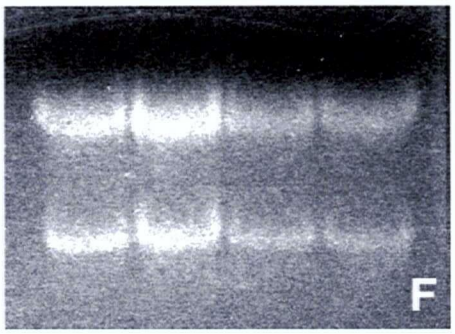
A

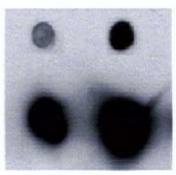

B


C


~350 bp
D


RNA
Standard
E
Antisense


F


Sense
Antisense
G

production of a specific RNA probe to distinguish between the two family members (Adams et al., 1996). Using this information, a chick probe corresponding to the final 300 bp, or the intracellular region of cSEMA5B, was used for the *in situ* hybridization and Northern experiments (figure 4A). Further sequence analysis demonstrated that this region is not only divergent between the class 5 semaphorins, but is distinct from other known semaphorin sequences.

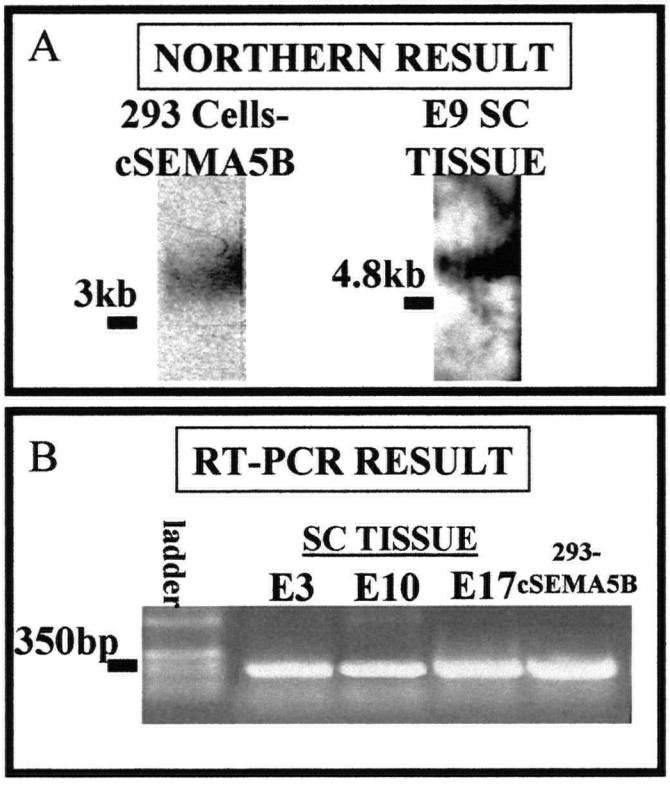
Following amplification of the desired probe sequence using PCR, the resulting product was sub-cloned into a Bluescript29-KS expression vector to produce both sense and anti-sense probes (figure 4B-D). Initial analysis of the probes was done by spotting total RNA, isolated from 293 cells expressing recombinant cSEMA5B, onto a nylon membrane, followed by a Northern blot procedure (figure 4F,G). The presence of strongly hybridized spots on the blot probed with the anti-sense RNA, demonstrated that these probes provided a suitable tool for use in the Northern studies (Figure 4F, G). In addition, a quantification protocol using dot blots, demonstrated that sufficient quantities of the DIG-labeled RNA probes were available for *in situ* hybridization studies (Figure 4E).

Northern Studies:

In order to determine whether cSEMA5B exists as a single transcript in chick, a Northern analysis was performed on spinal cord tissue obtained from an E9 chick. A single band at ~5kb suggests that cSEMA5B does exist as a single transcript (Figure 5A). Northern analysis on RNA isolated from 293 cells expressing a full-length recombinant form of cSEMA5B also displayed a single band of similar size, confirming the findings from chick tissue (Figure 5A). This single transcript product is consistent with Northern

Figure 5: Northern and RT-PCR Results

Chick SEMA5B is expressed as a single transcript of ~5kb as confirmed from total RNA isolated from cells expressing recombinant cSEMA5B and from E9 SC tissue (A). RT-PCR using primers specific to cSEMA5B confirms the expression of this semaphorin in spinal cord tissue isolated from chick ranging in age from E3 until E17 (B). HEK-293 cells expressing recombinant cSEMA5B were used as a positive control.


A
NORTHERN RESULT
293 Cells-
cSEMA5B
E9 SC
TISSUE
3kb
4.8kb
B
RT-PCR RESULT
ladder
SC TISSUE
E3
E10
E17
293-
cSEMA5B
350bp

analyses of other class 5 semaphorins in *Drosophila* and mice (Adams et al., 1996; Khare et al., 2000). In mice, Northern blot analysis of mRNA isolated from embryos revealed two Sema5A transcripts of 5.5 and 9.4 kb, while Sema5B is expressed as a single transcript of 5.9kb (Adams et al., 1996). In *Drosophila*, the class 5 semaphorin DSema5C is expressed as a single transcript ~5kb in size (Khare et al., 2000). As only a probe to the cytoplasmic region was used in these studies on cSEMA5B, it does not rule out the possibility for the existence of splice variants that lack this intracellular region.

RT-PCR studies:

As the Northern studies were technically difficult, and the sensitivity was low, RT-PCR was employed. Using primers specific to cSEMA5B, RT-PCR was performed on a variety of different chick tissues from a wide range of developmental stages. RT-PCR analysis demonstrated expression of cSEMA5B in the neural tube or spinal cord as early as E3 and as late as E17 (figure 5B). Expression of cSEMA5B at E3 was not confirmed using Northern blot analysis neither was any message observed in the *in situ* hybridization studies from chicks at this age. This is likely due to the greater sensitivity of the RT-PCR technique, and that less material is required. The spinal cord expression at E17 was also the latest stage examined for all of the studies presented, and corresponds with a stage when the majority of spinal cord development is complete (Eide and Glover, 1997). Other tissues examined included the tectum, retina, and regions of the gut (Data not shown). All of these areas were shown to express cSEMA5B, and confirmed the findings from both the Northern and *in situ* hybridization studies.

***In situ* Hybridization Studies:**

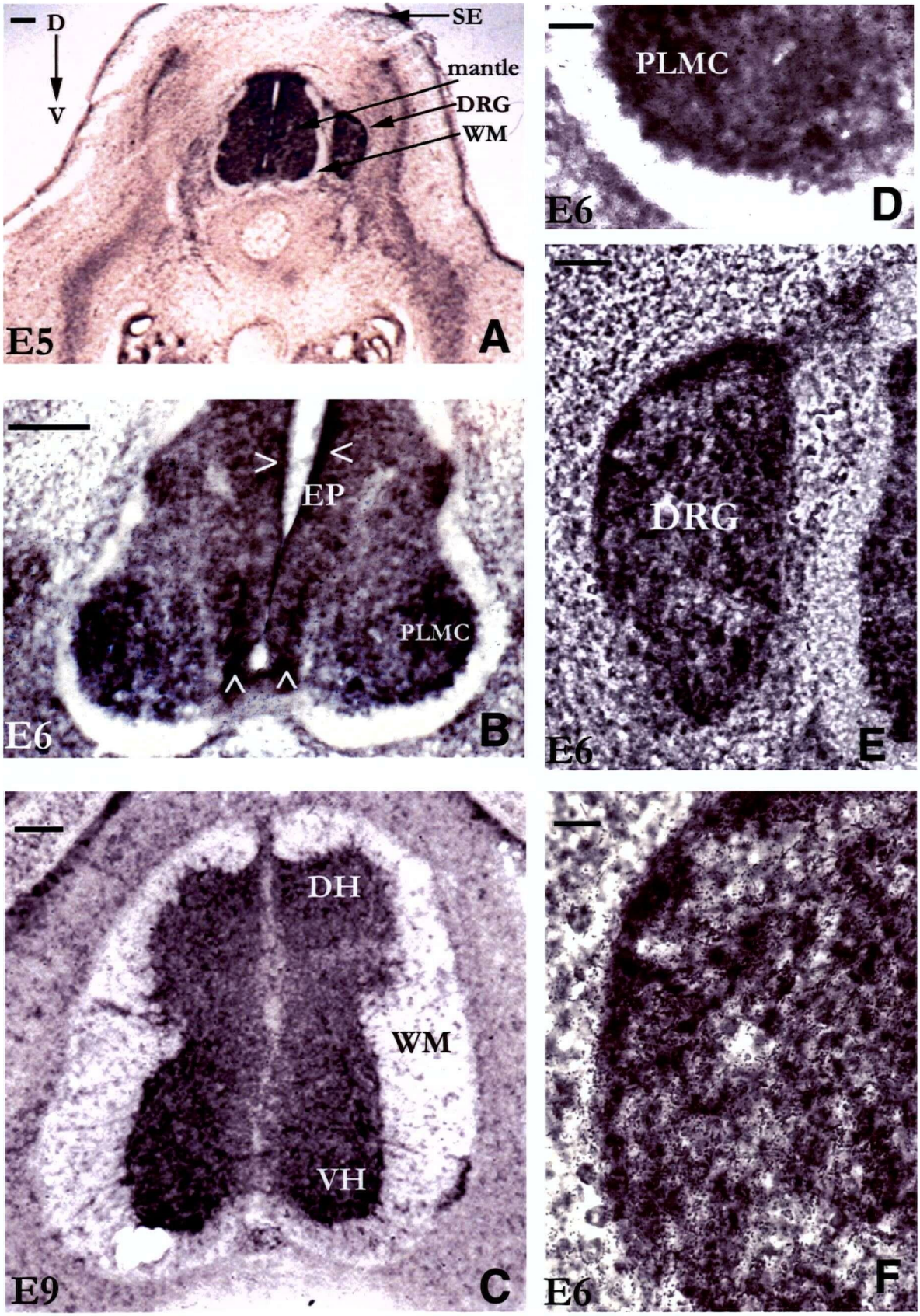
In order to gather more information as to the distribution of cSEMA5B message, *in situ* hybridization was utilized. Based on the results presented above, *in situ* hybridization studies were performed on chicks ranging in age from E2 until E11. From these *in situ* hybridization studies, cSEMA5B expression was first clearly observed in a variety of tissues at E5. This expression was broad and included the gut, epidermis, spinal cord, brain, olfactory and ventricular epithelium, and the retina. While many tissues express cSEMA5B, this thesis focuses on its expression in the developing nervous system.

cSEMA5B expression in the Spinal Cord:

Strong cSEMA5B expression is observed in the spinal cord at stage 26 (E5; figure 6A). At this age, the expression in the spinal cord is broad, being present in both dorsal and ventral regions including the mantle layer (figure 6A). Though this expression is broad, it is restricted to the grey matter with no observable message in the surrounding white matter (figure 6A). While the dorsal and ventral horns are not distinguished yet, a number of sensory axons have already reached the dorsal funiculus, an area associated with expression of cSEMA5B (figure 6A; Altman and Bayer 1984). There is also expression along the entire dorsal-ventral axis of the medial ependymal (EP; ventricular epithelium) layer, an area associated with a large population of mitotic cells (figure 6A; Bellairs and Osmond, 1998). By stage 28 (E6) however, the expression of cSEMA5B becomes more restricted in appearance, with the highest levels observed in large cells of the ventral horn associated with presumptive lateral and medial motor columns (figure 6B, D). Again, there is expression in the ependymal layer (figure 6B).

Figure 6: In situ hybridization studies in the spinal cord

In situ hybridization studies using a probe for cSEMA5B clearly confirm its expression in the spinal cord including the mantle layer, DRGs, and the dorsal surface epithelium (SE) at stage 26 (E5; A). At E6, the expression of cSEMA5B increases ventrally in presumptive lateral motor columns (PLMC) and is present along the ventricular ependymal (EP; epithelial) layer as indicated by the arrow heads (>; B). At E9 cSEMA5B message is highest in the ventral horn (VH), with lower levels in dorsal regions including the dorsal horn (DH; C). In contrast, the white matter (WM) is negative (C). At higher magnification, the ventral expression in the SC at E6 is clearly cellular in the presumptive lateral motor column (D). The expression in DRGs at E6 is uniform (E, F). Scale bar in (A,B,C) is 100μm; scale bar in (D,E,F) is 20μm.


D
V
SE
mantle
DRG
WM
E5
A
PLMC
E6
D
EP
PLMC
E6
B
DRG
E6
E
DH
WM
VH
E9
C
E6
F

The intensity of cSEMA5B expression in the ventral spinal cord continues to increase through E9 (figure 6C). The majority of cSEMA5B expression is associated with cells located in the lateral motor column region (figure 6C). While much of the spinal cord patterning has taken place at this stage, a number of primary afferent axons are still invading the dorsal grey matter with many reaching the lateral motor column at E10 (Shiga et al., 2000). Observations from E10 spinal cord sections indicate that this expression of cSEMA5B is maintained during these latter axon pathfinding events (data not shown).

cSEMA5B expression in DRG:

Expression of cSEMA5B was also apparent within dorsal root ganglia. This expression was evident as early as E5 (Figure 6E, F). Unlike the dynamic expression in the spinal cord, the expression in the DRGs remained uniform and constant throughout development. As there are a variety of different sensory neurons and glial cells present at all of the ages examined, characterization of the cell types expressing cSEMA5B awaits further analysis. Different populations of cells are localized to different areas within the DRG, and while high magnification on DRGs demonstrates that not all of the cells express cSEMA5B, the expression is uniform and not biased for a particular area associated with a specific cell type (figure 6F; Eide and Glover, 1997). This coincident expression of a semaphorin in both the spinal cord and DRG neurons is similar to the expression of Sema3A in embryonic chick (Shepherd et al., 1996).

Expression of cSEMA5B in retina:

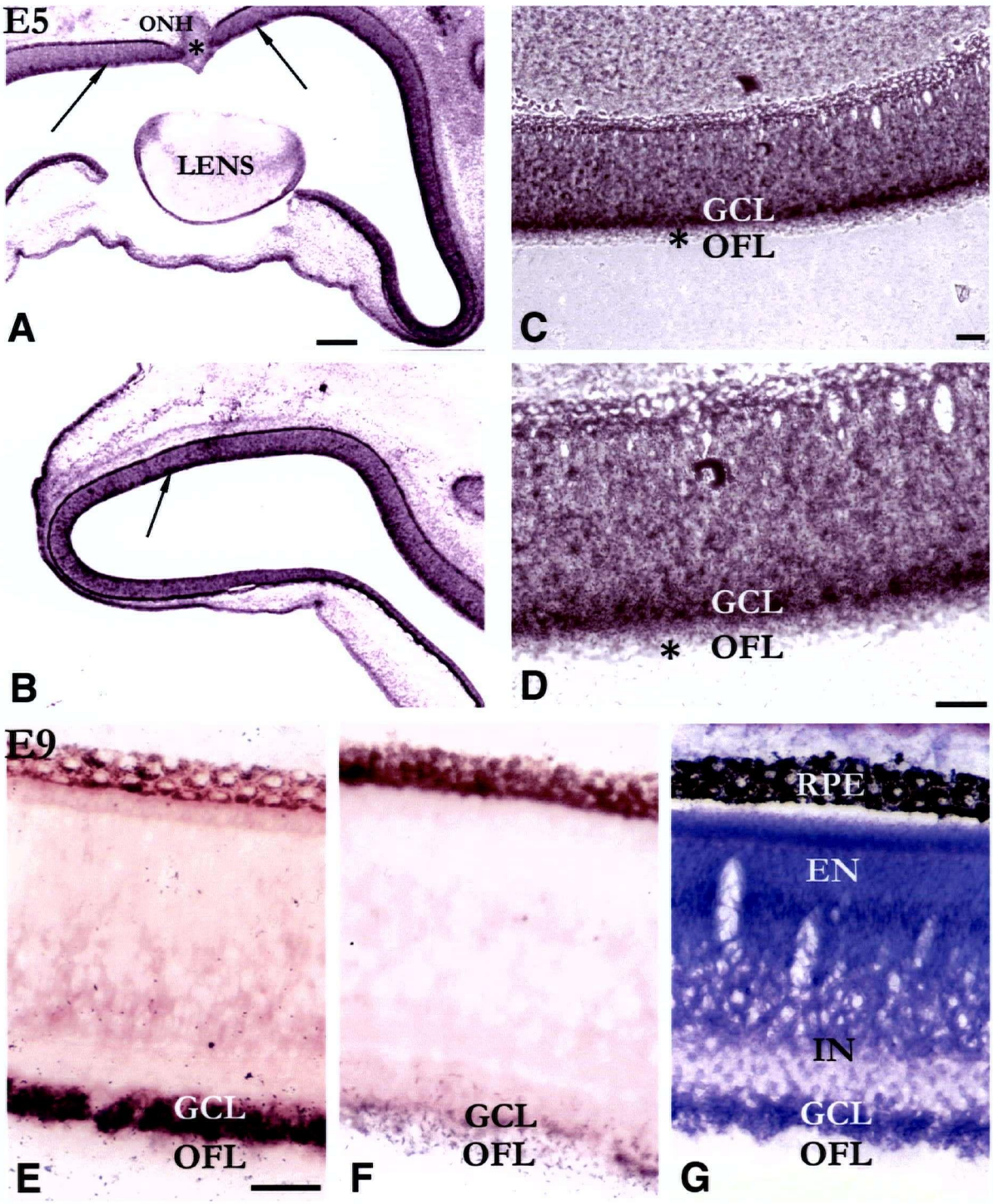
In addition to the spinal cord and DRGs, cells associated with the retina also express cSEMA5B. Expression can be clearly observed at stage 26 (E5), in an inner

layer of the retina, presumably along the retinal ganglion cell (RGC) layer (GCL; Figure 7). The expression is confined to the cell body layer and is not associated with the nerve fibre layer (figure 7C-F). Unlike the uneven distribution of cSEMA5B message within the spinal cord, the expression associated with the retina is uniform. Expression is absent in the optic nerve head (ONH) and along the optic nerve (ON; Figure 7A). This region shows exclusive expression of Sema5A in mice (Oster et al., 2003). The cells expressing cSEMA5B are likely ganglion cells, as the other populations of cells associated with the RGC layer, including some amacrine and Müller glial cells, do not have their nuclei exclusively in this region (Mey and Thanos, 2000). Interestingly retinal axons likely receive intruction from the radial glial cells of Müller which have been shown to express different guidance cues, for example the netrin receptor DCC (Gad et al., 2000). Retinal ganglion cells encounter other guidance cues, such as laminin, through interactions with the glial endfeet and basal lamina present in the nervefibre layer at the vitreo-retinal border (figure 15B; Stier and Schlosshauer, 1999). Interactions between radial glia and the retinal axon growth cones are assumed to occur within the optic stalk, along the optic tract, and within the optic tectum (Silver, 1984; Thanos and Mey, 2001).

At later stages including E9, this cSEMA5B expression becomes more intense and continues to be associated with the inner most cell layer of the retina (Figure 7E). At these older ages, the distribution of cSEMA5B message remains uniform along both the temporal/nasal axis and the rostral/caudal axis. At these stages, it is still not clear as to what cell-types are expressing cSEMA5B, but the expression overlaps the normal distribution of RGC and amacrine cells (Mey and Thanos, 2000).

Figure 7: In situ hybridization studies in the retina

cSEMA5B expression within the retina is relatively uniform in the nasal temporal plane. This expression at stage 26 (E5) is associated with the RGC layer (GCL) as indicated from the arrows (A, B, C, D). Notably, cSEMA5B expression is absent from the optic fibre layer (OFL) and the optic nerve head (ONH) as indicated with an asterisk (A, C, D). At a later stage, cSEMA5B expression is still associated with the GCL in the E9 retina (E). The sense probes do not have message associated with this layer (F). (G) is an example of a Nissl-stained retina used in histological analysis of the tissue. The associated retinal layers identified include the optic fibre layer (OFL), ganglion cell layer (GCL), inner nuclear layer (IN), external nuclear layer (EN), and retinal pigmented epithelium (RPE). Scale bar for (A, B) is 100μm; scale bar for (C-G) is 10μm.

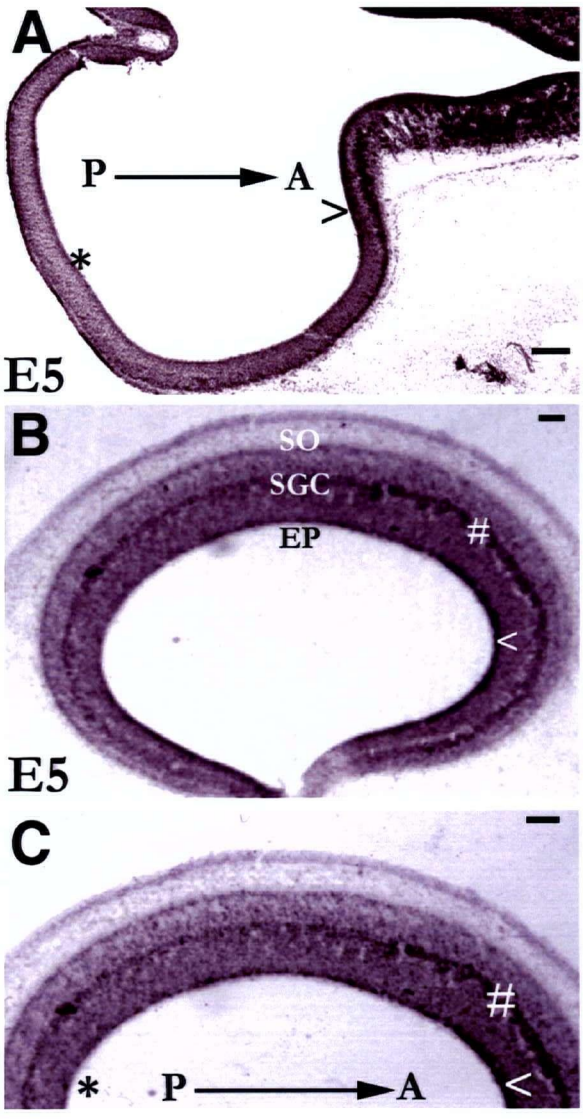

E5
ONH
*
LENS
A
B
C
GCL
* OFL
D
GCL
* OFL
E9
GCL
OFL
E
F
GCL
OFL
RPE
EN
IN
GCL
OFL
G

cSEMA5B expression in the Tectum:

At early stages of development, the tectum is the most prominent anatomical and functional structure of the avian brain. Results from the *in situ* hybridization studies demonstrate that cSEMA5B is expressed in the tectum at E5 (figure 8), just prior to the arrival of the first retinal fibres at E6, when maximal cell proliferation is occurring in the tectum (Halfter, 1987). Similar to the expression of cSEMA5B in the spinal cord, the distribution of message in the tectum is asymmetrical (Figure 8). The highest expression appears to be associated with anterior regions, getting less intense in posterior regions (Figure 8A). Anterior/ventral tectum represents the entry for incoming retinal afferents (figure 16C). In middle regions of the tectum, this expression occurs along the inner most ependymal (EP; neuroepithelium) lining of the ventricle, a layer highly associated with cell proliferation at this stage (figure 8A; Mey and Thanos, 2000). There is also additional expression in a central layer at E6 and E7, which later forms the stratum griseum centrale (SGC; figure 8B, C). This layer is comprised of large multipolar neurons, often referred to as ganglion cells, which are generated early in comparison to other neurons in the tectum (figure 8B, C; Mey and Thanos, 2000). While this layer represents the principal tectal efferent neurons, it also acts as a target for incoming retinal afferents growing along the superficial, stratum opticum layer (Mey and Thanos, 2000). Tectal expression was observed at later stages but is not included in this report. The expression in the tectum overlaps a period of maximum cell proliferation as well as innervation from retinal afferents.

Figure 8: In situ hybridization studies in the tectum

Expression of cSEMA5B is present at stage 26 (E5) along the ependymal (EP; epithelial) layer as indicated by arrowheads (>; A-C). The anterior expression (<) of cSEMA5B is greater than the posterior expression (*) along the EP (A-C). In sections from the dorsal tectum, there is additional expression of cSEMA5B in the presumptive stratum griseum centrale (SGC) layer as indicated (#; B, C). There is no expression along the stratum opticum (SO) the most superficial layer associated with optical fibres. Scale bar in A is 100μm; scale bar in (B,C) is 10μm.


A
P
A
*
E5
B
SO
SGC
EP
#
<
E5
C
#
*
P
A
<

Epithelial expression:

In all of the regions examined there is an obvious expression of cSEMA5B in the associated neuroepithelia. This expression is consistent with observations from mice and rat, where Semaphorin5B is ubiquitously expressed in most neuroepithelia in the brain and other structures (Adams et al., 1996; Skaliora et al., 1998). Observations from embryonic chick brain show that cSEMA5B is expressed in the ventricular ependymal (epithelium) layer at stage 26 (E5; Figure 9). The expression, as seen in the ependymal layer of the spinal cord, is relatively uniform along the anterior-posterior axis. Sections from rostral and caudal regions of the brain again display a similar pattern of expression within the ventricular epithelium (Figure 9A, B). This expression is consistent with brain sections from rat where SEMA5B is found uniformly distributed in most of the epithelia within the brain, including the ventricular zones (Skaliora et al., 1998).

High expression of cSEMA5B is also observed in the olfactory epithelium in transverse sections from E5 and E6 chick (Figure 10). During embryogenesis many studies have demonstrated that the olfactory epithelium is the site of origin of several types of cells which migrate along the olfactory nerve (ON) towards the brain (Dryer and Graziadei, 1994; Drapkin and Silverman, 1999; Wray, 2001). The expression of cSEMA5B decreases in the surrounding lateral olfactory tissue layers. These layers serve as target regions for a number of cells produced along the olfactory epithelium. Thus, this assortment of migrating cell populations and various neuronal pathfinding events associated with the olfactory epithelium during these stages correlates with expression of cSEMA5B.

Figure 9: In situ hybridization of ventricular epithelium

cSEMA5B expression in the ventricles at stage 26 (E5) is highest in the neuroepithelium as indicated by the arrowheads (A-C). This expression is uniform along anterior-posterior plane (A). A similar, uniform, epithelial-associated expression is seen at lower levels within the brain (B). Scale Bar in (A,B) is 100μm; scale bar in C is 20μm.

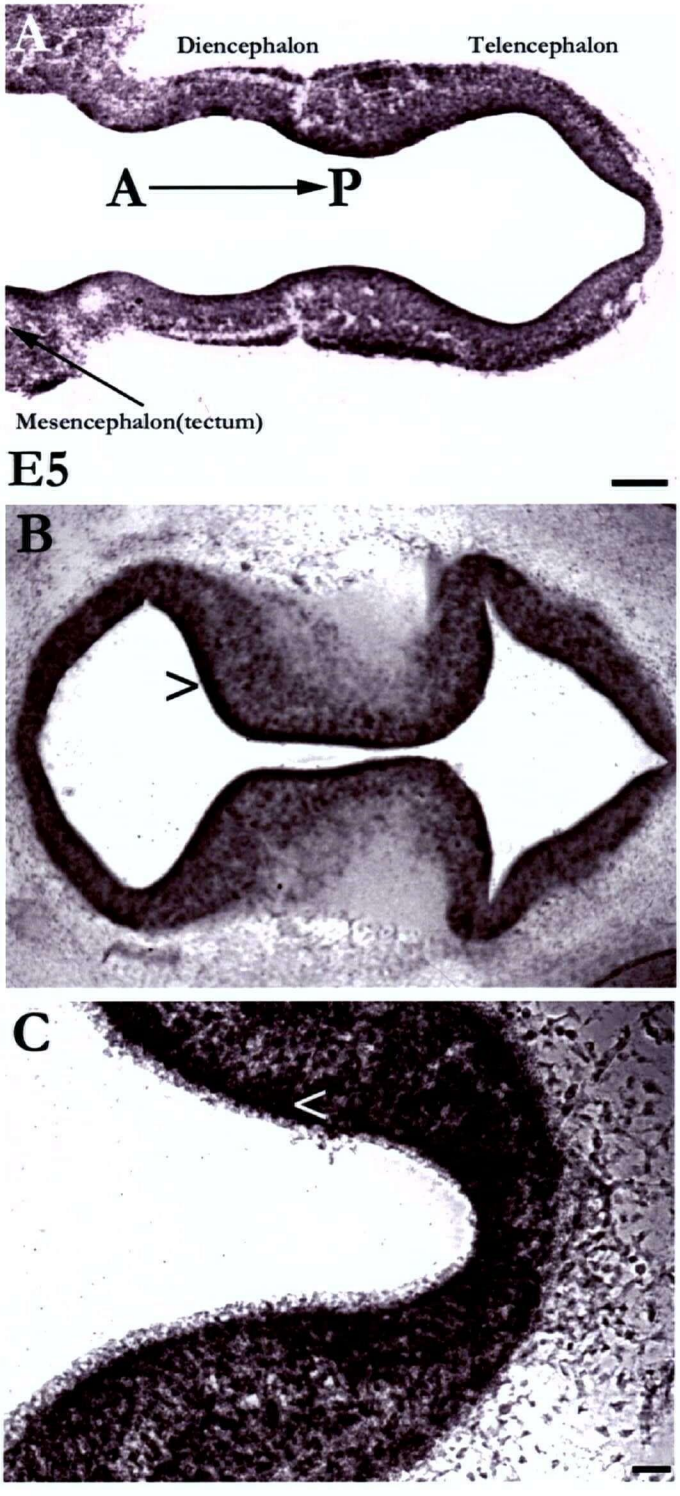
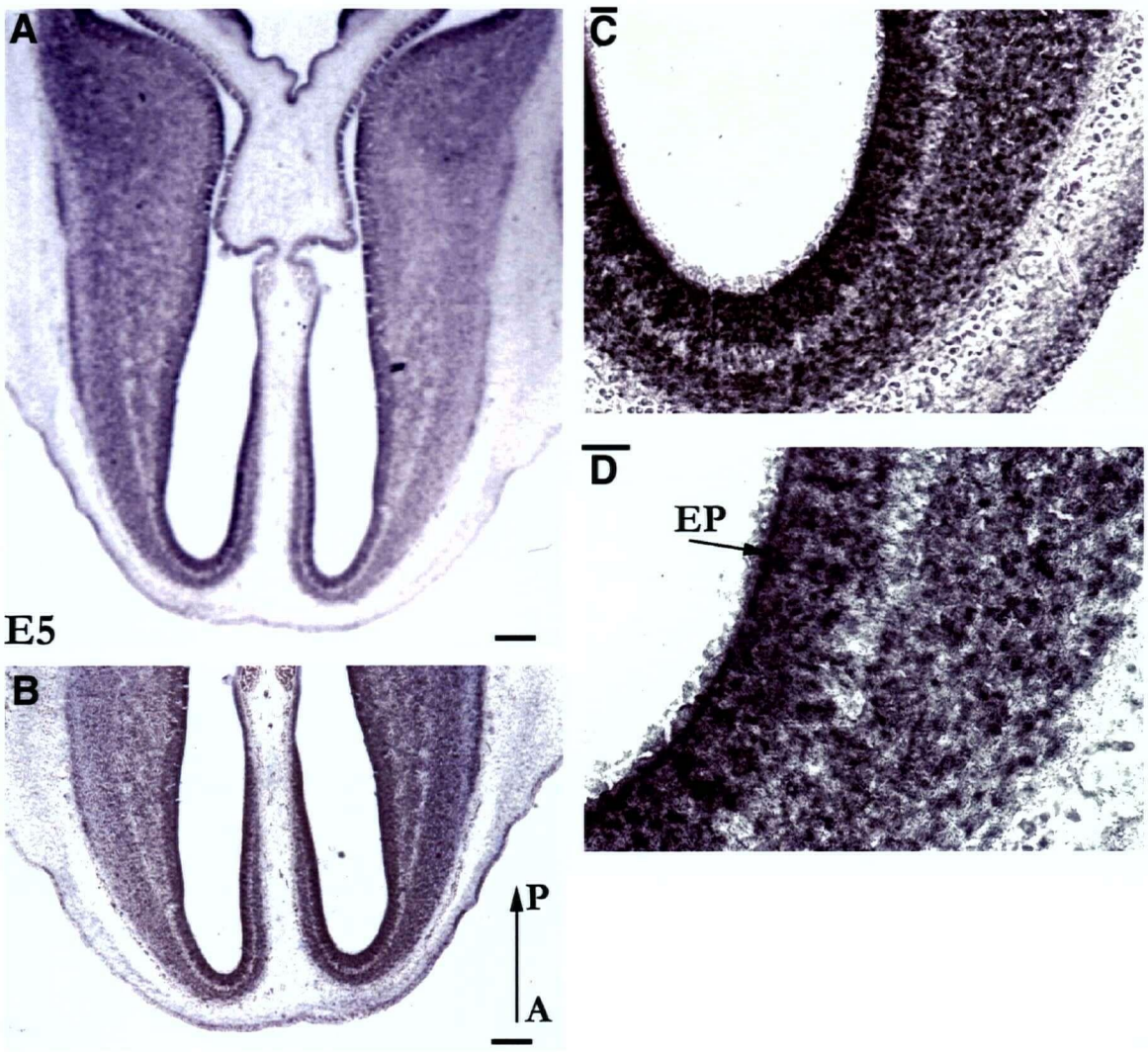

A
Diencephalon
Telencephalon
A
P
Mesencephalon(tectum)
E5
B
C

Figure 10: In situ hybridization studies in nasal regions

At stage 26 (E5), expression of cSEMA5B in the nasal epithelium is relatively uniform (A, B). At higher magnification, the cSEMA5B distribution is greatest in the inner epithelial layer, decreasing in the outer layers (C, D). Scale bar in (A,B) is 100μm; scale bar in (C,D) is 10μm.


A
E5
B
P
A
C
D
EP

In addition to the tissue regions mentions above, there was expression seen in the surface epithelium located dorsally to the spinal cord. This observation was consistent in both the *in situ* hybridization studies as well as from antibody studies (figures 6A & 12 A). Again, similar staining patterns for semaphorins have been observed from studies on secreted sema3A (Shepherd et al., 1996). There was also distribution of cSEMA5B expression in the epithelium associated with the gut (data not shown). A summary of the expression of cSEMA5B from all tissues examined clearly demonstrates the association of this expression with the neuroepithelia in these regions (Table I).

Immunohistological studies :

Using an affinity purified antibody (see materials and methods) raised against the N-terminal region of cSEMA5B, the distribution of cSEMA5B protein was analyzed (figure 3). Based on the expression of semaphorin from the *in situ* hybridization studies, chicks were examined from E5 through until E11 to determine whether the protein distribution mimicked that of RNA. Initial studies performed using serum from non-affinity purified antibody revealed expression in the spinal cord similar to the RNA distribution from *in situ* hybridization studies, and seemed to support both the findings from the *in situ* hybridization studies, as well as the use of this antibody in further studies (data not shown).

Westerns:

In order to confirm the specificity of the antibody, a series of western blots from a variety of different tissues were produced and analyzed. Initial blots suggested that the non-affinity purified antibody recognized a number of different antigens on the blot (data not shown). Based on these westerns the antibody underwent an affinity purification

Table I: Summary of cSEMA5B expression in all regions

A summary of the noted expression of cSEMA5B fro the in situ hybridization studies highlights the broad expression of this semaphorin in the developing neuroepithelium as well as within the CNS including the spinal cord and tectum. Abbreviations: N/C, not completed; n/a, not applicable.

Tissue/ Region	Age	Epithelial expression	Distribution	Non-epithelial expression	Distribution
Spinal cord	E5	yes	Along EP layer	yes	Dorsal and Ventral grey matter
	E6	yes	Along EP layer	yes	No change
	E7/E8	yes	Ventral EP layer	yes	Low dorsal, higher ventral
	E9/E10	none	n/a	yes	Ventral regions including LMC
Retina	E5	none	n/a	yes	Along RGC layer, no gradient
	E6	none	n/a	yes	No change
	E7/E8	none	n/a	yes	Increase in expression along RGC layer
	E9/E10	none	n/a	yes	No change
Tectum	E5	yes	Gradient along epithelial layer with higher anterior levels	yes	Along the SGC layer in dorsal tectum
	E6	yes	Gradient along epithelial layer with higher anterior levels	yes	Along the SGC layer in dorsal tectum
	E7/E8	N/C	N/C	N/C	N/C
	E9/E10	N/C	N/C	N/C	N/C
Ventricle	E5	yes	Along EP layer	none	n/a
	E6	yes	Along EP layer	none	n/a
	E7/E8	N/C	N/C	N/C	N/C
	E9/E10	N/C	N/C	N/C	N/C
Nasal	E5	yes	Along entire epithelial layer	yes	The expression in outer layers is lower, decreasing as you go further away from the epithelium
	E6	yes	Along entire epithelial layer	yes	The expression in outer layers is lower, decreasing as you go further away from the epithelium
	E7/E8	N/C	N/C	N/C	N/C
	E9/E10	N/C	N/C	N/C	N/C

procedure, outlined in the materials and methods (figure 11). Brain fractions and spinal cord fractions were isolated at embryonic days 8 and 9, and the protein fractions were separated on SDS-polyacrylamide gels. The resulting blots showed a significant decrease in the number of bands present. There were two prominent bands one at 90 kD and one at 130kD (the predicted molecular weight; figure 11D). Further isolation of the soluble fraction from the insoluble cytoskeletal fraction revealed a single band in the insoluble fraction (containing membrane fraction) at 130kD (figure 11E). This band corresponds with the predicted molecular weight for cSEMA5B and confirms the specificity of the affinity-purified antibody. The staining was then performed in non-detergent conditions in order to prevent any antibody from entering the cells.

The identity of the 90kD band remains unclear, however, a possible cleavage binding site within the fifth thrombospondin repeat would produce a secreted product of ~90kD in predicted size. The possibility for this product presents an interesting situation where cSEMA5B might exist in a transmembrane form or as a secreted molecule that may act long range.

cSEMA5B distribution in Spinal Cord:

After a series of trials using a variety of fixation procedures and antibody exposures, consistent labeling of cSEMA5B was obtained. Within body sections at embryonic days E6 and E7, the expression is observed in the spinal cord and the surface epithelium located dorsally to the spinal cord (figure 12A). A closer examination of the expression in the spinal cord at this stage suggests that it is broadly distributed in the spinal cord in both dorsal regions, including lamina II and III and in ventral regions including the presumptive lateral motor column (figure 12A). Though the expression is

Figure 11: Ab production and western blots

Isolated GST (A) and cSEMA5B (B) peptides were separated on a 12% polyacrylamide SDS gel and stained with coomassie blue to confirm the correct size, ~26kDa and 35kDa respectively. Following Ab purification through a GST and cSEMA5B column, the final elutions containing cSEMA5B Abs, were confirmed by separating the heavy and light chain Ab peptides on a 12% polyacrylamide SDS gel (C). Total protein was isolated from E9 brain and Spinal cord tissue using RIPA buffer (D). Affinity purified cSEMA5B Ab recognizes two distinct bands at 90kD and 130kD (D). Separating the soluble fractions (right lane) from the insoluble fractions containing the membrane (middle two lanes) reveals a 130kD band in the insoluble component of similar size to the cSEMA5B expressing 293 control cells (left lane: E).

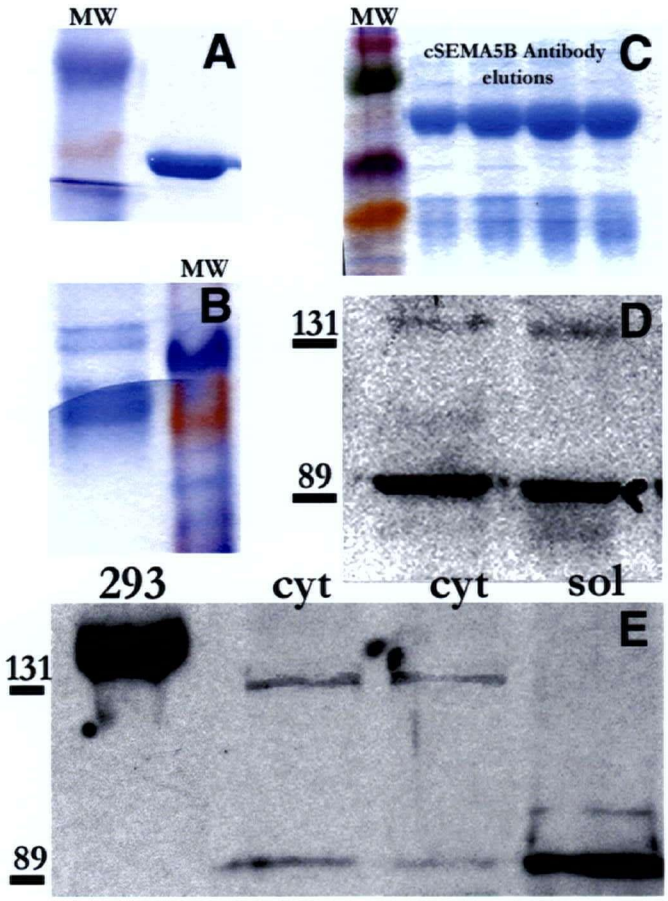
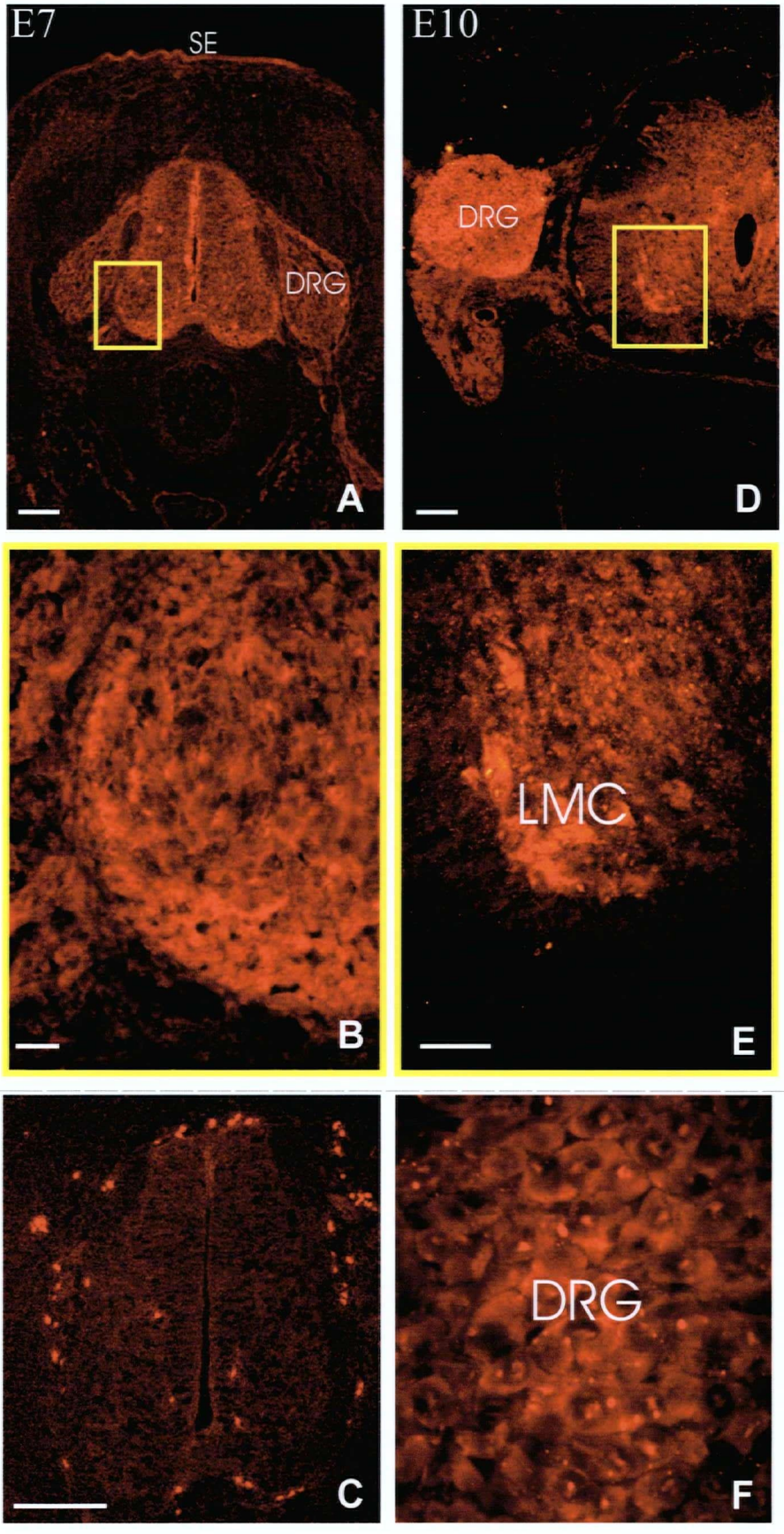

MW
A
MW
cSEMA5B Antibody elutions
C
MW
B
131
D
89
293
cyt
cyt
sol
E
131
89

Figure 12: Semaphorin 5B distribution in Spinal Cord

At E7, the distribution of cSEMA5B protein in the grey matter is wide spread (A) with highest levels in the ventral regions (B). The dorsal surface epithelium (SE) also stains for cSEMA5B. E10 Spinal cord sections stained without detergent show staining in the spinal cord with highest levels in a subset of cells in the same location as presumptive motor neurons (D, E). The staining associated with DRGs is relatively uniform at all stages examined (A, D, F). Sections stained with the pre-immune serum show no significant staining (C). Scale bars in (A, C, D) are 100μm; scale bar in (B, E, F) are 10μm.

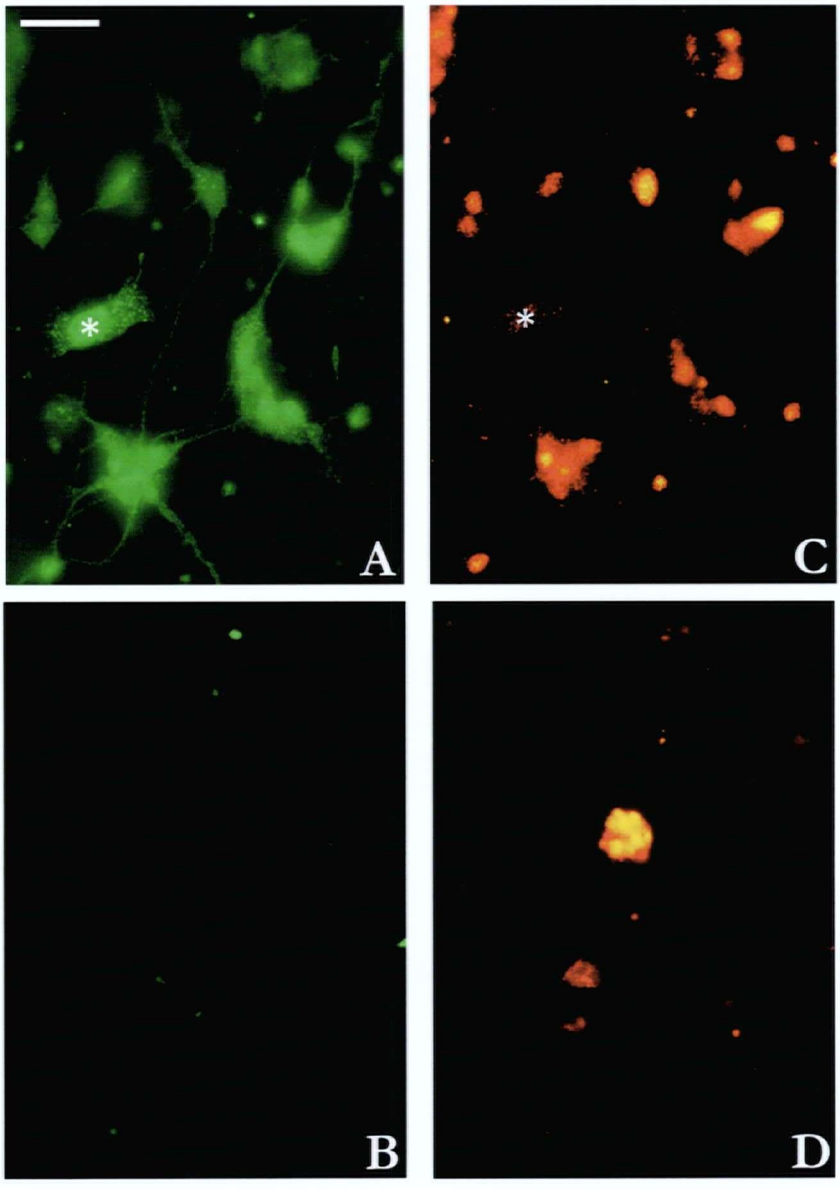

E7
SE
DRG
A
E10
DRG
D
B
LMC
E
C
DRG
F

broad, the levels of cSEMA5B are higher in ventral regions, as is evident from the more intense levels of fluorescence in this region of the spinal cord (figure 12A, B). Similar to the regions identified in the *in situ* hybridization studies, the expression is highest in cells located within the presumptive lateral motor column. In addition, there is some staining associated with the ependymal layer along the dorsal ventral axis.

As the spinal cord continues to develop, there are significant changes in the distribution of cSEMA5B. The dorsal expression of semaphorin diminishes, particularly in medial regions corresponding to lamina III. The ventral expression at this age remains strong at E8 and E9 along with staining along the ventral ependymal layer (figure 13B; data not shown). At later stages including E10 and E11, the expression continues in ventral regions however, becoming localized to a population of large diameter cells in the ventral horn. The identity of these cells is unclear, but overlaps with the normal distribution of motor neurons (figure12 D, E; Eide and Glover, 1997). In addition, spinal cord cells that have been dissociated were also labeled with cSEMA5B antibody along with a neuronal marker NeuN to further characterize these cells (figure13). From these experiments it is clear that many of the cells labeled with anti-cSEMA5B antibody are neuronal, as they are also recognized by the NeuN antibody (figure 13 A, B, D, E). Interestingly, a small population of cells that express cSEMA5B are not NeuN positive, and based on morphology appear to be glial in origin (figure 13 A, B). When the antibody is pre-incubated with cSEMA5B fusion protein prior to staining, the cells no longer stain for cSEMA5B further confirming the Ab's specificity (figure 13 C, F).

Figure 13: Dissociated Spinal Cultures

Anti cSema5B antibody (A) labels neuronal cells (C) as well as glial cells (*). Spinal cords were isolated from E8 chick and subjected to trituration with a small bore pipette. The resulting suspension of cells was plated on coverslips coated with poly-L-lysine and laminin. Cells were doubled labeled using anti Sema5B antibody (green; A) and a neuronal anti NeuN antibody (red; C). Cells stained with Abs that are pre-incubated with cSEMA5B peptide, are devoid of cSEMA5B staining (B). These same cultures stain for NeuN (D). Scale bars in (A-D) are 10μm.


A
C
B
D

The high levels of cSEMA5B in this region of the spinal cord suggests that it might be involved in either the sorting of motorneurons or in the maintenance of proper patterning within the spinal cord.

Distribution of cSEMA5B in DRGs:

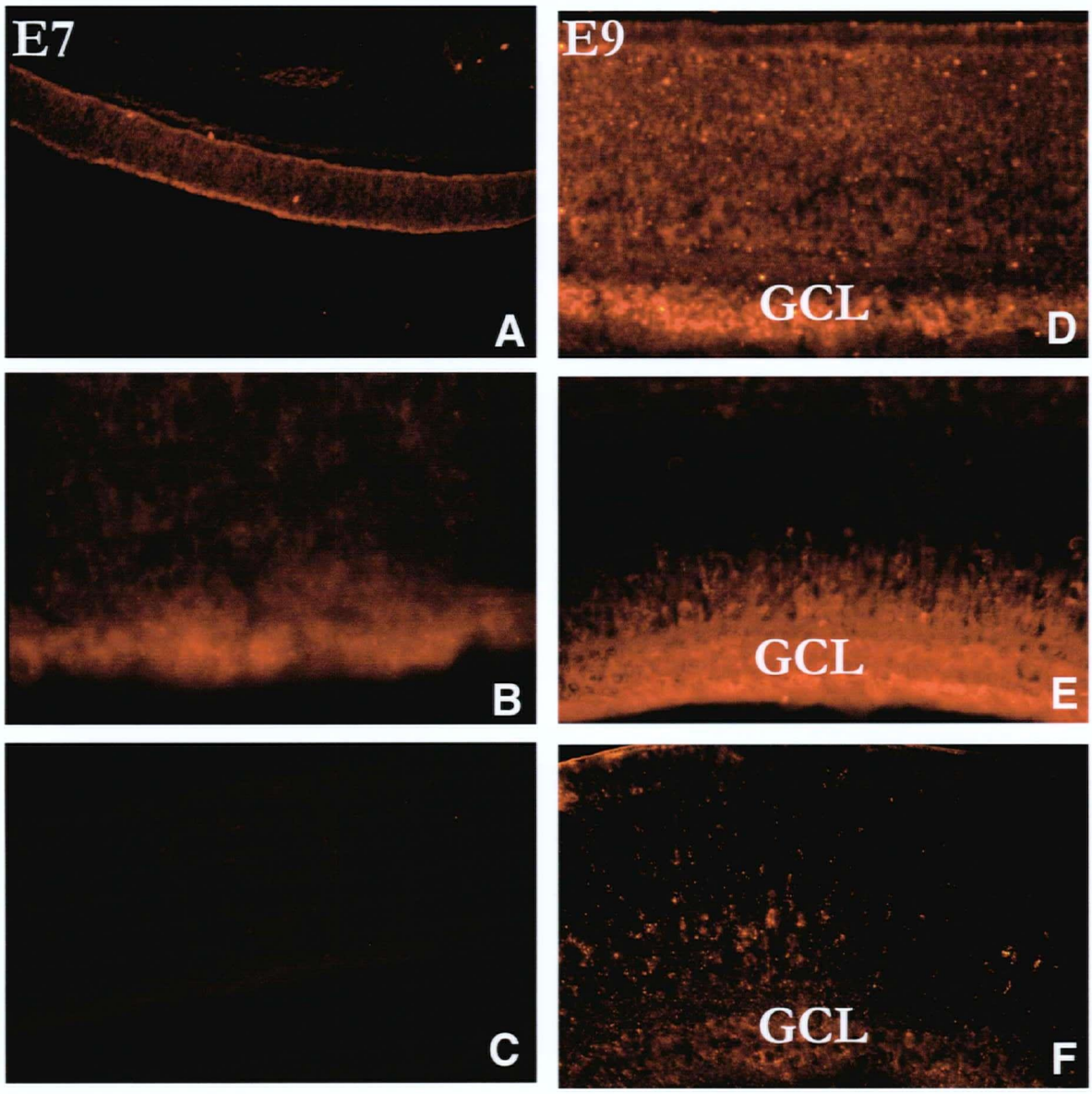
The *in situ* hybridization studies showed that expression of cSEMA5B in the DRGs is uniform and maintained from E5 through E10. The distribution of cSEMA5B within DRGs, as demonstrated by immuno-histochemical studies using anti-cSEMA5B antibody, is consistent with the distribution of cSEMA5B message (compare figures 6A, E; 12A, F). Again, cSEMA5B is highly expressed in DRGs at all stages examined. DRG ganglion were isolated from E8 chick and were dissociated and labeled using cSEMA5B antibody. A strong axonal and cell body punctate staining confirms the presence of cSEMA5B in the DRG neurons (data not shown). At E6 when DRG expression is clearly apparent, there are different sized classes of sensory neurons that occupy different spatial domains within the DRG. However, there was no apparent restriction of the labeling with either population of neurons, leaving the identity of these neurons unknown.

cSEMA5B distribution in the retina:

The distribution of cSEMA5B protein in the retina is consistent with the RNA studies and is associated with the retinal ganglion cell layer. This expression is identified at E7 and continues through E9 and E10 (figure 14). While the distribution of this semaphorin in the retina remains relatively uniform throughout development, the levels of expression appear to change (figure 14 A, E). While not quantified, it is clear that the intensity of fluorescence increases with an increase in age, particularly at E9 and E10

Figure 14: Semaphorin 5B distribution in the Retina

The distribution of cSEMA5B is identified in the RGC layer as early as E7 (A, B). At later stages (E10), cSEMA5B expression remains uniform and is associated with the RGC layer (D, E, F). At these later stages, the intensity of the staining increases as compared to E7. Staining using pre-immune serum from rabbit was used as a control (C, F). The staining is visualized with an anti-rabbit, Cy-3 secondary Ab. Scale Bar in A is 100μm; Scale bar in B is 10μm; Scale bars in (C-F) are 10μm.


E7
A
B
C
E9
GCL
D
GCL
E
GCL
F

(figure 14D, E). Though the distribution of cSEMA5B associated with the ganglion cell layer would suggest that the staining is neuronal, this has not been confirmed from dissociated cultures.

Discussion:

Ten years after the identification of the first semaphorin in the grasshopper limb (Kolodkin et al., 1992, 1993), our understanding of this family of proteins has greatly advanced. While much effort has gone into the characterization of the secreted vertebrate semaphorins, recent work has begun to characterize vertebrate transmembrane semaphorins. The results presented in this thesis, in conjunction with some important functional studies from our lab, have allowed us to gain some insights into the role of one transmembrane semaphorin in chick, SEMA5B. What is particularly intriguing about this semaphorin is the presence of a typically repulsive SEMA domain and a region of thrombospondin repeats associated with neuronal outgrowth. The possibility of two regions associated with opposite guidance responses located on the same protein makes this semaphorin an interesting choice for study.

These studies have demonstrated that the expression of cSEMA5B in chick is consistent with expression of this semaphorin in other organisms including mice and rat. Within the developing nervous system, the majority of cSEMA5B expression is associated with the neuroepithelium. This expression occurs along the epithelium of olfactory system, in the ventricular zone of the brain and spinal cord, and in the tectal epithelium (table I). During the ages examined, all of these regions are associated with the proliferation of a number of different cell types, many of which are neuronal. While much of the literature on semaphorins suggests that they are involved in axon guidance, a number of studies have implicated a role for semaphorins in the migration of a variety of different cell types (Hu et al., 1996; de Castro et al., 1999; Sugimoto et al., 2001; Ginzburg et al., 2002; Comoglio and Trusolino, 2002; Tsai et al., 2002; Tamamaki et al.,

2003). As most semaphorins are inhibitory guidance cues, one can speculate that perhaps in these tissues, this semaphorin may be required for the induction of migration of these cells away from their proliferative centers, and into the surrounding tissue.

In addition to this broad epithelial expression of cSEMA5B, there are also a variety of cells within the DRGs, spinal cord, and retina which express cSEMA5B and are not associated with an epithelial layer. Expression of cSEMA5B in these regions overlaps with the normal distribution of neuronal cells and correlates with a number of key axon guidance decisions and processes. Indeed, evidence collected from a number of functional in vitro studies demonstrates that different neuronal populations associated with these regions are inhibited by cSEMA5B. Based on our knowledge on semaphorin function from *in vivo* and *in vitro* studies, these results support a role for this semaphorin in neuronal development in the chick.

Role of SEMA 5B in developing spinal cord:

The expression of cSEMA5B within the grey matter of the spinal cord is dynamic, being first expressed broadly in both dorsal and ventral regions, but later being restricted to ventral regions. This dynamic expression correlates with some key axon guidance decisions in these regions. At this stage of development various populations of sensory and motor neurons make projections either to or away from the developing spinal cord. Primary DRG sensory neurons follow highly stereotyped pathways in reaching their specific target areas in the spinal cord. The first sensory afferents reach the spinal cord at embryonic day 4.5 (E4.5), just prior to the first observed expression of cSEMA5B in both dorsal and ventral grey matter (Vaughn and Grieshaber, 1973; Altman and Bayer, 1984). These incoming sensory afferents then grow rostrocaudally along the dorsalateral regions

of white matter, waiting for about 48 hrs before finally invading the grey matter at its most dorsal aspect (Vaughn and Grieshaber, 1973; Altman and Bayer, 1984). As semaphorins are generally characterized as inhibitory guidance cues, the expression of cSEMA5B in the dorsal grey matter at this stage may contribute to the prevention of these sensory afferents from invading the dorsal grey matter. Other semaphorins, including Sema3A, have also been implicated in the guidance of sensory afferents during these pathfinding processes (Tanelian et al., 1997; Pasterkamp et al., 2000).

While all of the sensory afferents enter the spinal cord at the dorsal horn, different neuronal populations are instructed to terminate at different regions in the spinal cord based on their sensory function (figure 15). These DRG neurons are divided into distinct populations that express different markers and serve different sensory modalities. The neurotrophin(NT)-3-responsive, TrkC positive, large-diameter DRG afferents involved in proprioception, project to the ventral spinal cord to synapse on the dendrites of motor neurons (figure 15 (A, E10); Pasterkamp et al., 2000; Fu et al., 2000). The small-diameter, TrkA positive, nerve growth factor (NGF) responsive DRG neurons that are implicated in thermoreception and nociception, synapse most dorsally in laminae I and II, while those implicated in mechanoreception terminate in laminae III-IV (figure 15 (A, E10); Pasterkamp et al., 2000; Zhang et al., 1994). This dorsal migration of TrkA neurons has been associated with the presence of the repulsive Semaphorin 3A expressed in ventral regions of the spinal cord at this point in development (Fu et al., 2000). Indeed, in vitro studies from chick DRGs have shown that TrkA neurons are inhibited by semaphorin 3A, which even causes collapse of their growth cones in vitro (Luo et al., 1993). In contrast, TrkC neurons do not respond to sema3A, which correlates with their

Figure 15: A Model for cSEMA5B function in guidance

Spinal Cord (A):

A schematic representation of the dynamic cSEMA5B distribution within the developing spinal cord highlights a possible role for this semaphorin in axon guidance from E4.5 to E10 (A). Sensory afferents first arrive at the dorsal grey matter just prior to cSEMA5B expression at E5. As DRG axons are inhibited by cSEMA5B *in vitro* this broad expression in the grey matter may contribute in preventing these axons from invading dorsal regions, leading to axonal growth along the rostral/caudal axis through E6.5. As the spinal cord develops the dorsal expression of cSEMA5B decreases and coincides with the entry of sensory axons into the dorsal funiculus at E6.5/E7. Variable inhibitory phenotypes of DRG axons to cSEMA5B *in vitro*, suggests that these neurons have differential responses to this semaphorin. These studies demonstrate that many axons ceased growth after contact with cells expressing cSEMA5B, while other axon avoided these cells. DRG axons may target to different regions of the spinal cord depending on their response to cSEMA5B (see E10). At later stages of development, the ventral expression is associated with large diameter cells in the lateral motor column at lamina IX. Again, functional *in vitro* studies on sympathetic neurons demonstrated that they are strongly inhibited by cSEMA5B. This inhibitory response to cSEMA5B may contribute to the ventral exit of motor neurons as well as preventing sympathetic axons from invading the ventral spinal cord. Different anatomical structures associated with the spinal cord include: the laminae I-IX, ventral horn (VH), dorsal horn (DH), white matter (WM), dorsal root ganglia (DRG), sympathetic ganglia (SG).

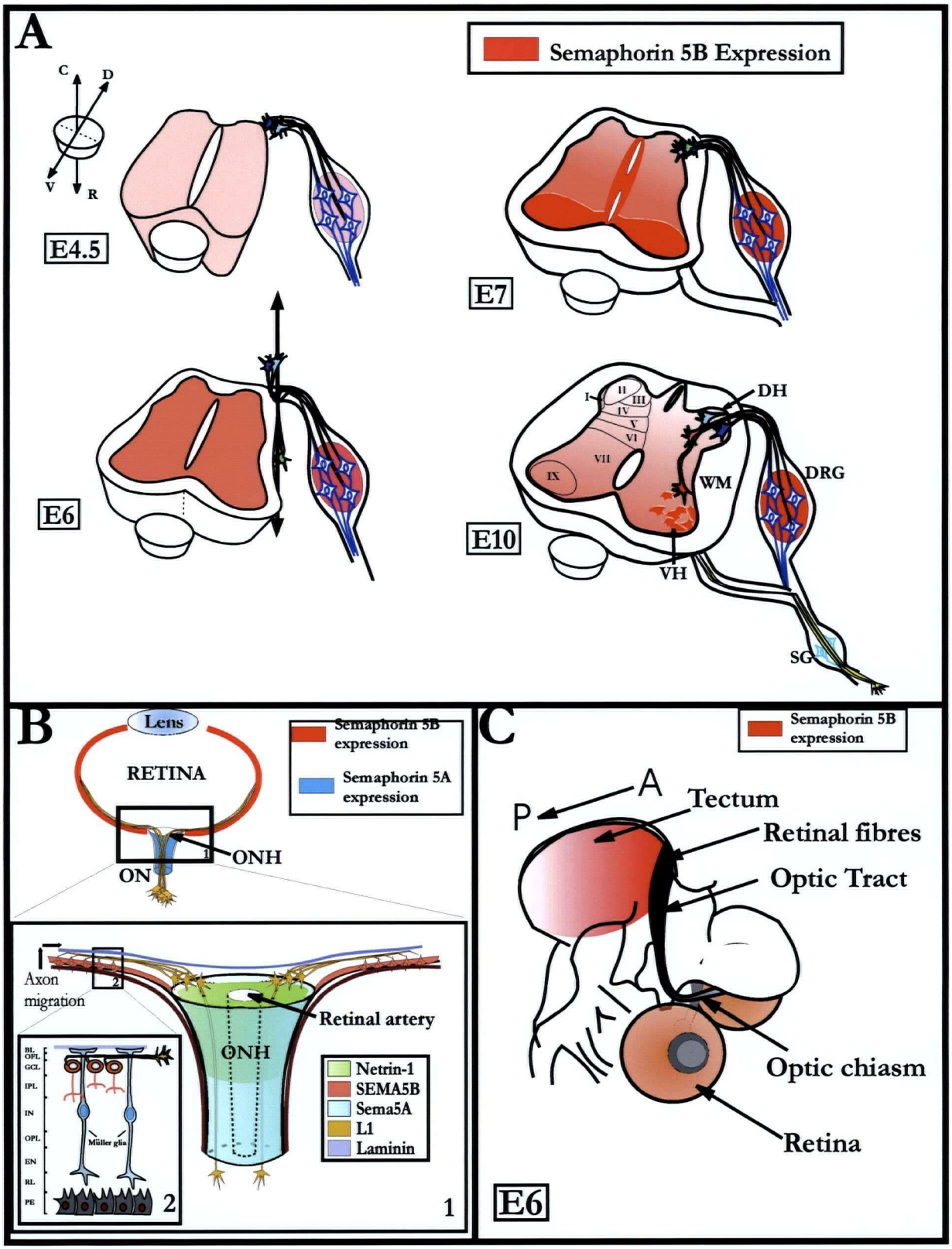
Retina (B):

A schematic representation of cSEMA5B expression in the retina, along with the expression of other known guidance cues at the optic nerve head highlights a role for this semaphorin in axon guidance in the retina (B). The expression of cSEMA5B at E5 corresponds with the migration of retinal ganglion axon from the ganglion cell layer (GCL), to the optic fibre layer (OFL) and towards the optic nerve head (ONH; Inset 2). Retinal axons which express the known plexin interacting protein, L1, are directed away from a layer of cSEMA5B inhibition and grow along a layer of basal lamina containing laminin which promotes neurite outgrowth. Expression of cSEMA5B is absent from the retinal axons and its presence in the GCL, in conjunction with the results from *in vitro* studies, suggests that it may actually help to prevent axons from straying away from the OFL into other retinal layers. Upon reaching the optic nerve head, axons encounter new guidance cues including netrin-1 and possibly cSEMA5A (based on the expression of Sema5A in mouse; Inset 1). The attractive guidance cue netrin-1 is expressed by a ring of epithelial cells surrounding the ONH and helps to attract these incoming retinal axons to this structure. Interestingly in the presence of laminin the normally attractive netrin switches to an inhibitory guidance cue which helps to steer axons into the optic nerve head and away from the retina through the optic nerve. The expression of Sema5A along the ONH and ON is thought to act as an inhibitory sheath which encases the retinal axons along their path towards the chiasm (Oster et al., 2003). This continuous inhibition from SEma5A is supported from studies which demonstrated that the inhibitory response of RGC axons to Sema5A is maintained in the presence of L1 and netrin-1 (Oster et al., 2003). Thus, the possibility of this mutually exclusive expression of class 5 semaphorins

may contribute to the guidance of retinal axons. Different retinal layers are identified in Inset 2: basal lamina (BL), optic fibre layer (OFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (IN), outer plexiform layer (OPL), external nuclear layer (EN), receptor layer (RL), pigment epithelium (PE).

Tectum (C):

A schematic representing the expression of cSEMA5B in the tectum displays the increasing posterior to anterior gradient. This expression of cSEMA5B overlaps with the arrival of the first retinal fibres at E6, in the ventral/anterior region of the tectum. A gradient of expression suggests that different populations of retinal afferents may be directed to a particular region of the tectum depending on their expression of the receptor for cSEMA5B. Those neurons which are inhibited by cSEMA5B the most will travel to anterior regions, while neurons unresponsive to cSEMA5B may continue to the posterior tectum.


A
Semaphorin 5B Expression
C
D
V
R
E4.5
E7
E6
E10
DH
WM
DRG
VH
SG
I
II
III
IV
V
VI
VII
IX
B
Lens
RETINA
ONH
ON
Semaphorin 5B expression
Semaphorin 5A expression
Axon migration
Retinal artery
ONH
Netrin-1
SEMA5B
Sema5A
L1
Laminin
BL
OFL
GCL
IPL
IN
OPL
EN
RL
PE
Müller glia
2
1
C
Semaphorin 5B expression
A
P
Tectum
Retinal fibres
Optic Tract
Optic chiasm
Retina
E6

ability to migrate ventrally in the presence of this inhibitory semaphorin. Studies from mice lacking Sema3A do not show any major guidance errors associated with these neurons, suggesting that other factors contribute to this process, including cSEMA5B (Taniguchi et al., 1997).

Support for the involvement of cSEMA5B in these guidance events is based on the dynamic expression of this semaphorin in the grey matter and from functional *in vitro* studies in the lab. As demonstrated in the *in situ* hybridization and antibodies studies, the dorsal expression of cSEMA5B declines at approximately stage 31 (E6.5-E7). This decrease in dorsal expression corresponds with the first entry of $TrkA^+$ afferent collaterals into the dorsal gray matter (figure 15A; Pasterkamp et al., 2000; Fu et al., 2000). Thus a decrease in this inhibitory semaphorin might help to facilitate the entry of sensory axons into dorsal regions of the grey matter. Contrastingly, there is a continuous ventral expression through E11 (table I). As the spinal cord develops, this ventral expression becomes more discretely localized to a small population of large diameter cells that overlap with the normal distribution of motor neurons. Motor neurons undergo sorting and must pathfind to specific targets either in the periphery. This discrete cSEMA5B expression in motor neurons may function in guiding the motorneurons from the spinal cord at these later stages of spinal cord development (E8 and later; Eide and Glover, 1997).

Functional Studies:

A number of *in vitro* studies in our lab have demonstrated that cSEMA5B inhibits the growth of E7 DRG neurons. What is particularly interesting is that DRG neurons show variable guidance behaviors in response to cSEMA5B. In the presence of small

groups of 293 cells expressing cSEMA5B, some of the DRG neurons avoid these cSEMA5B expressing cells, while some of the DRG neurons do not avoid the cells but rather cease their growth after contacting them. The existence of these multiple phenotypes suggests that perhaps different DRG neurons respond differently to cSEMA5B. Further tests looking at the different neuronal populations *in vitro* would be able to determine this. While the response of DRG neurons to cSEMA5B is somewhat variable, the same is not true of sympathetic neurons. Based on the expression studies, these neurons do not express cSEMA5B (figure 12 D). From the functional *in vitro* studies, it is obvious that these neurons are strongly inhibited by cSEMA5B, completely avoiding the cSEMA5B expressing cells.

The contrast in these neuronal responses to cSEMA5B may play an importance in the chick. Incoming DRG neurons, even though they do not invade the dorsal grey matter at E5, are not completely repelled from it but rather grow rostrally/caudally until entering 48hrs later. This creates a scenario where incoming DRG axons are not repelled away from the dorsal gray matter, but rather they may cease their growth until they receive further instruction. As the levels of cSEMA5B decrease, then perhaps these axons can continue to migrate into the gray matter and synapse in their target regions. Discrete ventral expression in different cells may prevent certain DRG axons from forming a synapse with these cells. In addition, the expression of cSEMA5B in ventral regions may result in the pathfinding of motor axons away from the spinal cord to the periphery, and its presence in the spinal cord may prevent the sympathetic neurons from incorrectly invading these regions. A model for a possible role of cSEMA5B in the spinal cord is presented (figure 15A).

Role of SEMA5B in retina:

While the expression of cSEMA5A has not been described in chick, the results from expression studies in mice suggest a situation where mutually exclusive expression of these two class 5 semaphorins may correlate with sorting and pathfinding of retinal ganglion cells (RGC) at this stage. In mice, retinal distribution of Sema5B has not been described, however studies on the retinal expression of Sema5A have demonstrated that it is specifically localized to the optic nerve head (ONH) and along the optic nerve (ON; Oster et al., 2003). Studies on cSEMA5B in chick have demonstrated that it is broadly expressed along the GCL in retina but is absent from the optic fibre layer, ONH, and ON (figure 7A). Taking the results from both organisms suggests that their expression is mutually exclusive. These results are supported from studies in mice and rat describing the expression of these two class members are sometimes mutually exclusive.

The expression of cSEMA5B occurs at a time when axons are in the midst of a number of important axon guidance decisions. The first fibers leave the eyeball and grow along the optic stalk at about stage 21 (E 3.5; Thanos and Mey, 2001). Axons first arrive at the chiasm from stage 22-24 (E3.5-E4), then advance through the contralateral optic tract towards the tectum, where they first arrive at the anterior pole at E6 (Halfter, 1987). Within the retina differentiated ganglion cells extend axons exclusively within the innermost retina layer, the optic fiber layer, thereby avoiding misrouting into outer retina layers (Stier and Schlosshauer, 1999). These retinal axons likely receive instruction from the radial glial cells of Müller until the growthcones approach the glial endfeet present in the nerve fibre layer at the vitreo-retinal border (Stier and Schlosshauer, 1999). These axons are then directed to grow towards the optic fissure, likely receiving their instruction

from both the glial endfeet and the basal lamina present (Halfter and Schurer, 1998). Interactions between radial glia and the retinal axon growth cones are assumed to occur within the optic stalk, along the optic tract, and on the optic tectum (Silver, 1984; Thanos and Mey, 2001). During this period of growth within the retina, this instruction is the result of the interaction between the growth cones and a number of molecules which are required for proper guidance (Thanos and Mey, 2001). A role for semaphorins in the guidance of retinal ganglion cells was identified in *Xenopus*. Retinal ganglion cells expressing the neuropilin-1 receptor are responsive to Sema3A, and are guided to their correct projection by the presence of Sema3A in the optic chiasm (Campbell et al., 2001). In the chick, the role of semaphorins in guiding retinotectal axons remains elusive. Based on the expression of SEMA5B, along with observation from Sema5A expression in mice, it seems a likely possibility that the combination of these two semaphorins might work in concert to guide neurons from the retina and through the optic disk towards the chiasm (figure 15B).

Functional Studies:

As is the case with neurons associated with the spinal cord, a number of *in vitro* studies in the lab have examined the response of retinal ganglion neurons to cSEMA5B. In these studies it has been demonstrated that retinal ganglion cells avoid cells expressing cSEMA5B as compared to controls. In addition, Oster et al. (2003) demonstrated that these same neurons are inhibited by Sema5A, helping to support a role for this class of semaphorins as an inhibitory guidance cue for retinal afferents. A possible model for the role of cSEMA5B in the retina is presented (figure 15B).

Role of cSEMA5B in tectum:

Another known model for studying axon guidance is the development of retinotectal projections. The primary retinotectal projection of chicken maintains a specific, topographical order at structural levels (Thanos and Mey 2001). Within the tectum, a decreasing anterior to posterior gradient of cSEMA5B expression suggests that it may be involved in proper topographical projection of incoming retinal afferents. The existence of a gradient cues within the tectum was first proposed in Sperry's chemoaffinity hypothesis, based on his experiments with the retinotectal projection in frog (Sperry, 1963). Support for tectal gradients of guidance cues came from *in vitro* studies using a co-culture system of retinal explants and tectal membranes of anterior or posterior tectal origin (Halfter et al., 1981; Bonhoeffer and Huf, 1982; Bonhoeffer and Huf, 1985). These experiments demonstrated that temporal axons of the retina prefer to grow on anterior tectal membranes while those of the nasal retina did not. From a variety of studies it is clear that axons from retinal ganglion cells in the temporal regions of the retina will target to the anterior regions within the tectum, while axons from RGC in the nasal retina will target to the posterior tectum. Subsequent studies have gone on to identify a number of different molecules that are expressed in gradient in the tectum (reviewed in Thanos and Mey, 2001). Presently the most intensely studied family of guidance cues in the retinotectal projection is that of the ephrins, ligands for the Eph-related tyrosine kinase receptors (Knoll and Drescher, 2002). Initial experiments characterizing the ephrins demonstrated that they were expressed in a gradient fashion in the tectum, with high levels located in the posterior tectum (Cheng et al., 1995; Drescher

et al., 1995). High levels of Ephrin-A2 and Ephrin-A5 in the posterior tectum act as ligands for Eph-A3 expressed in the retina (Cheng et al., 1995; Drescher et al., 1995). The receptor Eph-A3 is also distributed in a gradient that increases from nasal to temporal in retinal axons (Drescher et al., 1995). The ephrins are inhibitory and thus, axons from temporal regions of the retina associated with high levels of Eph-3A are more inhibited by the ephrin ligands in the tectum, and are restricted to anterior regions of the tectum associated with lower ligand levels (Sefton and Nieto, 1997; Pittman and Chien, 2002).

In the case of cSEMA5B, the expression gradient occurs in the opposite direction of the ephrins, with highest levels in anterior regions at E6 (figure 8A). This expression of cSEMA5B correlates with the arrival of the first retinal afferents at the anterior/ ventral regions of the optic tectum at E6 (figure 15C). Retinal axons advance over the tectal surface and then descend into the stratum griseum et fibrosum superficiale (SGFS) to connect with tectal targets, from E7 to E12-13 (Thanos and Mey 2001). Expression of cSEMA5B within the tectum during these processes suggests that it may play a role in axon guidance. This is again supported from the *in vitro* studies in the lab which demonstrated that retinal ganglion afferents avoid cells expressing cSEMA5B. As the ligand for cSEMA5B is unknown, it is difficult to predict whether a subset of retinal neurons would likely respond to this semaphorin upon reaching the tectum. Also, it has not been demonstrated whether different retinal ganglion cell types have variable responses to cSEMA5B *in vitro*. Interestingly, many axons within the retina have presumably been pre-exposed to cSEMA5B before reaching the tectum, based on the retinal expression. This leads to the question as to whether this will affect their response

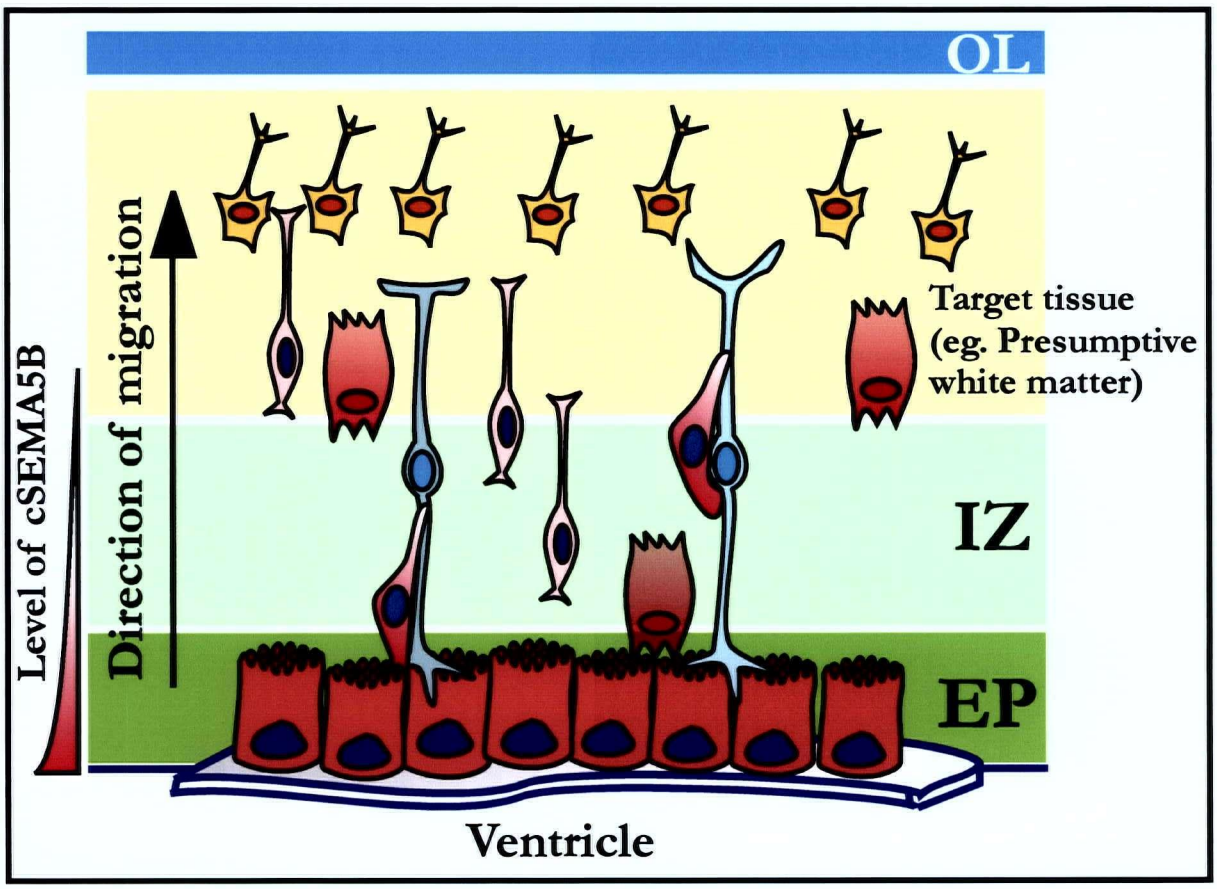
to cSEMA5B within the tectum? A model summarizing a possible role for cSEMA5B in the tectum is presented (figure15C).

Role of SEMA5B in the neuroepithelium:

In addition to a role in pathfinding, the expression of cSEMA5B in the neuroepithelium overlaps with normal areas of cell proliferation and sorting. Within most of these structures, new cells migrate from inner layers to outer layers. In all of these areas the expression of cSEMA5B is greatest along the inner layer, decreasing in the outer layers. This expression, in conjunction with the *in vitro* studies and known evidence on semaphorins, suggests that cSEMA5B may play a role in the migration of these cells from the proliferative inner layer. Recent evidence on the class 3 semaphorins have implicated them in the migration of glial cells associated with the optic nerve (Sugimoto et al., 2001; Tamamaki et al., 2003). These studies demonstrate that the migration of glial precursors along the rat optic nerve is guided by gradients of diffusible cues produced in the optic chiasm, including Sema3A (Sugimoto et al., 2001). Within this population of glial precursors, it was demonstrated that different cell types would selectively respond to different cues, and thus some were responsive to Sema3A while others were not (Sugimoto et al., 2001). Thus, the possibility that cSEMA5B is able to contribute to the migration of neuronal and glial precursors within these proliferative epithelial layers, is likely. In order to examine this aspect of cSEMA5B function, further studies *in vitro* using assays to assess cell migration might help to support this hypothesis. A model for the role of cSEMA5B in cell migration within the various epithelia is presented (figure 16).

Figure 16: A Model for cSEMA5B function in migration

A schematic representation of cSEMA5B function during cell migration from the various neuroepithelia. Cell migration plays a major role for proper CNS development in vertebrates. Many neuronal progenitors, post-mitotic neurons, and glial progenitors born in proliferative zones within the nervous system, must migrate away from these regions towards their final destination in the cortex and other areas of the CNS. A number of key players involved in this migration process have been identified. The broad expression of cSEMA5B in a number of proliferative zones suggests that it contributes to this process of cell migration in these tissues. As it has been identified as an inhibitory guidance cue, cSEMA5B expression along the inner most ependymal layer (EP) may help to drive migration away from ventricular regions towards the target tissue. The levels of cSEMA5B decrease in more superficial layers including the intermediate zones (IZ), target regions, and outer layers (OL).


OL
Target tissue
(eg. Presumptive
white matter)
IZ
EP
Level of cSEMA5B
Direction of migration
Ventricle

Future Directions:

The experiments presented in this thesis have described the expression of cSEMA5B from E5 to E11. Though they have provided us with key insight into the role and expression of this semaphorin, there are a number of experiments which would help in better characterizing its expression. While sections from different levels of tectum have demonstrated that it exists in an increasing posterior to anterior gradient, a clear gradient in whole tectum has not been established. Thus, performing whole-mount *in situ* hybridizations would help to define the expression of cSEMA5B in an intact chick. In addition, further characterization of the different cell types expressing cSEMA5B would help to better define its role. This can be completed using a variety of markers to known populations of cells including a variety of glial and neuronal markers. Finally, a better understanding or the role of class 5 semaphorins in chick, would come from similar studies on chick Semaphorin5A.

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