REGULATION OF NEURAL CONNECTIVITY BY
THE EphA4 RECEPTOR TYROSINE KINASE

by

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The University of Melbourne
For

Harry Davis Penrice

1920-1997
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Thank You!

Jason.
DECLARATION

This is to certify that:

(i)  *This thesis comprises only my original work except where indicated in the preface.*

(ii) *Due acknowledgement has been made in the text to all other material used.*

(iii) *This thesis is less than 100, 000 words in length, exclusive of tables, figures and bibliography.*

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The University of Melbourne
Regulations that govern the Doctor of Philosophy degree at the University of Melbourne require the candidate to provide an estimation of the contribution that he/she has made to the thesis presented for the fulfilment of the aforementioned degree. I therefore assess my contribution to this thesis as follows:

Chapter 1: 100%

Chapter 2: 100%

Chapter 3: 90% in collaboration with Ursula Greferath, Jonathan Messenger, Mirella Dottori, Lynne Hartley, Mark Murphy and Andrew Boyd

Chapter 4: 100%

Chapter 5: 90% in collaboration with Robert Moyer and Simon Koblar

Chapter 6: 100%

Therefore my overall contribution to the work presented in this thesis is greater than 95%. Some of the work presented in thesis has been published or submitted for publication in scientific journals and conference proceedings as detailed below:

Scientific journal articles:


Conference proceedings:


# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>Avidin and biotinylated horseradish peroxidase complex</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and a metalloprotease</td>
</tr>
<tr>
<td>AES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>AF6</td>
<td>ALL1 fused gene from chromosome 6</td>
</tr>
<tr>
<td>AOB</td>
<td>Accessory olfactory bulb</td>
</tr>
<tr>
<td>ApoER2</td>
<td>Apolipoprotein E receptor 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BDA</td>
<td>Biotinylated dextran amine</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CV</td>
<td>Cerebral cortex layer 5</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus callosum</td>
</tr>
<tr>
<td>CG</td>
<td>Cingulum</td>
</tr>
<tr>
<td>CNR</td>
<td>Cadherin-related neuronal receptor</td>
</tr>
<tr>
<td>COS</td>
<td>CV-1 origin SV40</td>
</tr>
<tr>
<td>CP</td>
<td>Cerebral peduncle</td>
</tr>
<tr>
<td>CPG</td>
<td>Central pattern generator</td>
</tr>
<tr>
<td>CRMP</td>
<td>Collapsin response mediator protein</td>
</tr>
<tr>
<td>CST</td>
<td>Corticospinal tract</td>
</tr>
<tr>
<td>CTP</td>
<td>Cortical plate</td>
</tr>
<tr>
<td>CX</td>
<td>Cerebral cortex</td>
</tr>
<tr>
<td>dATP</td>
<td>2’-deoxyadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>2’-deoxynucleoside 5’-triphosphate</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>Dab1</td>
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<tr>
<td>DAPI</td>
<td>4’-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dorsal column</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colorectal cancer</td>
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<td>DF</td>
<td>Dorsal funiculus</td>
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<tr>
<td>DiI</td>
<td>1,1’-dioctadecyl-3, 3’, 3’-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DME</td>
<td>Dulbecco’s modified Eagles’s medium</td>
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</table>
DNA  Deoxyribonucleic acid
DNase  Deoxyribonuclease
DTT  Dithiothreitol
DPN  Deep peroneal nerve
E  Embryonic day
EC  External capsule
ECM  Extracellular matrix
EDL  Extensor digitorum longus
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
Eph  Erythropoietin-producing-hepatoma cell line
EphA4^{+/−}  Wild type mice
EphA4^{−/−}  EphA4 deficient mice
Ephrin  Eph family receptor interacting protein
ETS  E26 transformation specific
FAK  Focal adhesion kinase
FCS  Foetal calf serum
FDL  Flexor digitorum longus
FGF  Fibroblast growth factor
FHL  Flexor hallucis longus
GPI  Glycosylphosphatidylinositol
GFAP  Glial fibrillary acidic protein
H&E  Haematoxylin and eosin
HB9  Homeobox 9
HBSS  Hank’s balanced salt solution
HEM  HEPES-buffered Eagle’s medium
HEPES  N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]
HGF/SF  Hepatocyte growth factor/Scatter factor
Hox  Homeobox
IB  Immunoblot
IC  Internal capsule
ICP  Inferior cerebellar peduncle
Ig  Immunoglobulin
InC  Inferior colliculus
IP  Immunoprecipitation
<table>
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<tr>
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<td>IZ</td>
<td>Intermediate zone</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>Kuz</td>
<td>Kuzbanian</td>
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<tr>
<td>LG</td>
<td>Lateral gastrocnemius</td>
</tr>
<tr>
<td>LIM</td>
<td>Lin-11, Isl-1, Mec-3</td>
</tr>
<tr>
<td>LMC</td>
<td>Lateral motor column</td>
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<td>LMC(_M)</td>
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<td>mRNA</td>
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<td>MASA</td>
<td>Mental retardation, aphasia, shuffling gait and adducted thumbs</td>
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<tr>
<td>MCP</td>
<td>Middle cerebellar peduncle</td>
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<td>Mena</td>
<td>Mammalian enabled</td>
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<td>MG</td>
<td>Medial gastrocnemius</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>Medial lemniscus</td>
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<td>MLF</td>
<td>Medial longitudinal fasciculus</td>
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<td>MMC</td>
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<td>MOPS</td>
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<td>Marginal zone</td>
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<td>Number of animals examined</td>
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<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>P</td>
<td>Postnatal day or Statistical probability</td>
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<td>pAC</td>
<td>Posterior tract of the anterior commissure</td>
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<td>PB</td>
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<td>PBS</td>
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<td>Posterior commissure</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PD</td>
<td>Pyramidal decussation</td>
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<td>PDZ</td>
<td>Postsynaptic density protein, discs large, zona occludens</td>
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<td>PL</td>
<td>Peroneous longus</td>
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<td>PNA</td>
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<td>PRN</td>
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<td>Retinoic acid</td>
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<td>RasGAP</td>
<td>Ras GTPase-activating protein</td>
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<td>RGS</td>
<td>Regulator of heterotrimeric G protein signalling</td>
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</tr>
<tr>
<td>ScN</td>
<td>Sciatic nerve</td>
</tr>
<tr>
<td>SCPD</td>
<td>Superior cerebellar peduncle decussation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology-2</td>
</tr>
<tr>
<td>SHEP1</td>
<td>SH2-domain-containing Eph-receptor-binding protein 1</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SHP2</td>
<td>SH2-domain containing protein tyrosine phosphatase</td>
</tr>
<tr>
<td>SOL</td>
<td>Soleus</td>
</tr>
<tr>
<td>SPN</td>
<td>Superficial peroneal nerve</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate</td>
</tr>
<tr>
<td>ST</td>
<td>Striatum</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>TBS-Tween</td>
<td>Tris buffered saline-Tween20</td>
</tr>
<tr>
<td>TFP</td>
<td>Transverse fibres of the pons</td>
</tr>
<tr>
<td>TMRD</td>
<td>Tetramethylrhodamine dextran</td>
</tr>
<tr>
<td>TOAD</td>
<td>Turned on after division</td>
</tr>
<tr>
<td>TN</td>
<td>Tibial nerve</td>
</tr>
<tr>
<td>TUC</td>
<td>TOAD/Ulip/CRMP</td>
</tr>
<tr>
<td>TWH</td>
<td>Thymocyte winged helix</td>
</tr>
<tr>
<td>Ulip</td>
<td>Unc-33-like phosphoprotein</td>
</tr>
<tr>
<td>V/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>Vax1</td>
<td>Ventral anterior homeobox 1</td>
</tr>
<tr>
<td>V3</td>
<td>3rd ventricle</td>
</tr>
<tr>
<td>VH</td>
<td>Ventral horn</td>
</tr>
<tr>
<td>VSCT</td>
<td>Ventral spinocerebellar tract</td>
</tr>
<tr>
<td>VLDLR</td>
<td>Very low-density lipoprotein receptor</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome proteins</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
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ABSTRACT

Interactions between the Eph family of receptor tyrosine kinases, and their ligands, the ephrins, are required for the normal development and maintenance of many patterns of connectivity within the nervous system. Eph receptors and ephrins are expressed widely throughout both the developing and mature nervous system where they function as important regulators of cell migration and axon guidance. The studies presented in this thesis examine the role of one particular member of the Eph receptor family, EphA4, in regulating mechanisms that underlie the development and maintenance of certain neural connections within the nervous system.

This thesis demonstrates that the EphA4 receptor is expressed within specific regions of the developing and mature nervous system, some of which are associated with the control of locomotor activity. Consistent with these observations are the locomotor defects exhibited by animals with a targeted disruption of the EphA4 gene. These animals exhibit abnormal bilateral limb movements and have severe disruptions of a number of major axonal pathways. One of these disrupted axonal pathways, the corticospinal tract (CST), is a particularly important mediator of locomotor activity. This thesis reveals that EphA4 is expressed on the axons that comprise the CST. It demonstrates that although EphA4 is not required for the initial development of the CST, repulsive interactions between EphA4-bearing CST axons and ephrinB3, a ligand for EphA4 that is expressed at the midline of the spinal cord, appear to prevent CST axons from aberrantly recrossing the spinal midline during development. Furthermore, this thesis reveals that EphA4 is also expressed within the intermediate and ventral zones of the spinal grey matter where it may serve to confine CST terminations to the dorsal horn of the spinal grey matter during the latter stages of CST maturation in the spinal cord.

In addition to defining the EphA4 mediated mechanisms that underlie certain aspects of CST development, this thesis also shows that EphA4 is important for mechanisms that underlie the correct development of the topographic arrangement of particular motoneuron pools in the spinal cord. It reveals that the correct positioning of the \textit{tibialis}
anterior motor pool is dependent upon mechanisms mediated by the EphA4 receptor, as animals deficient in EphA4 gene expression exhibit a major displacement of the tibialis anterior motor pool in the lumbar enlargement of the spinal cord. Importantly, this thesis also reveals that the ability of motor axons to project correctly to their destination is, under certain circumstances, independent of motoneuron pool position as the aberrantly displaced motoneurons that comprise the tibialis anterior motoneuron pool in EphA4 deficient animals still project axons out of the spinal cord at the appropriate levels and innervate the tibialis anterior muscle of the lower hindlimb correctly.

Lastly, this thesis reports the results of a preliminary examination into the role of the EphA4 receptor in mechanisms that underlie the directed migration of neuronal progenitors from the subventricular zone (SVZ) of the lateral ventricle to the olfactory bulbs within the mature central nervous system. This thesis demonstrates that cells derived from the SVZ of the lateral ventricle of the mature brain express EphA4. However, it also shows that the directed migration of neuronal progenitors from the lateral ventricle SVZ to the olfactory bulbs of the mature brain is not dependent upon EphA4, as the migratory route taken by these neuronal progenitors appears normal within the brains of mature EphA4 deficient animals. Thus, it is suggested in this thesis that other guidance receptor/ligand interactions, aside from those mediated by EphA4, are more important for directing the guidance of neuronal progenitor populations migrating from the lateral ventricle SVZ to the olfactory bulbs in the mature central nervous system.
CHAPTER 1: MECHANISMS THAT UNDERLIE THE DEVELOPMENT AND MAINTENANCE OF NEURAL CONNECTIVITY
Chapter 1: The development and maintenance of neural connectivity

1.1 INTRODUCTION

From simple reflex actions to complex thoughts, emotions and memories, neural circuits control all functions of the mature nervous system. Neural circuits function correctly because the pattern of connections between their component neurons is formed appropriately. A fundamental aim of neurobiological investigation is to determine how these precise patterns of neural connectivity are established during development and are maintained throughout maturation. The complexity of neural circuits within the nervous system of advanced vertebrates is astounding to say the least. In the human brain for example, there are approximately $10^{12}$ neural cells forming over $10^{15}$ correct synaptic connections with other neural cells. In order to understand how these precise patterns of neural connectivity are established we need to know what mechanisms control the migration of neural cells to their correct position within the nervous system and what signals direct the guidance of their processes to their eventual targets. In this chapter, the mechanisms underlying the development and maintenance of patterns of neural connectivity will be introduced. Initially, the molecular interactions required for the correct development of neural circuits will be discussed with specific emphasis given to the functions of the Eph family of receptor tyrosine kinases, and their ligands, the ephrins, in regulating morphogenic events in the nervous system. The roles of these molecular interactions in mediating the development of certain neural circuits involved in regulating motor activity will then be considered. Finally, this chapter will examine some of the molecular interactions that underlie the maintenance of connections within the mature nervous system.

1.2 REGULATION OF NEURAL MIGRATION AND AXON GUIDANCE IN THE DEVELOPING NERVOUS SYSTEM

Migrating neurons and extending axons are initially guided to their destinations by activity-independent mechanisms, consisting of a series of extracellular guidance cues, before activity-dependent mechanisms (reviewed in Goodman and Shatz, 1993)
promote the refinement of neural circuits into their final patterns of connectivity. Many families of extracellular ligands have been identified to function as molecular guidance cues by providing the substrate bound, short and long-range diffusible signals that are required to guide migrating neurons and axons to their correct destination. Guidance cues bind to and activate their receptors expressed on the surface of migrating neurons and their axons. Activation of these receptors transduce signals to the intracellular environment that result in cytoskeletal rearrangement and directed neuronal/axonal movement. Signals provided by guidance cues are interpreted by the intracellular machinery of migrating neurons and axons as being either attractive/permissive or repulsive/inhibitory influences and, given that there are often multiple influences acting in concert at any one time to guide migrating neurons and axons, it is the net effect of these influences that determines the direction in which a migrating neuron or extending axon moves.

Many of the molecular cues that guide the migration of neurons and the extension of their axons have been the focus of several recent detailed reviews (Tessier-Lavigne and Goodman, 1996; Tear, 1998; Hatten, 1999; Mueller, 1999; Krull and Koblar, 2000; Kaprielian et al., 2001; Song and Poo, 2001). These molecules include, but are not limited to, members belonging to the extracellular matrix molecule, netrin, slit, hepatocyte growth factor/scatter factor, reelin, cell adhesion molecule, semaphorin and ephrin families. Through interacting with their receptors, these families of guidance cues regulate many of the mechanisms that underlie the development and maintenance of connectivity in the nervous system.

1.2.1 The Eph receptors, and their ligands, the ephrins, are major regulators of neural cell migration and axon guidance in the nervous system

The largest known family of receptor tyrosine kinase proteins (reviewed in van der Geer et al., 1994; Hubbard, 1999; Hubbard and Till, 2000; Schlessinger, 2000) is that of the Eph receptors. Interactions between Eph receptors, and their membrane-bound ligands, the ephrins, mediate both repulsive and adhesive guidance events and are important for
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the correct guidance of migrating cells and axon projections to their final destinations in multiple regions of the nervous system (reviewed in Flanagan and Vanderhaeghen, 1998; Wilkinson, 2000a; Klein, 2001; Wilkinson, 2001).

1.2.1.1 Identification and structure of Eph receptors and ephrins

The first Eph receptor identified, EphA1, was cloned from a low-stringency cross-hybridization screen of a cDNA expression library for tyrosine kinases and was named for its high level of expression in an erythropoietin-producing-hepatoma cell line (Hirai et al., 1987). Subsequent Eph receptors were identified by screening cDNA expression libraries with antiphosphotyrosine antibodies, oligonucleotides, heterologous probes and also by polymerase chain reaction (PCR) amplification of conserved tyrosine kinase domains (see Wilkinson, 2000a for a complete list of references for the isolation and cloning of all known Eph receptors and ephrin ligands). Currently 14 Eph receptors have been identified in the vertebrate genome and they are divided into two subclasses, EphA (EphA1-EphA8) and EphB (EphB1-EphB6), based upon sequence homology and ligand binding specificity. Sequence analysis reveals that all members of the Eph family share several conserved domains among themselves and with other receptor tyrosine kinase proteins (Figure 1.1). They are characterized by a distinctive extracellular region consisting of 2 fibronectin type III domains (Pasquale, 1991), a cysteine rich motif (Labrador et al., 1997; Lackmann et al., 1998) and an N-terminal ligand-binding domain that consists of 11 antiparallel β-sheets with an extended loop that is most likely the site of ligand interaction (Himanen et al., 1998). Similar to most other receptor tyrosine kinases, they also have a single transmembrane domain and a highly conserved cytoplasmic tyrosine kinase domain. However, unlike most other receptor tyrosine kinases, Eph receptors also have a conserved sterile alpha motif (SAM) domain located at the very end of the cytoplasmic tail that may function as a potential site of receptor hetero- and homo-oligomerization (Stapleton et al., 1999).

The ligands for the Eph receptors, the ephrins (Eph family receptor interacting proteins), were first identified using soluble receptor affinity methods (Bartley et al., 1994). Currently 8 ephrin family members have been identified in the vertebrate genome, and, as with the Eph receptors, they are also divided into two subclasses based
upon sequence homology and binding specificities (Figure 1.1). All ephrins share a relatively short extracellular domain whose sequence is quite variable but consists of 4 conserved cysteine residues that probably correspond to a receptor-binding region (Gale et al., 1996a; Nicola et al., 1996; Kozlosky et al., 1997). Members belonging to the ephrinA subclass (ephrinA1-ephrinA5) are attached to the cell membrane via a C-terminal glycosylphosphatidylinositol (GPI) group and consequently lack a cytoplasmic domain. In contrast, members belonging to the ephrinB subclass (ephrinB1-ephrinB3) have a transmembrane domain and a short cytoplasmic region consisting of a highly conserved 33 amino acid C-terminal sequence that includes 5 tyrosine residues (Beckmann et al., 1994; Davis et al., 1994; Shao et al., 1994; Bennett et al., 1995; Bergemann et al., 1995; Gale et al., 1996a).

Eph receptors and ephrinB ligands also possess a postsynaptic density protein, discs large, zona occludens (PDZ)-binding domain at their C-terminal end that could function as a binding site for PDZ proteins or other proteins that contain PDZ domains (Hock et al., 1998b; Torres et al., 1998; Bruckner et al., 1999; Buchert et al., 1999; Lin et al., 1999). PDZ domains are important sites of attachment between proteins that can facilitate the assembly and localization of protein complexes (reviewed in Garner et al., 2000). They are important for the assembly of Eph or ephrin complexes that allow interactions with other regulatory molecules (Hock et al., 1998b; Bruckner et al., 1999; Cowan et al., 2000) and also for the localization of these Eph or ephrin complexes to specific sites within the cell such as membrane raft microdomains (Bruckner et al., 1999) and synapses (Torres et al., 1998).

1.2.1.2 Eph receptor/ephrin binding interactions and affinities

Receptor-ligand binding interactions between Eph receptors and ephrins occur with varying affinities (Bartley et al., 1994; Beckmann et al., 1994; Cheng and Flanagan, 1994; Davis et al., 1994; Bennett et al., 1995; Bergemann et al., 1995; Brambilla et al., 1995; Cerretti et al., 1995; Kozlosky et al., 1995; Brambilla et al., 1996; Gale et al., 1996a; Gale et al., 1996b; Sakano et al., 1996; Monschau et al., 1997; Park and Sanchez, 1997). Generally, receptors belonging to the EphA subclass bind only ephrinA ligands and receptors belonging to the EphB subclass bind only ephrinB ligands.
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Figure 1.1
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Binding interactions within the two subclasses, however, are promiscuous, with each ephrin being able to bind to multiple receptors and vice versa (Figure 1.2). An exception to the rule that Eph/ephrin binding interactions are restricted to members belonging to the same subclass is the EphA4 receptor, which not only binds ephrinA ligands but also ephrinB2 (Gale et al., 1996b) and ephrinB3 (Gale et al., 1996a) as well (Figure 1.2). It is possible that additional undiscovered ephrins exist since none of the ephrins identified thus far interact with the EphB5 receptor, and the only interaction demonstrated for the EphA1 receptor is its low-affinity binding to ephrinA1 (Gale et al., 1996b).

The promiscuity of binding interactions between Eph receptors and the ephrins may reflect functional redundancy in the in vivo interactions of these molecules, serving to ensure a high degree of fidelity in patterns of neural connectivity whose development is mediated by Eph/ephrin interactions. However, the variable affinities that most Eph receptors have for different ephrins within a particular subclass may also facilitate specific regulation of migrating cells and axons within the nervous system. For example, ephrinA5 has previously been demonstrated to bind to EphA3 with higher affinity than does ephrinA2 (Lackmann et al., 1997; Monschau et al., 1997) and it has been suggested that it is this variable binding affinity that regulates a differential responsiveness of EphA3-bearing axons to these particular ephrins (Monschau et al., 1997). Additional evidence exemplifying the functional role of specific Eph/ephrin interactions arises from the phenotypes that occur in animals that have disrupted Eph/ephrin genes. The almost identical phenotypes observed in EphA4 and ephrinB3 deficient animals for example (Dottori et al., 1998; Coonan et al., 2001; Kullander et al., 2001a; Kullander et al., 2001b; Yokoyama et al., 2001), suggest that EphA4/ephrinB3 interactions have very specific in vivo functions that cannot be compensated for by other members of the Eph/ephrin family.

1.2.1.3 Activation of Eph receptors and ephrins

Similar to most other receptor tyrosine kinase proteins, the binding of ephrin ligands to Eph receptors results in Eph receptor oligomerization and activation (Hubbard and Till, 2000; Schlessinger, 2000). Soluble monomeric ephrins cannot activate Eph receptors
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Figure 1.2
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unless they themselves are artificially clustered (Davis et al., 1994). This finding suggests that ephrins need to be attached to the cell membrane so that they can cluster prior to or during Eph receptor binding. Consequently, interactions between Eph receptors and ephrins mediate cell contact-dependent signalling mechanisms. Significantly, signal transduction during Eph/ephrin interaction is not mediated solely through the Eph receptor alone. Clustering of ephrinA and ephrinB ligands upon binding to Eph receptors can also lead to signal transduction via the ephrin intracellular domain. For members of the ephrinB subclass, activation results in the phosphorylation of conserved tyrosine residues in the intracellular domain by a cytoplasmic tyrosine kinase (Henkemeyer et al., 1996; Holland et al., 1996; Bruckner et al., 1997). Signalling through the intracellular domain of ephrinA subclass members is not yet fully understood but could involve the aggregation of these ephrins with lipid-conjugated signal transduction molecules located in membrane raft microdomains (Davy et al., 1999; Davy and Robbins, 2000). Consequently, interactions between Eph receptors and ephrins have the capacity to mediate bi-directional signalling mechanisms in adjacent populations of migrating cells or axons, thus creating situations where each component of an Eph/ephrin interaction acts as both a ‘receptor’ and a ‘ligand’. Unidirectional activation of ephrinB ligands is also likely since the EphB6 receptor has an inactive kinase domain and thus may act only as a ligand (Gurniak and Berg, 1996). In addition, alternative-splicing events can result in the production of membrane-bound or soluble truncated Eph receptors that may act either as unidirectional agonists or antagonists (Sajjadi et al., 1991; Ciossek et al., 1995; Connor and Pasquale, 1995; Valenzuela et al., 1995; Gurniak and Berg, 1996; Talukder et al., 1997).

1.2.1.4 Eph receptor and ephrin expression

Eph receptors and the ephrins exhibit widespread expression throughout many tissues of the developing vertebrate embryo (reviewed in Wilkinson, 2000a). In particular, many Eph receptors and ephrins exhibit highly significant patterns of expression in the developing and mature nervous system (Lai and Lemke, 1991; Henkemeyer et al., 1994; Carpenter et al., 1995; Fox et al., 1995; Mori et al., 1995; Flenniken et al., 1996; Gale et al., 1996b; Kilpatrick et al., 1996; Martone et al., 1997; Connor et al., 1998; Iwamasa et al., 1999; Rogers et al., 1999a; Rogers et al., 1999b). In many cases, the expression
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of Eph receptors and ephrins is reciprocally compartmentalized (Gale et al., 1996b), suggesting that Eph/ephrin interactions occur primarily at the interfaces of expression domains. There are also regions, such as the somites, branchial arches and the retina, where the expression of interacting Eph receptors and ephrins overlap (Flenniken et al., 1996; Connor et al., 1998). Regions of overlapping expression appear to be functionally important as this can facilitate persistent Eph receptor activation (Connor et al., 1998) which, in the case of retinal ganglion cell (RGC) axons, may be important for the correct development of topographic patterns in retinocollicular projections (Hornberger et al., 1999). Taken together, the distinct and sometimes overlapping expression patterns of Eph receptors and ephrin ligands in the developing and mature vertebrate nervous system suggest that Eph/ephrin interactions are required for many aspects of neural development.

1.2.1.5 Roles of Eph/ephrin interactions in the stabilization of tissue patterns and the guidance of migrating cells

Interactions between Eph receptors and ephrins are important regulators of tissue patterning in the vertebrate hindbrain. The vertebrate hindbrain consists of a number of repeated morphological segments, called rhombomeres (reviewed in Lumsden, 1990). The establishment of precise patterns of gene expression and cellular organization within the hindbrain involves the restriction of cell intermingling at the interfaces of adjacent rhombomeres thus allowing each rhombomere to maintain a distinct and homogenous identity (Fraser et al., 1990). One mechanism that restricts cell intermingling between hindbrain segments involves the complementary expression of Eph receptors and ephrins in adjacent rhombomeres (Xu et al., 1995; Xu et al., 1999). Following mosaic activation of Eph receptors or ephrinB ligands, signals transduced through both interacting proteins induced cell sorting in hindbrain segments (Xu et al., 1999), presumably through mediating cellular de-adhesion and repulsive migration. Furthermore, the restriction of cellular intermingling at rhombomere boundaries is dependent upon bi-directional activation of Eph receptors and ephrinB ligands as unidirectional activation of either component fails to restrict cell intermingling in the hindbrain (Mellitzer et al., 1999).
In addition to stabilizing tissue patterns in the hindbrain, interactions between Eph receptors and ephrins also appear to be important for the segmentation of somites. Somites are paired blocks of paraxial mesoderm located along the vertebrate body axis that differentiate into dermis, bone and muscle (reviewed in Hirsinger et al., 2000). EphA4 and ligands for EphA4, belonging to both the ephrinA and B subclasses, are expressed in a characteristic segmented pattern in the anterior and posterior presomitic mesoderm respectively (Nieto et al., 1992, Bergemann et al., 1995; Krull et al., 1997; Wang and Anderson, 1997; Durbin et al., 1998). The spatially restricted expression pattern of these molecules suggests that Eph/ephrin interactions may be important for pattern formation during somatogenesis. Indeed, disruption of Eph/ephrin signalling results in abnormal somite pattern formation (Durbin et al., 1998) and it is possible that, similar to their role in the hindbrain, Eph/ephrin interactions are also important regulators of somitic boundary formation.

Eph receptor and ephrin interactions are also important regulators of neural crest cell guidance. Neural crest cells form at the dorsolateral edge of the neuroepithelium and migrate to specific destinations to form most neurons and glia of the peripheral nervous system as well as most of the cartilaginous and skeletal tissue in the head (reviewed in Christiansen et al., 2000). Repulsive signals mediated by Eph/ephrin interactions appear to restrict migrating neural crest cells to specific routes during development (Robinson et al., 1997). In the cranial region, neural crest cells migrate in three streams from the midbrain and hindbrain to the first, second, third and posterior branchial arches where they form elements of the cranial ganglia and differentiate into specific patterns of bone and cartilage (Kontges and Lumsden, 1996). Interactions between Eph receptors and ephrins contribute to the restriction of cell intermingling between these streams. Recent studies have shown that EphA4 and EphB1 receptors are expressed only in the third-arch neural crest cells while ephrinB2 expression is confined to second-arch neural crest cells (Smith et al., 1997). These reciprocal expression patterns result in the activation of Eph/ephrin signalling cascades that, presumably through repulsive mechanisms, prevent third-arch neural crest cells from migrating into the second-arch (Smith et al., 1997).

Eph/ephrin interactions also have additional roles in the guidance of migrating neural crest cells in other areas. In the trunk of developing embryos, the repeated segmental pattern of dorsal root and sympathetic ganglia is dependent upon the migration of neural
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crest cells through the anterior sclerotome of each somite (Rickmann et al., 1985). The Eph ligands, ephrinB1 in the chick, or ephrinB2 in the mouse, are expressed in the posterior half of somitic sclerotome and serve as repulsive cues for migrating neural crest cells that express EphB2 and other EphB receptors (Krull et al., 1997; Wang and Anderson, 1997). However, given the apparent normal migration of neural crest cells in animals with a targeted disruption in the gene that encodes for ephrinB2 (Wang et al., 1998), it is highly likely that other repulsive interactions, such as those mediated by peanut lectin-binding glycoprotein and collagen type IX, can also mediate neural crest cell guidance through the anterior regions of the somitic sclerotome (Krull et al., 1995; Ring et al., 1996).

1.2.1.6 Guidance of developing axonal tracts by Eph/ephrin interactions

Developing axonal tracts are directed to their appropriate destinations by an array of molecular guidance cues. The Eph receptors and ephrins are a major family of axonal guidance cues that mediate the contact-dependent repulsion/inhibition of developing axons. Although in vitro studies have demonstrated that Eph/ephrin interactions can cause axonal growth cone collapse and neurite growth inhibition (Drescher et al., 1995; Winslow et al., 1995; Meima et al., 1997a; Meima et al., 1997b; Ohta et al., 1997; Wahl et al., 2000) it is the generation of mutant animals with targeted gene disruptions that has demonstrated an in vivo role for these molecules in the guidance of developing axonal tracts along specific routes by preventing their growth into inappropriate areas.

Targeted disruption of EphB2 resulted in the loss of the posterior tract of the anterior commissure (pAC), a major commissural connection in the cerebral cortex (Henkemeyer et al., 1996). In EphB2 deficient animals, rather than crossing the midline of the brain adjacent to the thalamus as expected, the axons of the pAC fail to cross the midline, and aberrantly terminate in the ventral thalamus. In contrast, disruption of the EphB2 kinase domain, while leaving the extracellular domain intact, does not perturb pAC development. Furthermore, analysis of EphB2 expression in the area surrounding the pAC revealed that EphB2 is not expressed on developing pAC axons, but rather in the adjacent ventral territory where they normally do not enter. Consequently, the correct development of the pAC requires the extracellular domain of EphB2 to be
present in the surrounding territory, but signalling through EphB2 is not required. Given that a ligand for EphB2, ephrinB1, is present on pAC axons, it is likely that ephrinB1 transduces repulsive signals to pAC axons following activation by EphB2 expressed in the adjacent territory. The anterior commissure is also perturbed in EphA4 deficient animals (Dottori et al., 1998; Kullander et al., 2001b) and it is also likely that EphA4, similar to EphB2, directs guidance of axons in the anterior commissure by non-cell-autonomous, kinase-independent mechanisms (Kullander et al., 2001b).

Another commissural tract of the cerebral cortex, the corpus callosum, is disrupted in homozygous animals with a targeted disruption of the EphB3 gene (Orioli et al., 1996). In EphB3 deficient animals, callosal axons reach the midline of the brain normally but fail to cross appropriately, instead forming large Probst’s bundles. Analysis of EphB3 expression reveals that it is present in the tissue adjacent to the developing commissure, in contrast to ephrinB ligands for EphB3, which are expressed by developing callosal axons (Orioli et al., 1996). Consequently, similar to the mechanism proposed to guide the pAC, it is likely that EphB3 expressed in the tissue adjacent to developing callosal axons serves to restrict these axons to a specific path. In addition to the axonal defects described for EphB2 and EphB3 single gene deficient animals, double gene deficient animals that have disruptions to both the EphB2 and EphB3 genes exhibit synergistic axonal aberrances in both the pAC and also the corpus callosum (Orioli et al., 1996). Given the overlapping expression domains of EphB2 and EphB3, the enhanced severity and frequency of aberrant projections in the EphB2/EphB3 deficient animals provides direct evidence for functional redundancy among Eph receptors (Orioli et al., 1996).

The EphB2/EphB3 receptors are also important for the correct development of key components in the vestibular apparatus, a sensory organ that is essential for balance and coordinated movement (Cowan et al., 2000). The vestibular apparatus has both afferent and efferent axon tracts connecting sensory hair cells of the inner ear with structures in the hindbrain. Inner ear efferent axons, that are thought to modulate sensory input from the ear, project from the hindbrain to both the ipsilateral and contralateral ear. In certain EphB2/EphB3 deficient animals, contralaterally projecting inner ear afferent axons exhibit temporary guidance abnormalities during development that are not evident at birth (Cowan et al., 2000), suggesting that although EphB2/EphB3 may be important,
their absence can be compensated for by other Eph receptors to ensure that the fidelity of axonal connections is maintained.

EphB receptors and their ephrinB ligands are also required for the correct development of axonal projections in the retina. During development, RGCs project their axons to the optic disc situated in the centre of the retina where they later form the optic nerve. A number of EphB receptors and ephrinB ligands are expressed either uniformly or in dorsoventral gradients within the developing retina (Birgbauer et al., 2000). Animals that have disruptions to both the EphB2 and EphB3 genes exhibit RGC axonal guidance defects within the retina, with dorsal RGC axons being more severely affected than ventral RGC axons. Additionally, RGC axon guidance defects were rescued in mutants lacking EphB3 and the kinase domain of EphB2 expression, suggesting that EphB receptors may act in a kinase-independent manner to mediate axonal guidance, possibly by activating signalling responses in ephrin expressing RGC axons (Birgbauer et al., 2000). Additionally, in the Xenopus visual system, interactions between EphB receptors and ephrinB ligands are also important for the ipsilateral routing of developing RGC axons in the optic chiasm (Nakagawa et al., 2000). However, given that the development of ipsilateral and contralateral RGC projections is almost synchronous in mammals (unlike in Xenopus where the ipsilateral RGC projection develops after the contralateral RGC projection) the roles of EphB/ephrinB interactions in regulating RGC axon guidance in the mammalian optic chiasm still remain unclear.

Perturbations of specific axonal tracts are also evident in EphA8 deficient animals (Park et al., 1997). Neurons in the midbrain that project caudally from the superior colliculus and across the midline to the inferior colliculus express EphA8. In EphA8 deficient animals, these axons fail to project across the midline, and instead project ipsilaterally through the hindbrain and spinal cord (Park et al., 1997). It is possible that ephrinA5, a ligand for EphA8 that is prominently expressed in the inferior colliculus, may prevent axons from the superior colliculus entering the spinal cord, thus facilitating the diversion of these axons across the midline to the contralateral side.

EphA4 has also been demonstrated to be essential for the correct guidance of certain developing axonal tracts within the spinal cord (Dottori et al., 1998; Coonan et al.,
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2001; Kullander et al., 2001b) and also the peripheral nervous system (Helmbacher et al., 2000). Its role in mediating the guidance of these axonal projections will be examined in greater detail in subsequent sections of this chapter.

1.2.1.7 Roles for Eph/ephrin interactions in the development of topographic maps

Many neural circuits in the nervous system are arranged topographically, that is, the spatial relationships between neuronal cell somas are maintained in their patterns of axonal termination. The Eph receptors and ephrins play important roles in the development of topographic maps through being expressed in gradients that allow graded repulsion of growing axons. Eph/ephrin interactions are important for the correct development of topographic patterns in projections from the retina to the lateral geniculate nucleus (Feldheim et al., 1998), from thalamus to cortex (Gao et al., 1998; Vanderhaeghen et al., 2000), hippocampus to lateral septum (Gao et al., 1996) and also from spinal motoneurons to muscles (Feng et al., 2000). The most well characterized topographic map in the nervous system is that of the retinocollicular system and its correct development is also largely dependent upon Eph/ephrin interactions (reviewed in Wilkinson, 2000b). In the retinocollicular map, RGCs within the retina project their axons to the optic tectum (OT) in fish, amphibians and birds or, in mammals, to the superior colliculus (SC). Axons from the temporal (posterior) retina project to the anterior SC, and axons from the nasal (anterior) retina project to the posterior SC (Figure 1.3A). Similarly, there is an orderly spatial map of RGC axons projecting from the dorsal retina to the ventral SC, and from ventral retina to dorsal SC (Figure 1.3C).

The topographic patterning of RGC axon terminations in the anterior/posterior axis of the SC appears to be partly dependent upon graded sensitivity of RGC axons to gradients of ephrinA expression in the SC. EphrinA2 and ephrinA5 are both expressed in gradients in the SC, ranging from low concentrations in the anterior SC to high concentrations in the posterior SC, with ephrinA5 exhibiting a steeper concentration gradient than ephrinA2 as there is little or no detectable ephrinA5 in the anterior SC (Cheng et al., 1995; Drescher et al., 1995; Monschau et al., 1997). These ephrins have distinct effects on nasal versus temporal retinal axons and act together to establish a gradient of repulsive cues along the SC. EphrinA2 is a potent inhibitor of temporal, but
not nasal RGC axons (Nakamoto et al., 1996). In contrast, when present at relatively high concentrations, ephrinA5 inhibits the growth of both temporal and nasal RGC axons (Drescher et al., 1995), however, at lower concentrations, RGC axons exhibit a graded nasal to temporal increase in growth inhibition (Monschau et al., 1997). The graded response of RGC axons to ephrins expressed in the SC appears to be a result of a combination of two distinct mechanisms (Figure 1.3B). Firstly, a receptor for both ephrinA2 and ephrinA5, EphA3 (in birds, EphA5 in mammals), is expressed in an increasing nasal to temporal gradient in the retina (Cheng et al., 1995; Feldheim et al., 1998). Consequently, temporal RGC axons (expressing high levels of EphA3/EphA5) are restricted by ephrinA2 expression to terminating within the anterior SC, whereas nasal RGC axons (expressing low levels of EphA3/EphA5), can grow further into the posterior SC until they encounter relatively higher levels of ephrinA2 and also ephrinA5, the latter acting as a stronger repellent (Monschau et al., 1997) for these axons. Additionally, given that ephrinA5 is also expressed at high levels in tissue that is immediately posterior to the OT or SC (Donoghue et al., 1996; Zhang et al., 1996; Brennan et al., 1997), ephrinA5 may also constitute a general repulsive barrier that prevents RGC axons overshooting the OT or SC. The second mechanism involves the uniform expression of Eph receptors, such as EphA4, and the decreasing nasal to temporal gradient of ephrinA5 expression, in the retina (Cheng et al., 1995; Marcus et al., 1996; Monschau et al., 1997; Connor et al., 1998). This overlap in expression causes persistent activation of EphA4 in nasal axons (Connor et al., 1998), which desensitizes these axons to the inhibitory signals of ephrinA ligands expressed in the anterior SC (Hornberger et al., 1999). The mechanisms that underlie receptor desensitization remain unclear, but may involve receptor degradation, downregulation of a component of the intracellular transduction pathway or even relocalization of the receptor away from the axonal growth cone. Thus, it appears that the differential sensitivity of RGC axons that results in the correct topographic organization of RGC projections in the retinocollicular system could, in part, involve a combination of graded levels of Eph receptor and ephrin expression in the retina and SC respectively, as well as uniform Eph receptor expression in the retina, which under certain circumstances, is overlapped by graded ephrin expression.

The generation of loss- and gain-of-function animal mutants has provided some
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Figure 1.3
confirmation of the mechanisms that are proposed to regulate the normal topographic organization of RGC terminations in the retinocollicular system. However, the generation of these mutants has revealed a complexity in the mechanisms underlying topographic patterning in the retinocollicular system that was not previously realized. In homozygote mutant animals lacking the gene that encodes for either ephrinA2 or ephrinA5, and in heterozygote and homozygote ephrinA2/ephrinA5 double mutant animals, some temporal RGC axons project more posteriorly in the SC than is normally expected (Figure 1.4B-D; Frisen et al., 1998; Feldheim et al., 2000). Indeed, in ephrinA5 deficient animals, but not the ephrinA2 deficient animals, temporal RGC axons transiently overshoot the posterior SC and extend aberrantly into the inferior colliculus (Figure 1.4C; Frisen et al., 1998). These defects are consistent with high levels of ephrinA ligands expressed in the posterior SC (and in the tissues immediately posterior to the SC) serving to inhibit the growth of RGC axons. In contrast to temporal RGC axons, in ephrinA5 deficient and the ephrinA2/ephrinA5 deficient animals, nasal RGC axons project to more anterior rather than posterior regions of the SC (Figure 1.4C-D; Feldheim et al., 2000). In ephrinA5 deficient animals, this effect is not as dramatic as is observed in the ephrinA2/ephrinA5 deficient animals, and could be explained by a loss of receptor desensitization in these axons, which is normally caused by ephrinA5 expression, resulting in nasal RGC axons in ephrinA5 deficient animals being more responsive than normal to ephrinA2 expressed in the SC. However, loss of receptor desensitization does not account for the dramatic anterior shift in the nasal RGC termination pattern observed in ephrinA2/ephrinA5 deficient animals (Feldheim et al., 2000). In these animals, the topographic organization of retinocollicular projections is almost completely abolished (Feldheim et al., 2000). Remarkably however, retinocollicular projections are still confined to the SC in ephrinA2/ephrinA5 deficient animals and do not overshoot its posterior borders (Feldheim et al., 2000). This effect is not consistent with the hypothesis that the topographic organization of retinocollicular projections is determined solely by specific expression gradients of Eph receptors and ephrins causing the arrest of RGC axons at specific ephrin concentration thresholds in the SC. This effect could be explained, however, by a model that proposes nasal and temporal RGC axons compete to terminate within the SC and success is biased by relative differences in the sensitivity of RGC axons to inhibitory or repulsive cues (Feldheim et al., 2000). During normal development, temporal RGC axons are confined
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Figure 1.4
to the anterior SC by ephrinA expression and compete out nasal axons that can enter the more posterior areas of the SC. In ephrinA2/ephrinA5 deficient animals, temporal RGC axons terminate in the posterior regions of the SC where they compete with nasal axons, which then lose preference for this region, and instead misroute to terminate in more anterior regions (Feldheim et al., 2000).

Support for a model predicting competition between RGC axons comes from the ectopic expression of elevated levels of EphA receptors in a subset of retinal axons (Brown et al., 2000). RGC axons expressing elevated levels of EphA receptors terminate more anteriorly in the SC as expected. However, RGC axons that don’t have elevated levels of EphA receptors also change their topographic position in the SC to terminate more posteriorly (Brown et al., 2000). This implies that RGC axons that do not express high levels of EphA receptor are competed out from their normal topographic position in the SC by RGC axons expressing elevated levels of EphA receptor (Figure 1.4E). Taken together, the results of genetic manipulation studies suggest that relative, not absolute, levels of EphA expression on RGC axons underlie their graded responses to ephrin gradients and that differential repulsion biases the success of axons competing for termination, rather than causing axonal inhibition/repulsion at particular levels of threshold activation.

In addition to regulating the topographic pattern of retinocollicular projections in the anterior/posterior axis, Eph/ephrin interactions may also be important for regulating the normal topographic pattern of the same projections in the dorsal/ventral axis. The mechanisms underlying the development of dorsal/ventral retinocollicular topographic patterns are not as well characterized as they are for the anterior/posterior axis but several members of the EphB/ephrinB subclass have complementary dorsal to ventral gradient expression patterns in the retina and SC, implying that they may play a role in guiding the topographic development of retinocollicular projections in the dorsal/ventral axis (Figure 1.3D). In the retina, EphB2 and EphB3 are expressed in an increasing dorsal to ventral gradient while ephrinB1 and ephrinB2 are expressed in a complementary increasing ventral to dorsal gradient (HOLASH and PASQUALE, 1995; KENNY et al., 1995; MARCUS et al., 1996; BRAISTED et al., 1997; HOLASH et al., 1997; CONNOR et al., 1998). Furthermore, a ligand for EphB2 and EphB3, ephrinB1, is
expressed in the SC in an increasing ventral to dorsal gradient. However, the gradient of ephrinB1 expression is contrary to it acting as an inhibitory/repulsive signal, since ventral RGC axons expressing high levels of EphB receptor project to dorsal regions of the SC expressing high levels of ephrinB ligand. Consequently, it is possible that EphB/ephrinB interactions may confer permissive/attractive signals to projecting RGC axons (Holash et al., 1997). Intriguingly, EphB5 (currently an orphan receptor) is also expressed in a centroperipheral gradient in the retina, suggesting that there may be regulation of topographic retinocollicular connections along this axis as well (Sefton et al., 1997).

1.2.1.8 Regulation of axonal branching patterns by Eph/ephrin interactions

In many neural circuits, the arrival of initial pioneering axons is followed by a period of axonal remodelling, in which axon collaterals are formed or eliminated, to create the mature termination pattern. Eph/ephrin interactions have been demonstrated to be important for the correct development of axon collaterals in a number of neural circuits. EphrinA ligands are important mediators of the initial outgrowth, branching and subsequent pruning of some hippocampal axons (Gao et al., 1999). Similarly, Eph/ephrin interactions may be important for the axonal branching patterns of cortical neurons. EphA5 is expressed in layers II/III and V of the cortex, whereas ephrinA5 expression is confined to layer IV (Castellani et al., 1998). As layer II/III neurons project their axons through to layer VI, they branch into layer II/III and V but not into layer IV, suggesting that ephrinA5 may inhibit collateral extension in some cortical neurons (Castellani et al., 1998). However given the apparent normal branching patterns of layer II/III neurons in ephrinA5 deficient animals (Yabuta et al., 2000) it is possible that the role of ephrinA5 in regulating branching patterns is functionally redundant. Additionally, it is also possible that the regulation of branching patterns of neurons in the cortex is area specific.

1.2.1.9 Eph/ephrin interactions can regulate adhesive events

It is highly likely that as well as mediating repulsive/inhibitory migratory events during the establishment and maintenance of connectivity in the nervous system, Eph/ephrin
interactions may also regulate adhesive/permissive events under certain circumstances. Indeed, the detection of co-expressed ephrinA5 and EphA7 in the lateral edges of the neural plate (Holmberg et al., 2000), which later fuse to form the neural tube, and the defective neural tube closure observed in ephrinA5 deficient animals (Frisen et al., 1998) suggests that these molecules may be important in mediating adhesion during neural tube development. Eph/ephrin interactions may also mediate adhesive/permissive responses in developing axons. As described previously in this chapter (section 1.2.1.7), EphB2 and ephrinB1 are expressed in dorsoventral gradients in the retina and SC respectively. However, given that RGC axons expressing high levels of Eph receptor project to regions of the SC expressing high levels of ephrin ligand, it is likely that these Eph/ephrins mediate adhesive/permissive interactions. There is currently no functional data to support this hypothesis but in vitro studies have shown that RGCs expressing high levels of EphB2 preferentially adhere to ephrinB1 substrates and RGCs expressing high levels of ephrinB1 preferentially adhere to EphB2 substrates (Holash et al., 1997).

In a similar situation, Eph/ephrin interactions have been demonstrated to be important for regulating the correct topographic projection of vomeronasal axons to the accessory olfactory bulb (AOB). In this network, apical vomeronasal axons expressing high levels of ephrinA5 project to the anterior AOB, which expresses high levels of EphA6 (Knoll et al., 2001b). In ephrinA5 deficient animals, some apical vomeronasal axons fail to project to regions of high EphA6 expression and instead project to regions of low EphA6 expression in the posterior AOB, possibly because ephrinA5 is required to mediate adhesive/permissive responses in these axons (Knoll et al., 2001b).

1.2.1.10 Biochemical mechanisms underlying Eph/ephrin mediated repulsion and adhesion

Interactions between Eph receptors and the ephrins appear to have functional roles in mediating both repulsive/inhibitory and adhesive/permissive cellular responses during neural development. The signal transduction mechanisms that underlie the ability of Eph/ephrins to regulate repulsive or adhesive events are only just beginning to be elucidated (reviewed in Kalo and Pasquale, 1999b; Mellitzer et al., 2000; Wilkinson, 2001).
Activation of Eph receptors leads to receptor oligomerization and the subsequent transphosphorylation of specific tyrosine residues in the cytoplasmic domain (Kalo and Pasquale, 1999a), some of which are involved in regulating tyrosine kinase activity (Choi and Park, 1999; Binns et al., 2000; Zisch et al., 2000). A number of these phosphorylated tyrosines serve as docking sites for Src-homology-2 (SH2)-domain-containing proteins (reviewed in Bruckner and Klein, 1998; Holland et al., 1998; Mellitzer et al., 2000), and in particular, two conserved phosphotyrosine residues located in the juxtamembrane region are particularly important sites of interaction between activated receptors and proteins containing SH2 domains (Ellis et al., 1996; Holland et al., 1997; Hock et al., 1998a; Zisch et al., 1998). Additionally, the cytoplasmic domains of activated Eph receptors may also directly phosphorylate intracellular effector proteins such as the neural cell adhesion molecule, L1 (Zisch et al., 1997), the Ras binding protein, ALL1 fused gene from chromosome 6 (AF6; Hock et al., 1998b), the docking protein p62\textsuperscript{dok} (Holland et al., 1997) and low-molecular weight phosphotyrosine phosphatases (LMW-PTP; Stein et al., 1998b).

Compared with Eph receptors, far less is known about the biochemical mechanisms that follow ephrin activation. It appears that some activated ephrinA ligands may be able interact with the Src-family tyrosine kinase Fyn in membrane raft microdomains to transduce intracellular signals (Davy et al., 1999). Similarly, little is known about which proteins recognize and interact with phosphotyrosine residues on activated ephrinB ligands. The SH2/SH3 domain adaptor protein Grb4, can associate with the cytoplasmic domain of tyrosine-phosphorylated ephrinB ligands and may be an important component of the ephrinB signal transduction pathway (Cowan and Henkemeyer, 2001). Furthermore, a novel cytoplasmic protein that binds ephrinB ligands via a PDZ domain, and has a regulator of heterotrimeric G protein signalling (RGS) domain, has recently been identified (Lu et al., 2001). This protein, PDZ-RGS3, can mediate signalling through activated ephrinB ligands by regulating the actions of cytoplasmic heterotrimeric G protein interactions (Lu et al., 2001).

What do we really know about the specific cytoplasmic interactions that regulate repulsion and adhesion following activation of Eph receptors and ephrins? As described previously, high levels of Eph receptor activation can exert repulsive/inhibitory effects on migrating cells and developing axons by eliciting the depolymerization of the actin
cytoskeleton that underlies the collapse of cellular filopodia (Krull et al., 1997; Wang and Anderson, 1997) and axonal growth cones (Meima et al., 1997a; Meima et al., 1997b). Indeed, several of the SH2-domain-containing proteins that are recruited by activated Eph receptors, including Fyn (Ellis et al., 1996), Src (Zisch et al., 1998), Nck (Stein et al., 1998b), Ras GTPase-activating protein (RasGAP; Holland et al., 1997; Hock et al., 1998a) and also the novel guanine nucleotide exchange factor, Ephexin (Shamah et al., 2001), are important components of the signal transduction pathways that control cytoskeletal rearrangement via the Rac/Rho/Cdc42 family of GTPases and Wiskott-Aldrich Syndrome Proteins (WASP). Moreover, Rho and its downstream effector, the serine/threonine kinase Rho kinase (ROCK), have recently been demonstrated to directly mediate axonal growth cone collapse induced by Eph receptor activation (Wahl et al., 2000).

In addition to causing the collapse of cellular filopodia and axonal growth cones via actin depolymerization, activated Eph receptors can also decrease cellular attachment to the extracellular matrix through inactivating integrins and focal adhesion kinase (FAK). Two SH2-domain-containing proteins that interact with activated Eph receptors have been implicated in the signal transduction pathway that leads to decreased integrin-mediated cellular adhesion. The first, SH2-domain-containing Eph-receptor-binding protein 1 (SHEP1), is recruited to interact with activated EphB2 and could bind to Ras GTPase family members to regulate integrin-mediated cellular adhesion (Dodelet et al., 1999). The second, SH2-domain-containing protein tyrosine phosphatase 2 (SHP2), is recruited to interact with activated EphA2 and induces the subsequent dephosphorylation of inactivation of FAK (Miao et al., 2000). Additionally, activation of EphA2 also leads to the inhibition of the Ras/mitogen-activated protein kinase (MAPK) signal transduction pathway (Miao et al., 2001). Given that the Ras/MAPK pathway promotes cell motility by phosphorylating and activating myosin light chains (Klemke et al., 1997), inhibition of this pathway by Eph receptor activation could also contribute to the repulsive/inhibitory effects of activated Eph receptors and ephrins on migrating cells and developing axons.

It would seem paradoxical that the cell contact-mediated, high-affinity, multivalent complexes formed by Eph/ephrin interactions can lead to the repulsion/inhibition of
migrating cells and developing axons. A particular group of proteases, the ADAMs (a disintegrin and a metalloprotease) are likely to be involved in terminating these Eph/ephrin interactions between juxtaposed cells so that cellular repulsion and axon retraction can occur. Following Eph/ephrin interaction, subsequent delayed activation of the ADAM protease, ADAM10/Kuzbanian (Kuz), causes localized ephrin cleavage from the cell surface (Hattori et al., 2000). This cleavage causes the disengagement of contacting cells, thus facilitating cytoskeletal collapse, and the subsequent repulsion/inhibition of migrating cells and developing axons.

As described previously, as well as mediating repulsion/inhibition, Eph/ephrin interactions also regulate particular adhesive/permissive responses in migrating cells and developing axons. Currently, the cytoplasmic events that occur during the regulation of adhesive/permissive responses by Eph/ephrin interactions are poorly understood. Increased integrin-mediated adhesion of cells caused by Eph/ephrin interactions is dependent upon two specific Eph receptor phosphotyrosine interacting sites (Stein et al., 1998a; Stein et al., 1998b; Huynh-Do et al., 1999). The interaction of Nck with the juxtamembrane interacting site results in the activation of the Jun kinase pathway and the subsequent activation of integrins (Stein et al., 1998a) via the Nck-interacting kinase (NIK) protein (Becker et al., 2000). Additionally, recruitment of LMW-PTP to a separate phosphotyrosine interacting site on activated Eph receptors is also linked to integrin activation (Stein et al., 1998b; Huynh-Do et al., 1999).

Given that Eph/ephrin interactions appear to transduce both repulsive/inhibitory and adhesive/permissive signals to migrating cells and developing axons, it is pertinent to consider the mechanisms that allow these distinctly different cellular responses to occur following Eph/ephrin activation. It may be that the nature of the response to Eph/ephrin activation is dependent upon the presence of different mediators of signal transduction within different cell types. Concordant with this is the ability of identical ephrin cues to promote neurite outgrowth in some neuronal cell types while inhibiting neurite outgrowth in others (Gao et al., 2000). Additionally, given that many Eph receptors and ephrins have overlapping expression domains, co-activation of Eph receptors and ephrins within the same cell could regulate different cellular responses. Moreover, the different binding affinities of different Eps and ephrins for each other, as well as the different levels of Eph/ephrin activation that could arise from these different binding
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affinities, may allow the assembly of quite different intracellular signal transduction complexes that could also modulate between a repulsive/inhibitory and adhesive/permissive cellular response. Also, distinct effects of Eph/ephrin interactions on migrating cells and growing axons could also be modulated by the degree of ephrin multimerization that mediates the level and/or nature of signals generated by Eph receptor activation (Gale and Yancopoulos, 1997; Stein et al., 1998b; Adams et al., 1999; Huynh-Do et al., 1999).

It appears that adhesive/permissive cellular responses arising from Eph/ephrin interactions probably also involve the simultaneous inhibition of signal transduction pathways that mediate repulsion/inhibition. Persistent Eph receptor activation, for example, can desensitize migrating cells and axons to repulsive/inhibitory ephrin cues present in the surrounding environment (Hornberger et al., 1999). Also, co-expression of a truncated form of EphA7 that lacks a tyrosine kinase domain (encoded by an alternative mRNA splice variant) with full length EphA7 can facilitate cellular adhesion following activation by ephrinA5 (Holmberg et al., 2000). Truncated EphA7 may act in a dominant-negative manner to inhibit repulsive/inhibitory signal transduction pathways mediated by full-length EphA7 receptors (Holmberg et al., 2000). Consistent with this proposal are the expression profiles of truncated EphA7 and full length EphA7 in the mature brain. Truncated EphA7 is expressed on cell somas and their proximal axons whereas full length EphA7 is expressed on the distal regions of axons, near the growth cones (Ciossek et al., 1995). Consequently, the segregation of EphA7 isoforms could facilitate adhesive interactions between neuronal somas while simultaneously allowing repulsive interactions to occur at the axonal growth cone.

Another mechanism that could facilitate adhesive/permissive cellular responses arising from Eph/ephrin interactions is the inhibition of metalloprotease-mediated ephrin cleavage. As described previously, delayed cleavage of ephrin ligands by Kuz causes the disengagement of contacting cells, thus facilitating cytoskeletal collapse and the subsequent repulsion/inhibition of migrating cells and developing axons (Hattori et al., 2000). Eph/ephrin interactions that mediate adhesive/permissive cellular events could do so by concomitantly inhibiting repulsive/inhibitory cellular responses through inactivating Kuz or its ephrin cleavage mechanism.
Cross talk between Eph receptors or ephrins and other classes of receptors could also allow Eph/ephrin interactions to mediate adhesive/permmissive cellular responses while simultaneously suppressing repulsive/inhibitory cellular events. Activation of fibroblast growth factor (FGF) receptors can phosphorylate ephrinB ligands independently of them binding to Eph receptors (Chong et al., 2000). Intriguingly, this cross talk between FGF receptors and ephrinB1 can result in the suppression of Eph/ephrin mediated cellular repulsion/de-adhesion (Chong et al., 2000). Additionally, Eph receptors can also interact with the orphan related-to-tyrosine-kinase (Ryk) receptor (Halford et al., 2000). Animals deficient in Ryk expression exhibit a very similar phenotype to EphB2/EphB3 deficient animals, that is, both mutants have a cleft palate (Orioli et al., 1996; Halford et al., 2000). The cleft palate in both these mutants is probably a result of the palatal shelves failing to fuse during development. Given that EphB2/EphB3 receptors can phosphorylate Ryk and may form in vivo complexes with Ryk as well (Halford et al., 2000), it is likely that cross talk between EphB2/EphB3 receptors and Ryk is essential for the correct mediation of particular events (such as those involved in the fusion of palatal shelves) that require adhesive/permmissive cellular responses.

### 1.2.2 Other receptors and ligands important for the regulation of neural migration and axonal guidance

As well as Eph/ephrin interactions, an ever-increasing number of other receptor/ligand interactions, which are able to mediate cytoskeletal rearrangement and cell motility, are also important in the mechanisms that establish and maintain connectivity in the nervous system (Figure 1.5).

#### 1.2.2.1 Extracellular matrix (ECM) molecules

Many ECM molecules belonging to the laminin, collagen, thrombospondin and tenascin families, as well as vitronectin, fibronectin and some proteoglycans can either promote or inhibit the migration of neurons and their axonal processes (reviewed in Bixby and Harris, 1991; Hynes and Lander, 1992; Schachner et al., 1994; Bovolenta and Fernaud-Espinosa, 2000). The receptors for ECM molecules are primarily derived from
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Figure 1.5
the integrin and immunoglobulin (Ig) receptor families, with some proteoglycans also able to act as receptors for ECM molecules as well (reviewed in Reichardt and Tomaselli, 1991; Bovolenta and Fernaud-Espinosa, 2000; De Arcangelis and Georges-Labouesse, 2000 and also see Zisch et al., 1992; Pesheva et al., 1993 and Taira et al., 1994). The \textit{in vitro} functions of ECM molecules and their receptors, and their \textit{in vivo} expression patterns in the nervous system, suggest important roles for these molecules in mediating neural migratory events, yet their \textit{in vivo} roles are still far from understood. Laminin A appears to be important for the correct guidance of specific photoreceptor axons (Garcia-Alonso et al., 1996), while Laminin 1 can serve to modulate the navigational behaviour of growth cones to other guidance cues (Hopker et al., 1999). Integrins appear to be important for the correct migration of cortical neurons (Anton et al., 1999; Dulabon et al., 2000) and also for the correct fasciculation and guidance of particular axonal populations (Lilienbaum et al., 1995; Baum and Garriga, 1997; Hoang and Chiba, 1998). Integrins have also been implicated in the regulation of migrating chains of neural precursors (Jacques et al., 1998). In humans, defects in the functions of ECM molecules and their receptors can result in abnormal development and disease (Novak and Kaye, 2000). Mutations in the \textit{Kallmann} gene for example, result in Kallmann’s syndrome, a disease characterized by developmental defects in the migration of particular neuronal populations. The \textit{Kallmann} gene encodes a small ECM molecule that is likely to provide a permissive substrate for migrating neurons during development (Franco et al., 1991; Legouis et al., 1991).

1.2.2.2 Cell adhesion molecules (CAMs)

CAMs are important mediators of neural migration and axonal guidance in the nervous system. Members of the CAM family are divided into two groups, the IgCAMs and the cadherins, with members of each group mediating cell adhesion events through both heterophilic and homophilic interactions. Members of the IgCAM family include L1, neural CAM (NCAM) and TAG-1 and are important for mediating guidance and fasciculation in developing axonal populations (reviewed in Walsh and Doherty, 1997; Van Vactor, 1998; Kamiguchi and Lemmon, 2000; Kaprielian et al., 2001). NCAM and polysialic acid (PSA)-NCAM interactions are important for regulating fasciculation/defasciculation in developing motor axon tracts (Tang et al., 1992; Tang et
al., 1994; Daston et al., 1996). The regulated expression of TAG-1 and L1 on particular populations of commissural axons is necessary for their correct guidance across the midline (Dodd et al., 1988; Fujimori et al., 2000; Imondi et al., 2000). Furthermore, L1 is particularly important for the correct development of axonal tracts descending from the cortex to the spinal cord (Dahme et al., 1997; Cohen et al., 1998). Indeed, in humans, mutations in the gene that encodes L1 causes MASA (mental retardation, aphasia, shuffling gait and adducted thumbs) syndrome, a neurological disease characterized by the inappropriate development of specific axonal tracts (Kenwrick and Doherty, 1998).

Members of the cadherin family of CAMs, including neural (N)-cadherin, are also important for the correct development of neural circuits (reviewed in Van Vactor, 1998; Redies, 2000; Tepass et al., 2000; Yagi and Takeichi, 2000). Consistent with a role in mediating axon guidance and fasciculation, cadherins are expressed on a number of populations of developing axons (Matsunaga et al., 1988; Redies et al., 1992; Shimamura et al., 1992; Arndt and Redies, 1996; Arndt et al., 1998; Arndt and Redies, 1998; Korematsu et al., 1998; Wohrn et al., 1998; Wohrn et al., 1999) and recently, N-cadherin has been demonstrated to be an important regulator of the outgrowth and guidance of specific RGC axon populations in vivo (Riehl et al., 1996; Inoue and Sanes, 1997). Cadherins are also important for the correct development of synaptic connections between neurons (Yamagata et al., 1995; Fannon and Colman, 1996; Uchida et al., 1996; Kohmura et al., 1998). Furthermore, given that synaptic activity can modulate certain properties of cadherins (Tanaka et al., 2000) and that cadherins, in turn, can regulate the activity of synapses (Tang et al., 1998; Yamagata et al., 1999; Manabe et al., 2000), cadherins may have important roles in the mechanisms that mediate synaptic activity and plasticity in the nervous system as well.

1.2.2.3 Hepatocyte growth factor (HGF)/Scatter factor (SF)

HGF/SF is a diffusible ligand for the c-Met receptor tyrosine kinase and functions as an axonal growth promoting and guidance cue in developing neural circuits (reviewed in Maina and Klein, 1999). During and after development, both HGF/SF and c-Met are differentially expressed in the nervous system and related tissues (Jung et al., 1994;
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Ebens et al., 1996; Thewke and Seeds, 1996; Achim et al., 1997; Thewke and Seeds, 1999; Powell et al., 2001). HGF/SF can function as a guidance cue and survival factor for particular populations of developing spinal (Ebens et al., 1996; Yamamoto et al., 1997; Novak et al., 2000) and cranial motoneurons (Caton et al., 2000), and also acts synergistically with nerve growth factor (NGF) to promote survival and neurite outgrowth in sensory neurons (Maina et al., 1997). It can also act as an autocrine factor to mediate neurite outgrowth in sympathetic neurons (Maina et al., 1998). HGF/SF may also be required for the correct migration of interneuron populations in the developing forebrain (Powell et al., 2001).

1.2.2.4 Netrins

The secreted netrins, and their receptors, such as the deleted in colorectal cancer (DCC) receptors, are important mediators of the mechanisms that underlie the correct development of neural connections (reviewed in Livesey, 1999; Kaprielian et al., 2001). Netrin and DCC are both expressed in the vertebrate nervous system in patterns that suggest a role in the guidance of migrating cells and developing axons (Cooper et al., 1995; Keino-Masu et al., 1996; Serafini et al., 1996; Gad et al., 1997; Livesey and Hunt, 1997; MacLennan et al., 1997; Shu et al., 2000; Barrett and Guthrie, 2001; Seaman and Cooper, 2001). Interactions between netrin and DCC are important for the attractive guidance of a number of commissural axon tracts across the midline of the spinal cord (Kennedy et al., 1994; Keino-Masu et al., 1996; Serafini et al., 1996; Fazeli et al., 1997) and hindbrain (Shirasaki et al., 1996). Additionally, netrin/DCC interactions are also important for the repulsive guidance of particular populations of trochlear (Colamarino and Tessier-Lavigne, 1995) and cranial motor axons (Varela-Echavarria et al., 1997). The bi-functionalilty of netrin-mediated interactions can be attributed to the activation of different receptors or receptor complexes on developing axons (Hong et al., 1999) and/or modulated levels of second messenger molecules in the neuronal cytoplasm (Ming et al., 1997; Song et al., 1997).

In addition to the spinal cord and hindbrain, netrin/DCC interactions are also important for the correct development of axonal tracts in the vertebrate forebrain. For example, both the corpus callosum and the hippocampal commissure are severely disrupted in
homzygous mutant mice deficient for netrin or DCC (Serafini et al., 1996; Fazeli et al., 1997; Barallobre et al., 2000). Moreover, chemoattractive netrin/DCC interactions may also be important for the correct development of corticofugal (Metin et al., 1997; Richards et al., 1997) and thalamocortical projections (Braisted et al., 2000). In the visual system, netrin/DCC interactions are important for the correct guidance of RGC axons through the optic disc into the optic nerve (Deiner et al., 1997) and at the optic chiasm midline (Deiner and Sretavan, 1999). Netrin/DCC interactions are also critical for the correct circumferential migration of basilar pontine neurons from the dorsal to the ventral midline in the hindbrain (Yee et al., 1999) and also for the correct outward migration of cells from the lateral ventricle subventricular zone to the striatum (Hamasaki et al., 2001). Furthermore, netrin mediated interactions also appear to regulate the correct migration of distinct neuronal populations in the cerebellar system (Alcantara et al., 2000) and are also important for the correct migration and survival of inferior olivary neurons (Bloch-Gallego et al., 1999). Additionally, given the widespread expression of netrin by neurons and oligodendrocytes in specific regions of the mature spinal cord, netrin mediated interactions may also be important for the maintenance of axon-oligodendroglial connections in the mature nervous system (Manitt et al., 2001).

1.2.2.5 Slits

The diffusible slit proteins were first characterized in Drosophila where they are expressed by midline glial cells and interact with roundabout (robo) receptors expressed on longitudinal commissural axons (reviewed in Brose and Tessier-Lavigne, 2000; Guthrie, 2001). Robo is upregulated on longitudinal commissural axons in the Drosophila nervous system after they have crossed the midline and subsequent interactions with slit prevent these axons from recrossing the midline later in development (Kidd et al., 1998a; Kidd et al., 1998b; Brose et al., 1999; Kidd et al., 1999). Multiple homologues of both robo and slit have been identified in vertebrates and their distinct and complementary expression patterns suggest that they mediate multiple events during the development of connectivity in the nervous system (Holmes et al., 1998; Itoh et al., 1998; Kidd et al., 1998a; Brose et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Yuan et al., 1999; Shu and Richards, 2001).
Slit proteins primarily function as repellent cues in the nervous system and can repel spinal motor axons (Brose et al., 1999) as well as hippocampal axons (Nguyen Ba-Charvet et al., 1999). Slit can also repel olfactory axons (Li et al., 1999; Nguyen Ba-Charvet et al., 1999) and can also function to repel the growth of, and inhibit fasciculation in, RGC axons (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000). Importantly, slit can selectively repel spinal commissural axons that have previously crossed the spinal midline (Zou et al., 2000) and may also serve to repel commissural cortical axons during corpus callosum development in the forebrain (Shu and Richards, 2001). As with most other neural guidance cues, slit is bifunctional and, along with repulsive events, can also mediate attractive/permissive events in the nervous system. Indeed, slit proteins can function as positive regulators of sensory axon elongation and collateral branching (Wang et al., 1999). In addition to slit/robo interactions mediating neural guidance independent of any other factors, the activation of robo by slit can cause robo to form complexes with other cell-surface guidance receptors. Slit binding to robo, for example, can result in robo forming a complex with DCC that silences netrin mediated attraction (Stein and Tessier-Lavigne, 2001).

Interactions between slit and robo are also important for the directed guidance of both cortical (Hu, 1999; Zhu et al., 1999) and olfactory bulb (Wu et al., 1999; Chen et al., 2001) neuronal populations in the embryonic and mature brain and their roles in mediating the guidance of some these cell populations will be reviewed in greater detail in subsequent sections of this chapter.

1.2.2.6 Semaphorins

Interactions between the semaphorins and their receptors, the neuropilins and plexins, mediate key events that underlie the correct development of connections in the nervous system (reviewed in Nakamura et al., 2000; Raper, 2000). Currently, the semaphorin family consists of over 20 different members that exist in diffusible, GPI-linked and transmembrane-containing protein forms (Semaphorin Nomenclature Committee, 1999). The semaphorins generally mediate repulsive/inhibitory events in the nervous system. They are able to induce the growth cone collapse and/or axonal repulsion of dorsal root ganglion (Luo et al., 1993; Messersmith et al., 1995) and cranial (Kobayashi
et al., 1997) sensory neurons. Semaphorins are also able to induce growth cone collapse and/or axonal repulsion in axons of sympathetic neurons (Adams et al., 1997), certain thalamic neurons (Bagnard et al., 2001), olfactory neurons (Kobayashi et al., 1997; de Castro et al., 1999), basilar pontine neurons (Rabacchi et al., 1999), hippocampal neurons (Chedotal et al., 1998), cortical neurons (Bagnard et al., 1998; Polleux et al., 1998) and spinal motor neurons (Varela-Echavarria et al., 1997). Animals that are homozygous for a targeted disruption of the gene that encodes for one of the most important semaphorins in the nervous system, semaphorin3A, exhibit some aberrant axonal projections in certain cranial and spinal nerves of the peripheral nervous system (Taniguchi et al., 1997). Despite this, it appears that the majority of neural pathways in semaphorin3A deficient animals develop normally (Catalano et al., 1998). This suggests that a certain amount of functional redundancy exists among individual members of the semaphorin family.

As described earlier, the receptors for the semaphorin family of guidance cues are the neuropilins and the plexins (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Winberg et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999). Consistent with the defects observed in the peripheral nervous system of semaphorin3A deficient animals, animals with a targeted disruption of the genes encoding for neuropilin family members also exhibit aberrant development of particular populations of cranial and spinal nerves (Kitsukawa et al., 1997; Giger et al., 2000). Furthermore, neuropilin-2 deficient animals also exhibit severe defects in axonal development within the anterior commissure, hippocampus and thalamus of the central nervous system (Giger et al., 2000).

Similar to most other neural guidance molecules, the semaphorins also appear to be bifunctional in that they are able to mediate events that, depending on the circumstances, can be either attractive/permisive or repulsive/inhibitory to neural components of the nervous system. Semaphorins can provide attractive guidance signals to axons of cortical neurons (Bagnard et al., 1998), the olfactory bulb (de Castro et al., 1999), the peripheral nervous system (Wong et al., 1999) and also to the apical dendrites of cortical pyramidal neurons (Polleux et al., 2000). Indeed, the specific
response of a particular population of neurons to semaphorin guidance signals present within the neural environment may be regulated by levels of intracellular cyclic nucleotides within the cytoplasm of responding neurons (Song et al., 1998).

In addition to their well-characterized roles as mediators of directed axonal growth, semaphorin/neuropilin interactions have also recently been shown to be important for the correct guidance of migrating populations of interneurons from the medial ganglionic eminence to their final destinations in the striatum and cortex during early brain development (Marin et al., 2001).

1.2.2.7 Reelin

Reelin, a secreted extracellular protein, is essential for the control of the migratory and neuron-positioning events that underlie the correct development of laminated brain structures in the cerebrum, cerebellum and the hippocampus, and also the correct positioning of other neuronal populations throughout the brain (D'Arcangelo et al., 1995 and reviewed in D'Arcangelo and Curran, 1998; Bar et al., 2000; Gilmore and Herrup, 2000; Rice and Curran, 2001). Reelin is developmentally expressed within specific areas of the cerebral cortex, cerebellum and hippocampus (D'Arcangelo et al., 1995) and, although the precise mechanism by which reelin regulates neuronal migration in the nervous system remains unclear, it is known to interact with two cell-surface lipoprotein receptors (or co-receptors), the very low-density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2; D'Arcangelo et al., 1999). Upon interacting with reelin, the VLDLR and ApoER2 appear to transduce intracellular signals by recruiting the cytosolic adaptor protein Disabled-1 (Dab1), which subsequently could be involved in mediating the downstream events that directly control cytoskeletal rearrangement and neuronal migration (Hiesberger et al., 1999; Howell et al., 1999). Indeed, the generation of mutant animals with disruptions in the gene that either encodes for Dab1 or for the genes that encode for both the VLDLR and ApoER2 confirm these proteins as participants in the reelin signalling pathway as both these mutant animals exhibit aberrant neuronal migration in the brain similar to that observed in reelin deficient animals (Howell et al., 1997; Ware et al., 1997; Trommsdorff et al., 1999). In addition, members of both the integrin and cadherin-
related neuronal receptor (CNR) families may also be involved in modulating reelin mediated signals during nervous system development (Senzaki et al., 1999; Dulabon et al., 2000).

1.2.2.8 Other molecules involved in the guidance of migrating neurons and their axons

A multitude of other molecules are also required for the development and maintenance of precise patterns of neural connectivity in the nervous system. These molecules are derived from a vast array of different families and include, but not limited to, factors such as the intracellular TOAD/Ulip/CRMP (TUC) proteins (Quinn et al., 1999), the intracellular signalling molecules p35 (Kwon et al., 1999) and mammalian enabled (Mena; Lanier et al., 1999) and the homeodomain transcription factors ventral anterior homeobox 1 (Vax1; Bertuzzi et al., 1999) and orthodenticle-like homeobox 1 (Otx1; Weimann et al., 1999). Indeed, large-scale, phenotype-based, genetic analyses have recently begun to identify multiple novel regulators of axon guidance and cell migration in the nervous system (Kraut et al., 2001; Leighton et al., 2001; Mitchell et al., 2001). Information gained from these genetic analyses, when combined with information derived from more “traditional” techniques directed at determining the functions of particular guidance cues and their receptors and the subsequent intracellular signalling mechanisms that these interactions elicit, may allow the elucidation of the mechanisms that underlie the establishment of connectivity within the nervous system.

1.3 MECHANISMS UNDERLYING THE DEVELOPMENT OF SELECTED CORTICAL AND SPINAL CIRCUITS INVOLVED IN REGULATING MOTOR ACTIVITY

1.3.1 The development of the major axonal pathway that connects the brain with the spinal cord-the corticospinal tract

The corticospinal tract (CST) is the longest axonal projection within the mammalian central nervous system and comprises the major pathway by which the cerebral cortex modulates peripheral motor activity via connections with neural networks within the
spinal cord (reviewed in Armand, 1982; Stanfield, 1992; Terashima, 1995; Joosten and Bar, 1999).

### 1.3.1.1 CST gross anatomy and function

In the rodent, axons comprising the CST originate from pyramidal neurons located in layer 5 of the motor and sensorimotor cortex and extend through the internal capsule, cerebral peduncle and medullary pyramids before the majority of axons decussate in the caudal medulla (i.e. cross the midline) and project dorsally to the spinal cord. Here, they descend through the ventral aspect of the dorsal funiculus and unilaterally terminate within the dorsal horn of the spinal grey matter at all levels of the spinal cord contralateral to their cells of origin (Figure 1.6). Although the major component of the corticospinal pathway is located within the contralateral dorsal funiculus of the spinal cord, there are also minor components located within the contralateral lateral (Schreyer and Jones, 1982) and ipsilateral dorsal and ventral (Vahlsing and Feringa, 1980; Liang et al., 1991; Brosamle and Schwab, 1997) funiculi of the spinal cord as well. For the purposes of this chapter, only those mechanisms underlying the development, maturation and function of the major contralateral CST component will be discussed.

The rodent CST appears to be most important for the fine motor control of limb movement during locomotion (Hicks and D’Amato, 1975; Donatelle, 1977). The CST originating from one side of the brain mediates motor control on the opposing side of the body through its terminations within the spinal grey matter. In the rodent, this motor control is somewhat indirect and exerted primarily through connections with specific neural networks located within the dorsal horn (Brown, 1971). However, given recent anatomical evidence suggesting that at least some CST axons may project to the ventral horn of the spinal grey matter to form direct synaptic connections with motoneurons (Kuang and Kalil, 1990; Liang et al., 1991; Curfs et al., 1996), it is highly likely that the rodent CST can also control motor activity directly in some circumstances.

### 1.3.1.2 The initial guidance of emerging corticofugal axons from the cortex

The mechanisms that underlie the initial guidance of emerging corticofugal axons from
Figure 1.6
the cortex are not well understood. Axons of cortical subplate neurons pioneer the route that corticofugal axons take to the internal capsule (Auladell et al., 2000 and for review Allendoerfer and Shatz, 1994; De Carlos and O'Leary, 1992; McConnell et al., 1989, 1994; Molnar and Cordery, 1999). It is possible that subplate axons function as a scaffold to guide subsequently projecting corticofugal axons out of the cortex, with bone fide corticofugal axons growing upon the earlier generated subplate axons (De Carlos and O'Leary, 1992; McConnell et al., 1994). However, given the apparent paucity of subplate axons past the level of the internal capsule, it is unlikely that these axons serve to guide corticofugal axons at any time other than during the very early stages of corticofugal tract development (De Carlos and O'Leary, 1992).

In addition to growing along a pre-formed scaffold of subplate axons, cortifugal axons may be guided out of the cortex by extracellular axonal guidance cues. Indeed, Semaphorin3A has been shown to direct the initial development of cortical efferent axons towards the deep white matter tracts by repelling these axons away from the superficial layers of the cortex (Polleux et al., 1998). Furthermore, netrin-1 is also able to influence the directed growth of cortical efferent axons and its activity within the internal capsule may serve to attract cortifugal axons emerging from the cortex during early neural development (Metin et al., 1997; Richards et al., 1997).

1.3.1.3 Other mediators of CST axon guidance through the white matter regions of the brain and spinal cord

Following their emergence from the cortex, axons comprising the CST traverse a long and sometimes complicated trajectory to the spinal cord. Although it is likely that a number of spatially and temporally regulated mechanisms underlie the appropriate guidance of CST axons to the spinal cord, these mechanisms are not currently well understood.

It is possible that astroglial cells may provide a permissive matrix or direct conduit to guide CST axons through appropriate areas in the nervous system. Vimentin is the major cytoskeletal component of immature glial cells (Dahl et al., 1981). Immunohistochemical analysis of vimentin distribution in the nervous system reveals
that it is transiently expressed within the medulla and spinal cord concomitantly with developing CST axons, and during subsequent development, this expression disappears at approximately the same time that glial fibrillary acidic protein (GFAP) expression appears (Joosten and Gribnau, 1989). The growth cones of developing CST axons frequently exhibit protrusions into vimentin immunopositive glial cell processes. Although vimentin immunopositive glial cell processes do not appear to form an obvious conduit that confines CST axons to a particular route, they are mainly arranged in longitudinal tiers, which, given the close interactions exhibited by glial cell processes and CST axons, suggests that astroglia could provide a scaffold in both the medulla and spinal cord to guide developing CST axons (Joosten and Gribnau, 1989).

In the region of the developing CST in the medulla and spinal cord, the greatest number of vimentin immunopositive glial cell processes occurs at the midline, in the form of a glial septum (Joosten and Gribnau, 1989). Whereas a prominent vimentin immunopositive glial septum is present in both the medulla oblongata and in the spinal cord, it is absent from the area where CST axons decussate to the contralateral dorsal column of the medulla at the spinomedullary junction (Joosten and Gribnau, 1989). Consequently, the glial septum may serve to prevent CST axons from crossing the midline of the nervous system except in the appropriate region of the spinomedullary junction (Joosten and Gribnau, 1989).

Although the presence of a glial septum within specific areas of the medulla and spinal cord may prevent CST axons from aberrantly crossing the midline in inappropriate regions, it does not appear to promote the decussation of CST axons at the spinomedullary junction. One mediator of axon guidance that appears to be very important for the correct development of the CST, especially at the spinomedullary junction, is the L1 IgCAM (Dahme et al., 1997; Cohen et al., 1998). Animals deficient in L1 expression exhibit a perturbed CST (Cohen et al., 1998). In particular, a substantial proportion of the CST axons in L1 deficient animals fail to decussate appropriately at the spinomedullary junction, projecting instead to the dorsal column of the medulla that is ipsilateral to their cells of origin (Cohen et al., 1998). Given that L1 is expressed on axons of the CST (Fujimori et al., 2000) and that a ligand for L1, CD24, is specifically expressed in the area of the CST decussation (Cohen et al., 1998), it
appears likely that the correct guidance of decussating CST axons within the spinomedullarly junction is dependent upon L1 mediated interactions.

NCAM also appears to be important for some aspects of the guidance of developing CST axons. The embryonic form of NCAM is likely to be involved in mediating the guidance of CST axons as they initially develop through the dorsal funiculus of the spinal cord (Joosten, 1994). Subsequent to this, the mature forms of NCAM become predominate, and are probably involved in mediating the fasciculation of later arriving CST axons along those initial CST axons that have pioneered the CST pathway earlier in development (Joosten, 1994). Indeed, it is the fasciculation of later arriving CST axons along pioneering CST axons (Schreyer and Jones, 1982; Gribnau et al., 1986; Joosten et al., 1987; Gorgels et al., 1989; Gianino et al., 1999) that most likely serves as the guidance mechanism that determines the trajectory of most CST axons during development.

1.3.1.4 Innervation of the spinal grey matter by CST axons

At any given level of the spinal cord there is a significant delay between the arrival of CST axons and their entry into the adjacent spinal grey matter (Donatelle, 1977; Reh and Kalil, 1981; Schreyer and Jones, 1982; Gribnau et al., 1986; Joosten et al., 1987). Although during spinal grey matter innervation some CST axons may change their direction and enter the adjacent grey matter directly (Joosten et al., 1994a), the innervation of adjacent spinal grey matter by CST axons mainly occurs by collateral branches formed interstitially along the length of the CST (O'Leary and Terashima, 1988; O'Leary et al., 1990; Joosten et al., 1994a; Kuang and Kalil, 1994; Bastmeyer and O'Leary, 1996).

The mechanisms that control the directed growth of CST collaterals into the spinal grey matter are not well understood but appear to be a property of the axon shaft rather than of the leading growth cone (Bastmeyer and O'Leary, 1996). CST collaterals are induced to form on their parent axons by spatially restricted increases in PSA expression (Daston et al., 1996). The subsequent guidance of these CST collaterals to their final destinations within the spinal grey matter is most likely mediated by a number of
diffusible as well as cell-attached molecular cues. Diffusible trophic activities derived from the spinal grey matter appear to specifically regulate target innervation of CST collaterals (Joosten et al., 1991). Indeed, this diffusible trophic activity is present at both the cervical and lumbar levels of the spinal cord (Joosten et al., 1991; Joosten et al., 1994a; Joosten et al., 1994b). The identity of the diffusible trophic factor(s) that mediates CST collateral guidance has not yet been elucidated, but given the fact that neurotrophin-3 (NT-3) can enhance sprouting of CST axons during development and following injury (Schnell et al., 1994), it is possible that this, or other members of the neurotrophin family, may be involved in the mechanisms that underlie the correct guidance of CST collaterals to their destinations in the grey matter of the spinal cord.

In addition to the roles of diffusible cues during CST collateral innervation of the spinal grey matter, cell-attached cues may also be involved in the mediation of this process as well. The dendritic fields of some motoneuron populations within the ventral horn of the spinal grey matter sometimes overlap the termination field of CST collaterals within the dorsal horn, suggesting a possible role for direct interactions between dendrites and CST collaterals in mediating the guidance of CST collaterals to their correct destinations (Curfs et al., 1993). Indeed, at an ultrastructural level, intimate contacts exist in the spinal dorsal funiculus between CST collaterals and neuronal dendrites that have radially extended into the dorsal funiculus from the adjacent grey matter (Gorgels, 1991). Taken together, these observations suggest that contact between CST collaterals and adjacent neuronal dendrites may facilitate a mechanism that allows the guidance of CST collaterals to their correct destinations within the spinal grey matter.

1.3.1.5 The reorganization of CST terminations within the spinal grey matter during maturation

Subsequent to CST collaterals innervating and spreading out within the spinal grey matter, the further reorganization of CST terminal arborizations during maturation allows the final CST termination pattern to develop in the spinal cord. In the rodent, when CST collaterals initially innervate the spinal grey from the dorsal funiculus, they do so by entering into the immediately adjacent grey matter (Donatelle, 1977; Reh and Kalil, 1981; Gribnau et al., 1986). During the subsequent period, CST collaterals then develop dorso- and ventrolaterally into other regions of the spinal grey matter.
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(Donatelle, 1977; Reh and Kalil, 1981; Gribnau et al., 1986). In the rodent, most CST collaterals appear to grow directly to their final destination within the dorsal horn of the spinal grey matter during development (Donatelle, 1977; Reh and Kalil, 1981; Gribnau et al., 1986). However, there are also a significant number of transient CST projections that, during early development, extend throughout the entire spinal grey matter area and are selectively eliminated, later in CST maturation, to allow the development of the mature CST projection pattern (Curfs et al., 1994). Although in the rodent the elimination of transient corticospinal projections during nervous system development is well characterized (Stanfield et al., 1982; Chung and Coggeshall, 1987; Joosten et al., 1987; Schreyer and Jones, 1988; Gorgels et al., 1989; Curfs et al., 1994; Uematsu et al., 1996) and (reviewed in Stanfield, 1992), the mechanisms that underlie the elimination and final reorganization of CST axons are poorly understood. Indeed, a role for extracellular guidance cues and their receptors in mediating the elimination and reorganization of CST terminations within the spinal grey matter during maturation has yet to be described.

1.3.2 The development of somatic motoneurons in the spinal cord and the guidance of their axons in the periphery

The direct control of motor activity arises from somatic spinal motoneurons within the ventral horn of the spinal grey matter that project their axons, via the spinal ventral roots, to skeletal muscles located in the periphery (Figure 1.7). Somatic motoneurons are positioned in longitudinal columns and are topographically organized both rostrocaudally and mediolaterally in relation to the position of the specific muscle which they innervate. The production of topographically specific connections between somatic motoneurons in the spinal cord and skeletal muscles at the periphery requires the coordinated generation of a diverse array of motoneuron subtypes from one common developmental origin (reviewed in Lumsden, 1995; Pfaff and Kintner, 1998; Eisen, 1999; Hughes and Salinas, 1999; Jessell, 2000; Jurata et al., 2000).

1.3.2.1 Regulation of generic motoneuron differentiation in the spinal cord

Neural progenitor cells in the ventrolateral spinal cord are induced to differentiate into generic motoneurons by signals derived from ventral midline structures i.e. the
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Figure 1.7
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notochord and floor plate (Yamada et al., 1993). The secreted protein, sonic hedgehog (Shh), is expressed by the notochord and floor plate at the time that these cellular structures are inducing generic motoneuron formation (Echelard et al., 1993; Roelink et al., 1995) and appears to be the major extrinsic signal that commits neural progenitor cells to a motoneuron fate. Specific concentrations of Shh induce motoneuron differentiation by mediating the expression of certain homeodomain transcription factors (Roelink et al., 1995; Ericson et al., 1996; Ericson et al., 1997; Briscoe et al., 1999; Briscoe et al., 2000). The ectopic expression of Shh, both in vivo and in vitro, can induce motoneuron differentiation in some neural progenitor cell populations (Echelard et al., 1993; Marti et al., 1995; Roelink et al., 1995; Tanabe et al., 1995). Moreover, elimination of Shh signalling by using function-inhibiting antibodies (Ericson et al., 1996) or by disrupting the gene that encodes Shh (Chiang et al., 1996), dramatically inhibits motoneuron differentiation in the ventrolateral spinal cord and confirms Shh-mediated signalling mechanisms as the major regulator of generic motoneuron differentiation in the ventrolateral spinal cord.

1.3.2.2 Regulation of somatic motoneuron column identity in the spinal cord

Once the population of generic motoneurons is established within the ventrolateral spinal cord, some of these motoneurons are induced to become somatic motoneurons by mechanisms mediated by the transcription factor, homeobox 9 (HB9; Arber et al., 1999; Thaler et al., 1999). Somatic motoneurons are positioned in distinct longitudinal motor columns that are orientated along the rostrocaudal axis of the spinal cord (Figure 1.8). The medial motor columns (MMC) extend along the entire length of the spinal cord and consist of motoneurons that supply the musculature of the trunk. In contrast, the lateral motor columns (LMC) are restricted to the cervical and lumbar expansions of the spinal cord and consist of motoneurons that innervate limb muscles (Landmesser, 1978a, b; Hollyday, 1980a, b). Additionally, both the MMC and LMC can be further subdivided according to the positional identity of the muscles they innervate. Motoneurons in the medial subdivision of the MMC (MMC_M) project to axial muscles that lie close to the vertebral column, while motoneurons in the lateral subdivision of the MMC (MMC_L) are restricted to the thoracic levels of the spinal cord and project to muscles of the
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Figure 1.8
ventral body wall (Gutman et al., 1993). Similarly, the LMC is segregated into medial (LMC_M) and lateral (LMC_L) subdivisions that project to ventrally (flexor) and dorsally (extensor) derived limb muscles respectively (Cruce, 1974; Landmesser, 1978a, b; Hollyday, 1980a, b; Bennett et al., 1983).

Signals derived from the paraxial mesoderm are important for controlling the columnar identity of motoneurons located at different rostrocaudal levels along the spinal cord (Ensini et al., 1998). The identity of motoneurons within different motor columns in the spinal cord is characterized by the combinatorial expression of various members of the Lin-11, Isl-1, Mec-3 (LIM) transcription factor family i.e. Isl-1, Isl-2, Lim-1, Lim-3 and Gsh-4 (Figure 1.9; Tsuchida et al., 1994; Sharma et al., 1998). All somatic motoneurons initially express Isl-1 and later, Isl-2 (Ericson et al., 1992; Tsuchida et al., 1994). Subsequent to this, regulated expression of the various LIM family members results in coexpression of Isl-1, Isl-2, Lim-3 and Gsh-4 defining the MMC_M, coexpression of Isl-1 and Isl-2 defining the MMC_L and LMC_M, and coexpression of Lim-1 and Isl-2 defining the LMC_L (Tsuchida et al., 1994; Sharma et al., 1998).

Loss- and gain-of-function studies have provided important confirmation of the importance of LIM family members in mechanisms that determine the columnar identity of motoneuron populations. In animals deficient in Isl-1 expression, motoneurons within the ventral spinal cord fail to fully differentiate and die shortly after they become post-mitotic (Pfaff et al., 1996). Furthermore, misexpression of Isl-1 in the spinal cord does not induce ectopic motoneuron generation, suggesting that Isl-1 is necessary but not sufficient for motoneuron differentiation (Tanabe et al., 1998). Inactivation of the genes that encode for both Lim-3 and Gsh-4 causes aberrant columnar organization of motoneurons within the spinal cord (Sharma et al., 1998). Indeed, ectopic expression of Lim-3 in all motoneurons of the spinal cord is sufficient to convert LMC motoneurons to MMC_M motoneurons (Sharma et al., 2000). Interestingly, Lim-1 deficient animals exhibit normal motoneuron columnar organization in the spinal cord, suggesting that alternative roles exist for LIM family members other that those that mediate the positional identity of motoneuron populations (Kania et al., 2000).

Although the columnar identity of motoneurons in the spinal cord appears to be induced by signals derived from the surrounding mesoderm, signals derived from within the
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Figure 1.9
spinal cord itself may also serve to confer the appropriate identity on developing motoneurons. The LMC, for example, is segregated into LMC\textsubscript{M} and LMC\textsubscript{L} subdivisions. Given that motoneurons belonging to each subdivision of the LMC are derived from neural progenitors that occupy the same rostrocaudal and dorsolateral positions within the developing spinal cord, it likely that signals derived from sources other than the surrounding mesoderm are required to induce distinct subdivisions of the LMC. During LMC development, LMC\textsubscript{L} motoneurons migrate past early-born LMC\textsubscript{M} motoneurons, which are a source of retinoic acid (RA) in the spinal cord. RA induces the further differentiation of LMC\textsubscript{L} motoneurons by mediating the downregulation of Isl-1 and the upregulation of the defining marker for LMC\textsubscript{L} motoneurons, Lim-1 (Sockanathan and Jessell, 1998). Therefore, local signalling interactions between post-mitotic motoneurons may represent an additional mechanism that facilitates the generation of multiple columnar identities among motoneurons within the spinal cord.

1.3.2.3 Regulation of somatic motor pool identity in the spinal cord

Each motoneuron column within the spinal cord is divided into discrete pools of motoneurons that innervate specific muscles at the periphery (Romanes, 1951, 1964; Landmesser, 1978b; McHanwell and Biscoe, 1981; Nicolopoulos-Stournaras and Iles, 1983; McKenna \textit{et al.}, 2000). The mechanisms that underlie the segregation of motoneurons into distinct pools are not well understood. Certain members of the E26 transformation specific (ETS) family of transcription factors are selectively expressed by specific motor pools within the LMC of the spinal cord (McKenna and Raper, 1988; Lin \textit{et al.}, 1998; Arber \textit{et al.}, 2000). Similarly, the thymocyte winged helix (TWH) transcription factor is also expressed by specific motor pools within the LMC of the spinal cord (Dou \textit{et al.}, 1997). Although ETS and winged helix transcription factors are expressed within specific subsets of motoneurons within the spinal cord, their precise role in mechanisms that determine the positional identity of specific motor pools is unknown.

Members belonging to the homeobox (Hox) family of transcription factors may also be involved in mechanisms that determine the positional identity of specific motor pools in the spinal cord. Indeed, mutant animals with genetic disruptions in the genes that
encode either Hoxc-8 or Hoxd-10 exhibit altered topographical arrangements of specific motor pools within the spinal cord (Carpenter et al., 1997; Tiret et al., 1998). Therefore, it is likely that transcription factors belonging to the Hox, LIM, ETS and winged helix families act in concert to specify the correct positional identity of specific populations of motoneurons within the spinal cord.

1.3.2.4 Mechanisms that guide motor axons to their correct destinations in the periphery

Immediately following specification, somatic motoneurons are instructed to innervate specific target muscles in the periphery using axonal guidance cues expressed in the surrounding environment (Lance-Jones and Landmesser, 1980b, a, 1981a, b; Ferguson, 1983; Tosney and Landmesser, 1984, 1985; Ferns and Hollyday, 1993; Matise and Lance-Jones, 1996). The precise guidance of developing motor axons is mediated by a complex array of both attractive/permisive and repulsive/inhibitory cues that act in concert to successfully direct motor axons to their targets (reviewed in Krull and Koblar, 2000; Jacob et al., 2001; Landmesser, 2001).

In addition to their role in mediating the correct positional identity of somatic motoneuron populations in the spinal cord, members of the LIM family of transcription factors also appear to directly influence the guidance of motor axons, possibly through regulating the expression of guidance cue receptors on the surface of developing axons (Lundgren et al., 1995; Thor and Thomas, 1997; Thor et al., 1999). Consistent with this are the abnormalities observed in peripheral motor axon guidance in Lim-1 deficient animals (Kania et al., 2000). In these animals, the LMC develops normally within the spinal cord. However, motor axons derived from the LMC1, that normally express Lim-1 and project to dorsally derived limb muscles, fail to do so in the mutant, and instead aberrantly project to both dorsally and ventrally derived limb muscles (Kania et al., 2000). Similarly, in homozygous mutant animals with disruptions in the genes that encode for various Hox family members, aberrant motor axon projections are also observed in the peripheral nervous system (de la Cruz et al., 1999; Wahba et al., 2001). Thus, as well as mediating positional identity among motoneurons within the spinal cord, the activity of various transcription factor families may also be important for
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regulating certain mechanisms that underlie the correct guidance of motor axons to their targets in the periphery.

The growth of spinal motor axons is initially directed away from the floor plate of the spinal cord out into the periphery. The directed growth of motor axons away from the floor plate may be a result of diffusible cues released by the floor plate that serve to repel motor axons from the midline (Guthrie and Pini, 1995). The exact nature of the molecular cue that serves to repel motor axons from the floor plate is unknown but it could belong to the semaphorin, slit or netrin families of axonal guidance cues. Semaphorin and slit can both repel ventrally projecting spinal motor axons in vitro (Varela-Echavarria et al., 1997; Brose et al., 1999). Furthermore, although a direct repulsive effect of netrin on ventrally projecting spinal motor axons has yet to be demonstrated, netrin is able to exert a repellent activity on trochlear motor axons (Colamarino and Tessier-Lavigne, 1995).

Motor axons exit the spinal cord by extending through the anterior half of the somatic sclerotome. Attractive/permissive and repulsive/inhibitory cues present within the anterior and posterior halves of the somatic sclerotome, respectively, mediate the directed growth of spinal motor axons (Keynes and Stern, 1984). The extracellular matrix molecule, chondroitin sulfate proteoglycan, is expressed within the posterior half of the sclerotome only (Oakley and Tosney, 1991), and consistent with a role in confining motor axons to the anterior sclerotome, can induce growth cone collapse of spinal motor axons (Ring et al., 1996). Similarly, T-cadherin is also expressed within the posterior half of the sclerotome and can inhibit motor axon outgrowth (Fredette et al., 1996). Furthermore, peanut agglutinin (PNA) binding glycoproteins are also expressed only within the posterior half of the sclerotome, and, like chondroitin sulfate proteoglycan and T-cadherin, can also exert a repulsive activity on specific axonal populations (Davies et al., 1990; Oakley and Tosney, 1991).

The Eph receptors and the ephrins, are also important mediators of spinal motor axon guidance through the anterior somatic sclerotome. EphrinB2 is expressed within the posterior half of the somatic sclerotome (Wang and Anderson, 1997; Koblar et al., 2000). Repulsive/inhibitory interactions between ephrinB2 in the posterior sclerotome
and EphB2 bearing spinal axons (Henkemeyer et al., 1994) may serve to confine spinal motor axons to the anterior sclerotome during the time that they are extending from the spinal cord out to the periphery (Wang and Anderson, 1997; Koblar et al., 2000). Similarly, EphA7 is expressed within the posterior half of the somatic sclerotome as well, suggesting that it could also serve to confine spinal motor axons to the anterior sclerotome during development (Araujo and Nieto, 1997).

After extending through the anterior somatic sclerotome, motor axons are directed to their final muscle destinations by a number of axonal guidance cues present in the surrounding environment. Semaphorin3A appears to be an important guidance cue for developing spinal motor axons as it can exert a potent repellent activity on these axons \textit{in vitro} (Varela-Echavarria et al., 1997). Additionally, HGF/SF also appears to be an important attractive/permissive cue for spinal motor axons (Ebens et al., 1996). Indeed, mutant animals with a disruption in the gene that encode for either semaphorin3A or HGF/SF both exhibit perturbations of spinal motor axon trajectory in the periphery (Taniguchi et al., 1997).

Eph receptors and ephrins, may also be important mediators of spinal motor axon guidance following their extension through the anterior somatic sclerotome into the periphery. The EphA7 receptor exhibits a characteristic developmental expression pattern within the limb that suggests it plays a role in mediating limb innervation (Araujo et al., 1998). Indeed, consistent with a role for EphA7 in mediating limb innervation, LMC motoneurons that project to the limb express ephrinA5, a ligand for EphA7 (Araujo et al., 1998). Furthermore, disrupting EphA7 mediated signalling mechanisms causes guidance defects in particular limb-innervating spinal motor axon populations (Araujo et al., 1998).

In order for motor axons to effectively respond to guidance cues, the fasciculation/defasciculation of motor axons needs to be regulated. As described previously, a major regulator of fasciculation/defasciculation among motor axons is PSA on NCAM. The association of PSA with N-CAM decreases the ability of N-CAM to regulate adhesion among adjacent axons (reviewed in Rutishauser and Landmesser,
1996; Rutishauser, 1998). Consequently PSA is required to regulate defasciculation among spinal motor axons that are responding to various axonal guidance cues at specific choice points in their development (Tang et al., 1992; Tang et al., 1994).

Thus, as is the case for the majority of axons within the nervous system, the mechanisms that underlie the guidance of spinal motor axons to their destinations in the periphery are mediated by an array of attractive/permititive and repulsive/inhibitory guidance cues, many of which we know little about, that act in concert to coordinate the correct development of these axons in the peripheral nervous system.

1.4 MAINTAINING NEURAL CONNECTIVITY IN THE MATURE NERVOUS SYSTEM

1.4.1 Neural progenitor cells are continually produced in discrete areas of the mature central nervous system

In the mature central nervous system, there are two main areas where neural progenitor cells are continually generated: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus (reviewed in Temple and Alvarez-Buylla, 1999; Gage, 2000; Alvarez-Buylla et al., 2001; Anderson, 2001; Morshead and van der Kooy, 2001; Temple, 2001). Neural progenitor cells (or neuroblasts) generated in the hippocampal SGZ or the lateral ventricle SVZ migrate to specific areas in the mature central nervous system, where they subsequently differentiate, and integrate into the existing neural network. In the case of neuroblasts generated in the lateral ventricle SVZ (Figure 1.10), these cells migrate in chains along a defined pathway, the rostral migratory stream (RMS), to the olfactory bulbs, where they differentiate into interneurons, and integrate into the existing neural circuit that controls olfaction (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Doetsch et al., 1997). At present, the factors involved in regulating neuroblast migration in the mature central nervous system are poorly understood.
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Figure 1.10
1.4.2 Regulation of neuroblast migration in the mature central nervous system

It appears that the same families of guidance cues that mediate the guidance of developing axons also mediate the guidance of migrating neuroblasts in the mature central nervous system.

1.4.2.1 CAMs

The migration of cells requires the dynamic regulation of their adhesive state, both during the initial detachment of the cells from their tissue of origin and also during the adhesion/de-adhesion processes that occur as the cell moves along a substrate. PSA-NCAM is an important mediator of the mechanisms that regulate the level of adhesion between migrating neuroblasts and their surrounding environment. NCAM deficient animals exhibit a dramatic reduction in the size of their olfactory bulbs and also accumulations of migrating neuroblasts along the RMS (Tomasiewicz et al., 1993; Cremer et al., 1994). The enzymatic removal of PSA from NCAM in normal animals produced similar defects to those observed in the NCAM mutants and further confirmed the importance of PSA-NCAM mediated interactions in the correct migration of neuroblasts along the RMS (Ono et al., 1994; Hu et al., 1996). The precise mechanism by which PSA-NCAM interactions regulate the migration of neuroblasts in the mature RMS is unknown but could involve PSA promoting adhesion, rather than de-adhesion, among migrating neuroblasts (Hu, 2000). Furthermore, given that mature NCAM deficient animals still exhibit a continuous, albeit morphologically disrupted, RMS that retains the ability to transport normal neuroblasts, it appears that PSA-NCAM is required more for mechanisms that maintain a migration-permissive environment, rather than for the active guidance of migrating neuroblasts, in the mature central nervous system (Chazal et al., 2000).

1.4.2.2 Slits

Certain factor(s) secreted from the septum repel neuroblasts migrating within the RMS (Hu and Rutishauser, 1996). These factors are members of the slit family of proteins and exert a potent repellent activity on neuroblasts migrating along the RMS (Wu et al.,
1999). The repellent activity of the slit proteins on migrating cells appears to be dependent upon a leucine-rich region located at the N-terminal of the slit molecule itself (Chen et al., 2001) and also upon heparan sulfate located on the cell-surface, which enhances interactions between slit and its receptor, robo (Hu, 2001). The in vivo importance of slit proteins in mediating neuroblast migration in the mature central nervous system has yet to be confirmed in slit deficient animals.

1.4.2.3 Eph receptors and the ephrins

The Eph receptors and ephrins are important regulators of cell migration events in the nervous system. The Eph receptors, EphB1, EphB2, EphB3 and EphA4, as well as their ligands, ephrinB2 and ephrinB3, are all expressed in areas associated with migrating neuroblasts within the lateral ventricle SVZ and RMS of the mature central nervous system (Conover et al., 2000). When Eph/ephrin signalling is inhibited in these areas, the chain migration of neuroblasts is severely disrupted (Conover et al., 2000). Additionally, inhibiting Eph/ephrin signalling also increases the rate of cellular proliferation within the lateral ventricle SVZ (Conover et al., 2000). Although the precise mechanisms by which Eph/ephrin interactions regulate neuroblast migration and proliferation are unknown, it certainly appears that Eph/ephrin mediated events will play an important role in the mechanisms that underlie the maintenance of neural connectivity in the mature central nervous system.

1.5 SUMMARY

The establishment and maintenance of anatomically and functionally correct neural circuits in the nervous system is controlled by an array of activity-independent and dependent mechanisms, all of which act in concert, to guide migrating neurons and their extending axons to the correct destination. Many of the mechanisms that mediate guidance events in the nervous system have been identified and functionally characterized, yet, given the many gaps that still remain in our understanding of the mechanisms underlying the establishment and maintenance of neural connectivity, it seems quite obvious that many more remain to be discovered in the future. It is hoped
that a comprehensive understanding of the mechanisms that underlie the establishment
and maintenance of neural connections will eventually lead to therapeutic strategies
directed at repairing connections within the nervous system that are damaged or lost as
a result of neural trauma or disease.

1.6 SPECIFIC ROLES FOR EphA4 IN THE
DEVELOPMENT AND MAINTENANCE OF NEURAL
CONNECTIVITY

The EphA4 receptor is important for the development of certain cortical and spinal
motor circuits and may also be important for the maintenance of particular connections
within the mature nervous system. EphA4 mediated interactions are very important
mediators of the guidance of CST collaterals extending into the grey matter of the spinal
cord. As noted earlier in this chapter (section 1.3.1.1), CST collaterals are normally
unilaterally confined as they project out of the dorsal funiculus into the adjacent grey
matter of the spinal cord. In EphA4 deficient animals however, axons of the CST that
project into the grey matter of the spinal cord aberrantly re-cross the spinal midline to
terminate ipsilateral to their cells of origin (Dottori et al., 1998; Kullander et al.,
2001b). This appears to cause severe behavioural abnormalities in these animals in that
they exhibit abnormal bilateral limb movements during locomotion (i.e. hopping gait).
Although EphA4 is critical for the correct development of the CST, the exact
mechanism(s) that underlie the control of CST development by the EphA4 receptor
remain unclear.

EphA4 mediated interactions are also important regulators of spinal motor axon
guidance in the peripheral nervous system. EphA4 is expressed on a specific
subpopulation of LMC_{L} motoneurons and their axons as they project to the dorsal
compartment of the developing limb (Ohta et al., 1996; Eberhart et al., 2000;
Helmbacher et al., 2000). Consistent with a role in confining EphA4-bearing motor
axons to the dorsal limb compartment, ligands for EphA4 i.e. ephrinA2 and ephrinA5
are concomitantly expressed within the ventral compartment of the developing limb
(Ohta et al., 1997; Eberhart et al., 2000). Indeed, the dorsoventral trajectory of spinal motor axons projecting to the limb is severely disturbed in EphA4 deficient animals (Helmbacher et al., 2000). In these animals, LMC_L motor axons that normally project to the dorsal limb compartment aberrantly alter their trajectory to project to the ventral limb compartment (Helmbacher et al., 2000). The aberrant limb innervation pattern observed in EphA4 deficient animals could be a direct result of the loss of repulsive Eph/ephrin mediated interactions that serve to confine LMC_L motor axons to the dorsal limb compartment (Ohta et al., 1997). Although EphA4 expression on spinal motor axons appears to be important for the correct guidance of these spinal motor axons to their destinations in the periphery, the function of EphA4 expression on specific motoneurons within the LMC_L of the spinal cord is unknown.

EphA4 mediated interactions may also be important for the directed migration of neuroblasts within the mature nervous system. As noted earlier in this chapter (section 1.4.2.3) EphA4 and two EphA4 ligands i.e. ephrinB2 and ephrinB3 are expressed within areas associated with migrating neuroblasts in the lateral ventricle SVZ and RMS of the mature central nervous system. (Conover et al., 2000). Although inhibiting Eph/ephrin interactions within the lateral ventricle SVZ severely disturbs neuroblast migration in this area (Conover et al., 2000), possibly as a result of disrupting EphA4 mediated signalling, the precise function of EphA4 in regulating the directed migration of neuroblast populations in the mature central nervous system is currently unknown.

1.7 THESIS AIMS

The investigations described in this thesis were undertaken with multiple aims directed at characterizing the functional role of the EphA4 receptor in the development of certain cortical and spinal motor circuits and in the maintenance of neural connections within the mature central nervous system. More specifically, the studies were done so with the express purposes of:

a) Demonstrating areas of the nervous system that express high levels of the EphA4 receptor so that those regions where EphA4 may be critical for correct development and function may be identified and examined.
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b) Characterizing the function of EphA4 in regulating the development of corticospinal tract terminations within the spinal cord.

c) Characterizing the function of EphA4 in regulating the position of somatic motoneuron pools in the spinal cord and also in guiding the motor projections emerging from these motoneuron pools to their destinations in the periphery.

d) Demonstrating the existence of EphA4 in neural progenitor populations derived from the mature brain and examining the role of EphA4 in mediating the *in vivo* proliferation and migration of neural progenitor populations in the mature central nervous system.
CHAPTER 2: IMMUNOLOCALIZATION OF EphA4
IN THE NERVOUS SYSTEM
Chapter 2: Immunolocalization of EphA4 in the nervous system

2.1 INTRODUCTION

Many Eph receptors exhibit widespread domains of expression throughout the developing and mature nervous system. The EphA4 receptor, in particular, is expressed throughout many areas of both the peripheral and central nervous system during development and following maturation. The expression domains of EphA4 are consistent with its role as a major regulator of the mechanisms that establish and maintain patterns of connectivity in the nervous system.

Much of what is known about the spatial distribution of EphA4 throughout the nervous system has come from \textit{in situ} hybridization analyses. \textit{In situ} hybridization analysis has revealed that in the developing and mature central nervous system, EphA4 is expressed within the cerebral cortex, striatum, thalamus and hippocampus of the fore and midbrain (Mori \textit{et al.}, 1995). It has also shown that in the hindbrain, EphA4 expression is initially confined to rhombomeres 3 and 5 very early in development (Gilardi-Hebenstreit \textit{et al.}, 1992; Nieto \textit{et al.}, 1992), while later in development, it has shown that EphA4 is also expressed outside rhombomeres 3 and 5, in specific motoneurons belonging to the trigeminal and facial motor nuclei (Kury \textit{et al.}, 2000). \textit{In situ} hybridization analysis has revealed that EphA4 is prominently expressed within the developing cerebellum (Mori \textit{et al.}, 1995). Furthermore, it has also revealed that EphA4 is expressed throughout the developing retina and in the SC of the midbrain (Connor \textit{et al.}, 1998). In the developing spinal cord, \textit{in situ} hybridization analysis has shown that EphA4 expression is largely restricted to motoneurons that comprise the LMCs (Nieto \textit{et al.}, 1992; Mori \textit{et al.}, 1995).

Some of the expression patterns that have been described for EphA4 by \textit{in situ} hybridization analysis have also been confirmed by immunohistochemical analysis. EphA4 expression has been immunohistochemically detected in the developing retina and cerebellum (Hornberger \textit{et al.}, 1999; Karam \textit{et al.}, 2000) and also in the cerebral cortex, striatum, thalamus and hippocampus of the mature fore and midbrain (Martone \textit{et al.}, 1997). Furthermore, immunohistochemical analysis has confirmed the presence of EphA4 on some motoneurons within the LMC of the developing spinal cord (Ohta \textit{et al.}...
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al., 1996; Iwamasa et al., 1999; Eberhart et al., 2000), and has also shown that EphA4 is expressed on the axons of these motoneurons as they project from the cervical and lumbar enlargements of the spinal cord to muscles present within the dorsal compartment of the developing limb (Soans et al., 1994; Ohta et al., 1996; Eberhart et al., 2000; Helmbacher et al., 2000). In the periphery of mature animals, immunohistochemical analysis has also revealed that EphA4 is localized at the junctions that exist between nerves and muscle i.e. the neuromuscular junctions (Lai et al., 2001).

As described above, many of the investigations that have documented EphA4 expression in the developing and mature nervous system have utilized in situ hybridization to detect the presence of EphA4-coding mRNA transcripts. In circumstances where EphA4 expression is examined in relation to axonal tract development, the site of protein expression at the axonal growth cone is often some distance away from the site of mRNA expression, which is usually located in the cytoplasm of the neuronal soma. In situ hybridization analysis of EphA4 expression does not, therefore, directly reveal the locality of functional EphA4 receptors on axons in the nervous system. Also, examining EphA4 expression by in situ hybridization may not necessarily provide a good indication of the levels of protein present as EphA4 proteins may be subjected to regulated cleavage, degradation or recycling events at the cell surface. Consequently, the most effective way to directly examine the expression of EphA4 in the nervous system is to immunohistochemically characterize its distribution.

Detailed immunohistochemical analysis of the spatial distribution of EphA4 is best carried out in sections of fixed, paraffin-embedded tissue because of their superior morphology. However, the examination of EphA4 distribution by immunohistochemical methods is hampered at the moment by the lack of effective anti-EphA4 antibodies that can detect the presence of EphA4 in fixed, paraffin-embedded tissue sections. Moreover, given the importance of EphA4 as a regulator of axon guidance, aside from its expression on particular limb innervating axons of the peripheral nervous system, little is known of what other axons in the nervous system express EphA4. In the investigation presented in this chapter, the generation of polyclonal antisera able to immunohistochemically detect the EphA4 receptor (especially on axons) in fixed, paraffin-embedded tissue is described. Using these antisera, neurons and axons
comprising the developing and mature mouse nervous system, which express high levels of EphA4 protein, are identified. Given their high levels of EphA4 protein expression, these neurons and axons may require EphA4 mediated mechanisms to develop and function correctly.
2.2 MATERIALS AND METHODS

2.2.1 Sequence alignment

Amino acid sequences corresponding to all known members of the Eph receptor family were obtained from the National Centre for Biotechnology Information (NCBI) GenBank database. Multiple sequence alignments were performed by MegAlign sequence analysis software (DNASTAR Inc., WI, USA) using the CLUSTAL alignment algorithm.

2.2.2 Production of anti-EphA4 antisera

Synthetic peptide antigens (Research Genetics Inc., AL, USA) were generated to correspond to amino acids 130-145 (DNDKERFIRESQFGKT) of the extracellular ligand-binding domain and also to amino acids 938-953 (TTLEAVHMSQDDLAR) of the intracellular SAM domain of the EphA4 receptor. Anti-EphA4 polyclonal antisera were prepared by immunizing rabbits with synthetic peptide antigens conjugated to keyhole limpet hemocyanin (Sigma, MO, USA) using standard immunological protocols (Cooper and Paterson, 2000).

2.2.3 In vitro transfection of EphA4

A complete mouse EphA4 cDNA construct cloned into the pCI-neo mammalian expression vector (Promega, Madison, WI, USA) was kindly provided by D. G. Wilkinson of the National Institute of Medical Research, London, UK. Transient transfections were performed on CV-1 SV-40 (COS) cells cultured in Dulbecco's modified Eagle's medium (DME; Gibco, NY, USA) supplemented with 10% (v/v) foetal calf serum (FCS), 0.0012% (w/v) penicillin and 0.0020% (w/v) streptomycin. Cells were transfected in 35 mm tissue culture plates at 60-80% confluency with 2 µg of DNA using the FuGene 6 transfection reagent (Roche, Basel, Switzerland) according to
manufacturer's instructions. Following transfection, cells were maintained for 48 hours at 37°C in a humidified incubator with 5% CO₂ prior to collection of expressed protein.

2.2.4 Immunoblot and immunoprecipitation analysis of protein extracts

For immunoblot analysis, adherent cells were harvested from the bottom of tissue culture wells by incubation with detaching solution \{10 mM EDTA; 150 mM NaCl; 0.01 M KH₂PO₄; 0.02% (v/v) KCl\} for 15 minutes at 4°C. Cell suspensions were then centrifuged for 5 minutes at 10,000 rpm at 4°C and the subsequent cell extracts were resuspended in 50 µl of 2 × SDS-PAGE loading buffer \{100 mM Tris-HCl (pH 6.8); 4% (v/v) SDS; 0.02% (w/v) bromophenol blue; 20% (v/v) glycerol\} with 100 mM DTT freshly added prior to being heated for 15 minutes at 95°C. Cell extracts were then cooled to room temperature before 150 mM of iodoacetamide (Sigma, MO, USA) was added. Cell extracts were then protected from the light and incubated for 30 minutes at room temperature prior to being centrifuged for 10 minutes at 13,000 rpm and separated on pre-cast 8-16% SDS-PAGE gradient gels (Gradipore, NSW, Australia).

For immunoprecipitation analysis, adherent cells were lysed in radioimmunoprecipitation buffer \{50 mM Tris-base (pH 8.0); 150 mM NaCl; 1% (w/v) NP-40; 0.5% (w/v) sodium deoxycholate; 0.1% (v/v) SDS\} containing protease inhibitor cocktail (Roche, Basel, Switzerland) for 30 minutes at 4°C. Cell extracts were then centrifuged for 10 minutes at 10,000 rpm at 4°C to allow supernatant recovery. Anti-EphA4 antisera/Protein A sepharose complexes were generated by incubation of antisera (1:300) with 10% slurry of Protein A sepharose (Pharmacia Biotech, Uppsala, Sweden) suspended in Tris buffered saline and Tween-20 \{TBS-TW - 150 mM NaCl; 10 mM Tris-HCl (pH 7.5); 0.1% (v/v) Tween-20\} for 3 hours at 4°C on a rotating wheel. The anti-EphA4 antisera/Protein A sepharose complexes were then washed 3 times with TBS-TW prior to the addition of cell extracts and then incubated overnight at 4°C on a rotating wheel. Following incubation, anti-EphA4 antisera/Protein A sepharose/antigen complexes were washed 3 times with TBS-TW prior to being resuspended in SDS-PAGE loading buffer and processed for SDS-PAGE separation as described previously.
Proteins were transferred from SDS-PAGE gels to Immobilon-P nylon membranes (Millipore Corporation, MA, USA) by wet transfer in Towbin's transfer buffer (39 mM glycine; 48 mM Tris-base; 0.037% (v/v) SDS; 20% (v/v) methanol) overnight at 4°C.

Transferred EphA4 protein was detected by incubating membranes with anti-EphA4 antisera (1:20, 000) diluted in 1% (w/v) bovine serum albumin (BSA)/0.02% (v/v) Tween-20/phosphate buffered saline (PBS) for 1 hour. The membranes were then washed 3 times in PBS, and incubated for 30 minutes with a secondary goat-anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:30, 000; Bio-Rad Laboratories, CA, USA) also diluted in 1% (w/v) BSA/0.02% (v/v) Tween-20/PBS. Following a final wash in PBS, immunoreactivity on the membrane was detected using the enhanced chemiluminescence detection system according to manufacturer's instructions (Pierce, IL, USA) and visualized on chemiluminescence film (Amersham Pharmacia Biotech, NJ, USA).

2.2.5 Immunohistochemical analysis of cells cultured in vitro

Cells transiently transfected with the EphA4 expression construct were cultured on top of 13 mm coverslips and fixed with 4% paraformaldehyde in PBS for 15 minutes. Cells were then washed with PBS and permeabilized in methanol for 10 minutes prior to being washed a further 3 times with PBS. Following this, cells were incubated in 1% (w/v) BSA/0.02% (v/v) Tween-20/PBS blocking solution for 30 minutes to block non-specific antisera interactions. Subsequent to blocking, cells were then incubated in anti-EphA4 antisera (1:200) diluted in the blocking solution for 30 minutes, washed 3 times with PBS and incubated for a further 30 minutes with a secondary Alexa Fluor 488 goat-anti-rabbit IgG conjugate antibody (1:700; Molecular Probes, OR, USA) diluted in blocking solution to detect specifically bound anti-EphA4 antiserum. All cell nuclei were routinely visualized at this stage by the addition of DAPI nuclear stain (1:1000; Molecular Probes, OR, USA) to the secondary antibody solution. Cells were then washed 2 times with PBS and once with water before coverslips bearing the adherent cells were placed onto microscope slides using fluorescent mounting medium (DAKO, CA, USA) and examined. The specificity of anti-EphA4 antiserum was demonstrated by
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the ability of synthetic peptide antigens (100 µg/ml added to the anti-EphA4 antiserum 30 minutes prior to primary incubation) to abolish anti-EphA4 antiserum immunoreactivity.

2.2.6 Immunohistochemical analysis of mouse embryonic and mature nervous tissue sections

The mice used in the investigation reported in this chapter were derived from a homogenous C57BL/6 genetic background. Female animals were mated overnight and pregnancy was confirmed by the presence of a vaginal plug. The day of insemination was defined as embryonic day 0 (E0). Animals were defined as mature after attaining 6 weeks of age. Timed-pregnant females were killed by cervical dislocation prior to embryos being removed by caesarean section, anaesthetized by hypothermia, and immersion fixed for 2-4 hours in 4% paraformaldehyde in PBS. Following fixation, embryos were then processed for paraffin embedding (Troyer, 1980), serially cut into 5 µm coronal/transverse sections, and mounted on microscope slides coated with 2% (v/v) 3-aminopropyltriethoxysilane (AES; Sigma, MO, USA). Mature animals were deeply anaesthetized with a solution (10 ml/kg administered into the intraperitoneal space) of ketamine (10 mg/ml; Delvet, NSW, Australia) and xylazine (1.3 mg/ml; Bayer, NSW, Australia) before being transcardially perfused with saline followed by 4% paraformaldehyde in PBS. The brain and spinal cord of perfused animals was then dissected free and processed for paraffin embedding as described for embryonic tissue.

Prior to commencing immunohistochemical staining, sections were first de-paraffinized and re-hydrated. Sections were then incubated for 20 minutes at room temperature in a 2% normal goat serum/2% FCS/0.02% Tween-20/PBS solution to block non-specific antisera interactions. Sections were then washed in PBS and incubated for 2 hours at room temperature in primary anti-EphA4 antisera (64EC or 65EC) diluted in blocking solution (1:200). Following primary incubation, sections were washed in PBS and specifically bound anti-EphA4 antiserum was detected by incubating sections for 30 minutes in a secondary horseradish peroxidase conjugated goat-anti-rabbit IgG antibody diluted in blocking solution (1:250; Zymed, CA, USA) which was visualized using a
diaminobenzidine (DAB; DAKO, CA, USA) reaction product. Subsequent to visualization, sections were dehydrated, counterstained with haematoxylin, and coverslipped using DPX (BDH Laboratory Supplies, Poole, UK). The specificity of anti-EphA4 antiserum was demonstrated by the ability of synthetic peptide antigens (100 µg/ml added to the anti-EphA4 antiserum 30 minutes prior to primary incubation) to abolish anti-EphA4 antiserum immunoreactivity.

2.2.7 Image presentation

Unless otherwise stated, images presented in this chapter and throughout the remainder of this thesis were photodocumented using an AxioCam digital camera (Carl Zeiss Vision GmBH, Germany) attached to a conventional compound microscope, processed using Photoshop 5.5 (Adobe Systems Incorporated, CA, USA) to equalize tone and contrast and assembled for documentation with Freehand 9.0 (Macromedia Incorporated, CA, USA).
2.3 RESULTS

2.3.1 The generation of polyclonal antisera directed against mouse EphA4

To immunohistochemically examine the expression of EphA4 throughout the developing and mature nervous system of the mouse, polyclonal antisera directed against synthetic peptide antigens corresponding to specific extra- and intracellular regions of the mouse EphA4 receptor were generated. In order to minimize the effects of cross-reactivity with other Eph receptors, synthetic peptide antigens corresponding to regions within the EphA4 receptor with low sequence homology to other Eph receptors were synthesized. These regions were within the extracellular ligand binding domain (Figure 2.1A) and the intracellular SAM domain (Figure 2.1B) of the EphA4 receptor. Antisera were generated in 2 rabbits for each of the EphA4 synthetic peptide antigens. The antisera directed against the extracellular EphA4 peptide antigen were named 64EC and 65EC and the antisera directed against the intracellular EphA4 peptide antigen were named 87IC and 88IC.

2.3.2 Characterization of anti-EphA4 polyclonal antisera

The ability of the anti-EphA4 polyclonal antisera to specifically detect EphA4 protein was first assessed by immunoblot analysis. Protein extracts derived from COS cells transiently expressing full-length EphA4 were separated on an SDS-PAGE gel and transferred to nylon membranes. EphA4 was detected using each of the anti-EphA4 polyclonal antisera that had been generated. In transfected cells (+), the 64EC and 65EC antisera both recognized a band of approximately 120 kDa that corresponded to the expected weight of the EphA4 receptor (Figure 2.2A). Accordingly, 64EC and 65EC did not detect any band of 120 kDa in untransfected (-) cells (Figure 2.2A). The 75 kDa protein bands detected by 64EC and 65EC in transfected cells most likely represent cleaved or degraded EphA4 protein products. Similar to 64EC and 65EC, the 87IC and 88IC antisera also detected EphA4 protein in transfected (+) cells, but not in
Figure 2.1
untransfected (-) cells (Figure 2.2A) It appeared however, that the affinity of 87IC and 88IC for the EphA4 protein was significantly lower than that of the 64EC and 65EC antisera.

The ability for the anti-EphA4 polyclonal antisera to immunoprecipitate EphA4 protein was also assessed. Each of the anti-EphA4 polyclonal antisera were used to immunoprecipitate EphA4 from protein extracts derived from COS cells expressing full-length EphA4. The 64EC and 65EC antisera both immunoprecipitated EphA4 protein (Figure 2.2B) of the expected molecular weight (i.e. 120 kDa). However, as expected from the previous immunoblot analysis, the 87IC and 88IC antisera did not immunoprecipitate EphA4 protein well (Figure 2.2B). The large bands detected at approximately 50 kDa in all the lanes shown in Figure 2.2B represent non-specific heavy chain Ig protein components.

Each anti-EphA4 polyclonal antiserum was also examined for its ability to detect endogenous EphA4 immunohistochemically. COS cells were transiently transfected to express the full-length EphA4 receptor. The 64EC and 65EC antisera both detected significant levels of EphA4 protein in transfected (Figure 2.3A,D) but not in untransfected (Figure 2.3B,E) cells. Furthermore, the specificity of the immunohistochemical reactions observed in Figure 2.3A,D was confirmed by the loss of specific staining patterns in cells that were incubated with anti-EphA4 antisera that had been pre-absorbed with synthetic peptide antigen prior to being used in the immunohistochemical reaction (Figure 2.3C,F). The 87IC and 88IC antibodies did not produce any signal above background intensity (Figure 2.3G-L). In Figure 2.3, the total cellular distribution present within each image is revealed by a DAPI stain shown as an inset. Given the success of immunohistochemically detecting EphA4 with the 64EC and 65EC polyclonal antisera, these antisera were used for all subsequent immunohistochemical reactions shown in this chapter.

2.3.3 EphA4 is expressed within specific areas of the developing and mature mouse nervous system

Immunohistochemical detection of EphA4 in paraffin-embedded tissue sections of the developing and mature mouse nervous system revealed a number of areas where EphA4...
Chapter 2: Immunolocalization of EphA4 in the nervous system

Figure 2.2
Chapter 2: Immunolocalization of EphA4 in the nervous system

Figure 2.3
Chapter 2: Immunolocalization of EphA4 in the nervous system

is specifically expressed at relatively high levels. In the developing nervous system, EphA4 was most notably expressed on motoneurons within the spinal cord (Figure 2.4A,B). At E14, the motoneurons that comprise the LMC within the grey matter of the spinal cord expressed high levels of EphA4 at both the cervical (Figure 2.4A) and lumbar (Figure 2.4B) levels. The specificity of the EphA4 immunohistochemical reactions shown in Figure 2.4A,B was confirmed by the loss of specific staining patterns in adjacent sections, which have been incubated with anti-EphA4 antiserum that has pre-absorbed with synthetic peptide antigen (Figure 2.4C,D).

In the mature nervous system, EphA4 expression was confined to a number of white matter tracts in varying locations throughout the brain and spinal cord. In the forebrain, EphA4 was expressed on axons within the lateral regions of the external capsule and also in the striatum (Figure 2.5A). EphA4 was also expressed within the cingulum overlying the corpus callosum (Figure 2.5B). Significant numbers of EphA4 immunopositive axons were also found in the internal capsule (Figure 2.5C). The approximate rostrocaudal position of the sections shown in Figure 2.5A-D is indicated by the dashed lines on the schematic brain shown in Figure 2.5E.

In the mature midbrain, considerable numbers of EphA4 immunopositive axons were observed within the medial lemniscus and cerebral peduncle (Figure 2.6A). Similarly, the axons that comprise the posterior commissure were also EphA4 immunopositive (Figure 2.6B). Large numbers of EphA4 immunopositive axons were observed within the lateral lemniscus, medial longitudinal fasciculus and the superior cerebellar peduncle decussation (Figure 2.6C). Furthermore, the axons that comprise the transverse fibres of the pons were also EphA4 immunopositive (Figure 2.6D). There was widespread expression of EphA4 throughout the ventral regions of the caudal midbrain and particularly on those axons within the middle cerebellar peduncle (Figure 2.6E).

In the mature medulla, there was also widespread expression of EphA4 (Figure 2.7A). The axons that comprise the pyramidal decussation were EphA4 immunopositive as was the paramedian reticular nucleus (Figure 2.7B). Additionally, superficial regions (arrows) in the dorsolateral (Figure 2.7C) and ventrolateral (Figure 2.7D) medulla corresponding to regions that contain the inferior cerebellar peduncle, rubrospinal tract
Figure 2.4
Figure 2.5
Chapter 2: Immunolocalization of EphA4 in the nervous system

Figure 2.6
Figure 2.7
and ventral spinocerebellar tract were also EphA4 immunopositive.

In the mature spinal cord, EphA4 expression was confined to areas that are comprised of axonal populations. All axons within the dorsal funiculus appeared to be EphA4 immunopositive (Figure 2.8A). Furthermore, some axons within the dorsal horn of the spinal grey matter (arrows) were also EphA4 immunopositive (Figure 2.8A). In the ventral horn of the spinal grey matter, in contrast to that observed in the developing spinal cord, there appeared to be very little EphA4 expression on motoneurons (arrows) within the LMC (Figure 2.8B). EphA4 immunoreactivity was also present on most axons comprising the spinal white matter and was also present on a subpopulation of axons (arrowheads) in the ventral horn of the spinal grey matter (Figure 2.8B).
Chapter 2: Immunolocalization of EphA4 in the nervous system

Figure 2.8
2.4 DISCUSSION

The investigation presented in this chapter has shown that EphA4 receptor protein is expressed at high levels within a number of regions of the developing and mature nervous system. In some cases, the expression pattern of EphA4 protein determined in this study corresponded to that described by *in situ* hybridization analyses in other reports, however, this study also demonstrated additional patterns of EphA4 protein expression (especially on axons) that have not previously been reported.

2.4.1 Characteristic expression pattern of EphA4 receptor protein in the mouse nervous system

The characteristic expression pattern of the EphA4 receptor in the developing and mature mouse nervous system is similar to that documented for the chick and rat (see selected citations in the introduction of this chapter), and is consistent with a role for EphA4 in regulating mechanisms that establish and maintain patterns of neural connectivity. In the mouse developing nervous system, EphA4 protein was most notably expressed on motoneurons within the LMC of the spinal cord. In the mature nervous system of the mouse, EphA4 was widely expressed throughout specific regions in the brain and spinal cord. Many of these regions, such as the internal capsule, cerebral peduncle, pyramidal decussation and the white matter of the spinal cord, correspond to areas that contain axonal tracts, such as the CST, that descend from the cortex to sub-cortical targets in the brain and spinal cord.

2.4.2 Novel areas of EphA4 protein expression in the mature mouse nervous system

The investigation presented in this chapter has not only shown EphA4 protein expression in areas of the nervous system that have previously been described by other studies, it has also revealed some novel areas of EphA4 protein expression that have not
previously been characterized. In the mature mouse nervous system for example, a number of axonal tracts were shown to be EphA4 immunopositive. Significant levels of EphA4 protein were noted on axons that comprise the posterior commissure, superior cerebellar peduncle decussation and the transverse fibres of the pons. All these axonal pathways have not previously been described to express EphA4 receptor protein.

2.4.3 EphA4 protein expression patterns in the mouse correlate with defects observed in EphA4 deficient mice

Most studies directed at characterizing EphA4 expression have been carried out in the chick and rat nervous system. However, many of the roles that EphA4 has in regulating the establishment and maintenance of neural connections in the nervous system have been identified as a result of defects observed in mutant mice with a targeted disruption of the gene that encodes the EphA4 receptor. This, in combination with the fact that variations do exist between Eph receptor expression patterns between different species of animals, have made it necessary, through studies such as that presented in this chapter, to characterize the expression of EphA4 in the normal mouse nervous system. This enables direct comparisons to be made between normal and EphA4 deficient mice and the direct elucidation of roles for EphA4 in regulating specific morphogenic events in the nervous system. Some of the areas noted in this chapter that express high levels of EphA4 protein correspond to areas of the nervous system where defects are observed in EphA4 deficient animals. Therefore, in these areas of the nervous system, it is likely that EphA4 plays a key role in regulating the specific mechanisms that underlie their correct development and maintenance. These roles for EphA4 will be examined in greater detail in the investigations described in subsequent chapters of this thesis.
CHAPTER 3: REGULATION OF CORTICOSPINAL TRACT TERMINATION PATTERNS BY EphA4
3.1 INTRODUCTION

The molecular mechanisms that guide axons within the developing CST from the cortex to their final targets in the spinal cord are yet to be elucidated. As described in chapter 1 of thesis (section 1.3.1), factors such as astroglial cells, neurotrophic factors and NCAMs have all been shown to play a role in the development of the CST. In addition to these factors, EphA4 is also an important regulator of CST development. Animals with a disruption in the gene that encodes EphA4 have been described previously in chapter 1 of this thesis (section 1.6). These animals display abnormal bilateral limb movements (i.e. a hopping gait), a reduced dorsal funiculus of the spinal cord, have a disrupted anterior commissure, and in some cases, exhibit abnormal hindlimb innervation. In addition to these defects, EphA4 deficient animals also exhibit aberrant projections of the CST. In the absence of EphA4, axons of the CST appear to aberrantly cross the spinal midline to terminate within inappropriate areas of the spinal cord grey matter ipsilateral to their cells of origin. This therefore results in a significant reduction in the number of CST axons that descend to the lumbar levels of the spinal cord (Dottori et al., 1998).

The initial characterization of the CST defects observed in EphA4 deficient animals were undertaken in mature animals after CST development was complete. Thus, the exact time(s) at which EphA4 became important for guiding the development of the CST remained unclear. Furthermore, the study that initially characterized the EphA4 deficient animals suggested a non-cell-autonomous role for EphA4 in guiding the development of CST axons (Dottori et al., 1998). It was suggested that, rather than EphA4 being expressed on developing CST axons, it was a ligand for EphA4, ephrinB3, that was expressed on developing CST axons. It was ephrinB3, therefore, that directed CST development by interacting with EphA4 present in the surrounding environment. However, even given this information, the exact function of EphA4 in regulating the correct development of the CST remained unclear. In the investigation presented in this chapter, CST development in EphA4 deficient animals was examined in detail in order to elucidate the exact mechanisms that underlie the EphA4 mediated development and maturation of the CST. This investigation reveals that EphA4 is not critical for the
guidance of the CST from the cortex to the spinal cord during early development, but instead, has highly specific roles in controlling the midline guidance and final reorganization of developing CST axons within the grey matter of the spinal cord.
3.2 MATERIALS AND METHODS

3.2.1 Animals

Animals deficient in EphA4 protein were generated using homologous recombination of a targeted vector in embryonic stem cells and have been fully described elsewhere (Dottori et al., 1998). Wild type (EphA4\textsuperscript{+/+}) and EphA4 deficient animals (EphA4\textsuperscript{-/-}) were bred to generate homozygous litters for each genotype. Mice were initially created on a C57BL/6 × 129/Sv background and have been backcrossed onto a C57BL/6 background for over 10 generations. Female animals were mated overnight and pregnancy was confirmed by the presence of a vaginal plug. The day of insemination was defined as embryonic day 0 (E0). Neonates were born on E19. The first 24 hours after birth was defined as postnatal day 0 (P0). Animals were defined as mature after attaining 6 weeks of age. The number of brains and spinal cords that were analyzed following injection of neuronal tracers is summarized in Table 1 for each developmental age investigated.

Table 1. Number of brains and spinal cords investigated by all neuronal tracer injections used in this investigation.

<table>
<thead>
<tr>
<th></th>
<th>E14</th>
<th>E15</th>
<th>E16</th>
<th>E17</th>
<th>E18</th>
<th>P2</th>
<th>P7</th>
<th>P14</th>
<th>Mature (anterograde)</th>
<th>Mature (retrograde)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EphA4\textsuperscript{+/+}</td>
<td>5</td>
<td>10</td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>8</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>EphA4\textsuperscript{-/-}</td>
<td>11</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

E is the embryonic age in days (day of insemination is E0) and P is the postnatal age in days (day of birth is P0).
3.2.2 DiI axonal labelling

The lipophilic fluorescent neuronal tracer 1, 1’-dioctadecyl-3, 3’, 3’-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, OR, USA) was used to label early developing corticofugal axonal populations in this investigation. DiI can be used to uniformly label axons in aldehyde-fixed tissue via a passive diffusion process through the plasma membrane (Godement et al., 1987; Honig and Hume, 1989) or alternatively, it can also be used to label axonal populations in living tissue where it is actively transported throughout the neuron (O’Leary and Terashima, 1988). DiI solutions used in fixed tissue {10% (w/v) in dimethylformamide} and living tissue {25% (w/v) in dimethylformamide} were injected into the presumptive dorsomedial motor cortex of experimental brains using a glass micropipette attached to a picospritzer (General Valve, NJ, USA). The use of a picospritzer allowed the volume of DiI solution injected into each brain to be kept constant and therefore permitted the valid comparison of labeled axonal populations between different brains. Injections were carried out under an operating microscope with the aid of a stereotaxic frame to ensure the consistency of the injection site. Only those brains that had similar injection sizes and location were used for comparison in this investigation. Following injection with DiI, brains were processed according to the requirements of the particular tissue being investigated.

3.2.3 DiI-labeling in foetal animals

Timed-pregnant females were anaesthetized with a solution (10 ml/kg administered into the intraperitoneal space) of ketamine (10 mg/ml; Delvet, NSW, Australia) and xylazine (1.3 mg/ml; Bayer, NSW, Australia) and sacrificed by cervical dislocation. Embryos were then removed from the mother by cesarean section and transcardially perfused with 4% paraformaldehyde in PBS. The brain was then dissected from each embryo and post-fixed in the same fixative solution overnight. Following post-fixation, single injections of a 10% DiI solution were made into the presumptive motor cortex of each experimental brain before it was returned to fixative. To allow for adequate diffusion of
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

the tracer, brains were stored in the dark at 37°C for varying lengths of time (4-12 weeks) depending on the age of tissue being investigated.

3.2.4 DiI-labeling in postnatal animals

Postnatal animals were anaesthetized by hypothermia prior to the operative procedure. An incision was made in the scalp and a craniotomy was performed in the area overlying the presumptive motor cortex. A single injection of 25% DiI was made into this region and the incision was closed using Vetbond tissue adhesive (3M animal care products, MN, USA). Animals were then allowed to recover from anaesthesia on a heating pad. Postoperative survival periods ranged from 24 hours to 3 days and animals received saline injections (administered subcutaneously) during this time to prevent dehydration. Following the survival period, the animals were anaesthetized with ketamine/xylazine and transcardially perfused with 4% paraformaldehyde in PBS. The brain and spinal cord was then dissected from each embryo and post-fixed in the same fixative solution overnight.

3.2.5 Histological processing and data analysis of DiI-labeled tissue

Immediately prior to sectioning, the dorsal surface of each brain was photographed to provide a record of the size and location of each DiI injection site. Brains and spinal cords were then embedded in 4% agarose and serial coronal/transverse sections of 100 μm were cut on a vibratome. Sections were either mounted immediately onto glass slides and coverslipped with a polyvinyl alcohol/glycerol based mountant (Heimer and Taylor, 1974) or collected in PBS and stored at 4°C. Sections were examined and photodocumented using a conventional epifluorescent microscope with a standard rhodamine filter (Olympus, Tokyo, Japan). For each brain examined, the course of the labeled axon projections was recorded on representative schematic sections taken from standard embryonic mouse brain atlases (Schambra et al., 1992; Franklin and Paxinos, 1997) thus enabling the precise anatomical comparison of labeled tracts at each developmental age investigated. Certain sections observed initially by conventional
epifluorescence were also subsequently examined using a BioRad MRC-1024 laser-scanning confocal microscope coupled to a Zeiss Axioplan microscope. Scanned digital confocal images of serial optical sections were compiled using Confocal Assistant software (BioRad, CA, USA).

3.2.6 BDA axonal labeling in juvenile and mature animals

CST axons were labeled in juvenile and mature animals using biotinylated dextran amine (BDA; Molecular Probes, OR, USA). BDA is an effective anterograde tracer that can be used to label axonal projections and their terminals (Veenman et al., 1992). Animals were anesthetized with a solution (10 ml/kg administered into the intraperitoneal space) of ketamine and xylazine. The head was positioned in a stereotaxic frame and a unilateral craniotomy (3-4 mm in diameter) was performed to expose the motor area of the cerebral cortex. Three (juvenile) or 7 (mature) 0.3 µl injections of a 15% (w/v) aqueous solution of BDA were made into the motor cortex at a depth of 0.5-1.0 mm below the pial surface. Injections were administered through a glass pipette attached to a Hamilton syringe. Following a 7 day survival period, animals were anaesthetized with ketamine/xylazine and transcardially perfused with saline followed by 4% paraformaldehyde in PBS. The brain and spinal cord was then dissected from each animal and post-fixed for 24 hours in 30% sucrose in fixative before being serially sectioned at 50 µm on a freezing microtome in the coronal/transverse plane. BDA visualization of the free-floating sections was then carried out using standard avidin and biotinylated horseradish peroxidase complex (ABC) histochemistry with a cobalt enhanced DAB reaction product. Sections were mounted on gelatinized slides, dried overnight, and coverslipped using DPX (BDH Laboratory Supplies, Poole, UK) prior to analysis.

3.2.7 Retrograde labeling and analysis of corticospinal neurons in mature animals

Corticospinal neurons in the cortex of mature mice were retrogradely labeled with the fluorescent tracer fast blue (EMS-POLYLOY GmBH, Groß-Umstadt, Germany). Mature mice were anaesthetized with a solution (10 ml/kg administered into the
intraperitoneal space) of ketamine and xylazine. Animals were then stabilized on a stereotaxic frame, the skin incised, and a laminectomy was performed to expose the cervical enlargement of the spinal cord between the 5th and 6th cervical segment. Unilateral injections of a 2% (w/v) aqueous solution of fast blue (0.3 µl per injection) were made into the dorsal horn of the spinal cord with a glass micropipette attached to a Hamilton syringe. Following a 7 day survival period, animals were anaesthetized with ketamine/xylazine and transcardially perfused with saline followed by 4% paraformaldehyde in PBS. The brain and spinal cord was then dissected from each animal and post-fixed for 24 hours in 30% sucrose in fixative before being serially sectioned at 50 µm on a freezing microtome in the coronal/transverse plane. The sections were mounted immediately on gelatinized slides, dried overnight and coverslipped with DPX prior to analysis. Injections were considered successful by confirmation of a unilateral injection site in the operated spinal cord. Qualitative and quantitative comparisons of cortical neurons were then made by mapping the locations of labeled cells in every 4th section of a series using a computer-linked digitizing system (MD3 Microscope digitizer and MD-Plot software; Minnesota Datametrics Corporation, MN, USA). Quantitative estimation of cell numbers are expressed in the text as mean value ± SEM and statistical probability (P) was determined with a Student's t-test (paired or unpaired, as appropriate).

3.2.8 Immunohistochemistry

At the time that the investigation presented in this chapter was being conducted the anti-EphA4 antisera described in the previous chapter had not yet been generated. EphA4 immunostaining of tissue sections in this investigation was therefore carried out using an anti-EphA4 rabbit polyclonal antiserum kindly provided by D. G. Wilkinson of the National Institute of Medical Research, London, UK. This antiserum has previously been shown to specifically bind to EphA4 (Irving et al., 1996) and no cross-reactivity was observed when this antibody was used to stain nervous system tissue taken from EphA4−/− animals (data not shown).
Nervous system tissue was initially collected in cold PBS before being immediately embedded in Tissue-Tek OCT medium (Sakura Finetek, CA, USA) and frozen over liquid nitrogen. Cryostat sections of the embedded tissue were cut at 20 µm in the coronal/transverse plane, mounted onto gelatin-coated slides, air dried and fixed in ice-cold acetone for 10 minutes. Following thorough washing in PBS, sections were incubated for 1 hour at room temperature in a 10% normal goat serum/0.1% Triton X-100/PBS solution to block non-specific antiserum interactions. Sections were then washed in PBS and incubated overnight at room temperature in primary antibody (1:500) diluted in blocking solution. To detect the bound EphA4 antisera, sections were incubated for 1 hour at room temperature with a secondary Alexa Fluor 594 goat-anti-rabbit IgG conjugate antibody (1:250; Molecular Probes, OR, USA) diluted in blocking solution. The sections were then washed, coverslipped with a polyvinyl alcohol/glycerol based mountant (Heimer and Taylor, 1974), and photodocumented. In experiments which combined both DiI labeling and EphA4 staining in the same section, DiI was first injected into the motor cortex of postnatal animals as described previously. Following an appropriate post-operative survival period, brains and spinal cords were dissected from operated animals and the tissue was embedded unfixed in OCT medium and sectioned on a cryostat at 50 µm so that DiI labeling could be photodocumented before EphA4 immunostaining was carried out.

3.2.9 *In situ* hybridization

*In situ* hybridization of tissue sections was performed as described previously (Lyons *et al.*, 1995) using ³³P-labeled riboprobes. Tissue was initially fixed overnight by immersion in 10% neutral buffered formalin (pH 7.0) before being embedded in paraffin and serially sectioned at 5 µm in the coronal/transverse plane. EphA4 mRNA expression was detected using an antisense probe synthesized with T7 polymerase from the *HindIII* linearized plasmid Bluescript KS. The Bluescript KS plasmid contained a 1.5 kb *EcoRI* fragment of 3′ untranslated and C-terminal coding sequences of EphA4. EphrinB3 mRNA expression was detected using an antisense probe generated first by cloning a polymerase chain reaction (PCR) amplified fragment of ephrinB3 cDNA (551-953 bp) into the *EcoRI* site of the Bluescript SK plasmid. The antisense probe was
then synthesized with T3 polymerase from the HindIII linearized plasmid. Sense EphA4 and ephrinB3 riboprobes were also generated to control for non-specific hybridization.

3.2.10 Histochemistry

Embryos were fixed by transcardial perfusion of saline followed by 4% paraformaldehyde in PBS. Tissue was then immediately post-fixed in fixative for a further 2-4 hours before being processed for paraffin embedding (Troyer, 1980). Serial coronal/transverse sections (10 µm) were obtained from paraffin-embedded tissue and stained with haematoxylin & eosin (H&E) using standard histological techniques.

3.2.11 Image presentation

Images presented in this chapter were prepared as described in chapter 2 of this thesis (section 2.2.8).
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

3.3 RESULTS

3.3.1 The early embryonic development of corticofugal axons appears normal in the brains of EphA4−/− animals

In brains taken from prenatal EphA4+/+ and EphA4−/− animals, injection of DiI into the presumptive motor cortex at E14-E18 enabled examination of the early development of corticofugal projections as they descended through the internal capsule and cerebral peduncle. At E14, significant numbers of axons were observed to project from the cortex to the internal capsule in the brains of both EphA4+/+ and EphA4−/− animals (Figure 3.1C,D). Labeled corticofugal axons of EphA4−/− brains exited the cortex normally in well fasiculated bundles and were present in a similar location and quantity (Figure 3.1F) to those observed in EphA4+/+ brains (Figure 3.1E). Coronal sections of EphA4+/+ (Figure 3.1A) and EphA4−/− (Figure 3.1B) brains stained with H&E at a level similar to that shown for Figure 3.1C-F reveal the spatial relationship of major neuroanatomical landmarks. The approximate rostrocaudal position of all sections shown in Figure 3.1A-F is indicated by the dashed line on the schematic brain shown in Figure 3.1G. One day later at E15, corticofugal axons descending from the cortex in EphA4+/+ and EphA4−/− brains began to project through the internal capsule (Figure 3.2A,B) and extend towards the cerebral peduncle. In all brains examined at E15, corticofugal axons within EphA4−/− brains (Figure 3.2D) were similar in location and quantity to those observed within EphA4+/+ brains (Figure 3.2C). By E16, significant reinforcement by later arriving axons of the developing corticofugal projection had occurred in both EphA4+/+ (Figure 3.3C) and EphA4−/− brains (Figure 3.3D). In all cases, closer examination of the corticofugal axons within the brains of EphA4−/− animals (Figure 3.3F) revealed that they were present in similar location and quantity to those observed in EphA4+/+ brains (Figure 3.3F). At E17, corticofugal axons in EphA4−/− brains had projected through the cerebral peduncle in all brains examined (Figure 3.4D) and showed no significant developmental abnormality when compared to the corticofugal axons labeled in EphA4+/+ brains of the same age (Figure 3.4C).
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Figure 3.1
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

Figure 3.2
Figure 3.3
Figure 3.4
At E18, similar to corticofugal axons in EphA4+/+ brains (Figure 3.5A), corticofugal axons in EphA4−/− brains have projected normally to the caudal regions of the cerebral peduncle (Figure 3.5B) where they are about to enter the medullary pyramids. Also at E18, cortical axons begin to extend to targets in the contralateral hemisphere, thus forming the corpus callosum. In all EphA4−/− brains examined at E18, cortical axons comprising the developing corpus callosum projected normally to the contralateral hemisphere in well fasiculated bundles and were present in a similar location (Figure 3.6D) to those observed in EphA4+/+ brains (Figure 3.6C).

### 3.3.2 The CST decussates normally in the medulla of EphA4+/− brains

In both EphA4+/+ and EphA4−/− animals, the developing CST reached the caudal medulla shortly after birth where the majority of its axons turned dorsally and crossed the midline in the transverse axis, projecting from the ventral pyramids of the caudal medulla to the dorsal columns, thus forming part of the pyramidal decussation. Transverse sections of the DiI-labeled pyramidal decussation at P2 revealed that there was no discernible difference between labeled axons in the EphA4+/+ (Figure 3.7C) and EphA4−/− (Figure 3.7D) medulla as they decussated and projected to the dorsal columns. Furthermore, examination of the fully developed CST in the mature brain also revealed that there was no significant difference between the BDA-labeled axons that comprised the pyramidal decussation in the medulla of EphA4+/+ and EphA4−/− animals. When compared to the labeled axons of the EphA4+/+ (Figure 3.8A,C), the labeled axons in the mature EphA4−/− medulla appeared to decussate appropriately (Figure 3.8B,D) with considerable numbers of axons projecting into the dorsal column (Figure 3.8D) at the transition from medulla to spinal cord.

### 3.3.3 The CST in the spinal cord of EphA4−/− animals aberrantly recrosses the midline

Following decussation in the caudal medulla, CST axons entered the cervical spinal cord of the EphA4+/+ and EphA4−/− animals and descended longitudinally through the ventral aspect of the dorsal funiculus. Consistent with prior investigations in the mouse (Gianino et al., 1999), CST axons labeled with DiI in both EphA4+/+ and EphA4−/−
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

Figure 3.5
Figure 3.6
Figure 3.7
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

Figure 3.8
animals had projected into the cervical enlargement of the spinal cord by approximately P2. Although the CST had entered the dorsal funiculus it had not yet begun to innervate the adjacent grey matter and no difference was observed between the EphA4+/+ and EphA4−/− animals with respect to the distribution of CST axons within the dorsal funiculus of the cervical spinal cord (data not shown).

The projection of CST axons from the dorsal funiculus into the grey matter of the cervical spinal cord had begun by P7 and was initially directed both ventromedially and dorsolaterally into the intermediate zone of the spinal grey matter. In the spinal cord of EphA4+/+ animals, these DiI-labeled projections were generally unilaterally confined (Figure 3.9A-C). However, in the spinal cord of all EphA4−/− animals examined at this age, large numbers of CST axons were observed to project bilaterally, aberrantly recrossing the spinal midline to project ipsilateral to their cells of origin (Figure 3.9D-F), and terminate in the lateral margins of the contralateral grey matter (arrows).

To confirm the existence of aberrant CST projections recrossing the midline of the spinal cord in EphA4−/− animals and to demonstrate that these projections were maintained after spinal cord maturation was complete, corticospinal neurons within the cortex of mature animals were retrogradely labeled to allow examination of their distribution. Unilateral injections of the fluorescent retrograde tracer fast blue into the dorsal horn of the cervical spinal cord of EphA4+/+ and EphA4−/− animals (Figure 3.10C,F) revealed that, identical to those corticospinal neurons labeled in EphA4+/+ animals (Figure 3.10A), retrogradely labeled corticospinal neurons in EphA4−/− animals were located within layer V of the motor cortex (Figure 3.10D) and appeared morphologically normal (Figure 3.10E) when compared with corticospinal neurons in EphA4+/+ animals. However, when the distribution of labeled corticospinal neurons was mapped across both cortical hemispheres, in contrast to the unilateral labeling pattern observed in the cortex of EphA4+/+ brains, where no neurons were ever observed in the cortex ipsilateral to the injection site, EphA4−/− animals exhibited labeling of corticospinal neurons in both cortical hemispheres (Figure 3.11; corticospinal neurons represented by filled circles). This, therefore, confirmed the existence of aberrant bilateral CST terminations in the spinal cord of EphA4−/− animals.
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

Figure 3.9
Figure 3.11
Quantification of the number of retrogradely labeled corticospinal neurons in the $EphA4^{+/+}$ and $EphA4^{-/-}$ cortex revealed that the number of labeled neurons contralateral to the injection site was not significantly different between the $EphA4^{+/+}$ ($n = 3$) and $EphA4^{-/-}$ ($n = 6$) animals ($P > 0.05$; unpaired Student's $t$-test). Moreover, labeled corticospinal neurons in $EphA4^{-/-}$ animals were not evenly distributed on both sides of the cortex. There were significantly fewer neurons labeled ipsilateral to the injection site ($344 \pm 126$) when compared to the numbers of labeled neurons present in the contralateral hemisphere ($562 \pm 172$; $P < 0.05$; paired Student's $t$-test; $n = 6$). Thus, although many CST axons aberrantly recross the spinal midline in $EphA4^{-/-}$ animals, the majority still terminate contralaterally as in the $EphA4^{+/+}$ animal.

### 3.3.4 Expression domains of EphA4 and a putative *in vivo* ligand, ephrinB3, suggest they are involved in regulating CST termination patterns

Analysis of the Eph/ephrin family of receptors and ligands has demonstrated very distinctive patterns of expression throughout embryogenesis. These patterns undergo spatial and temporal regulation and the analysis of the expression of both EphA4 and ephrinB3 (a putative *in vivo* ligand of EphA4) in this investigation may provide valuable clues as to their functional role in regulating the development of the CST. At E18 (a time just prior to the CST entering the cervical spinal cord) the detection of EphA4 mRNA by *in situ* hybridization analysis within the cortical plate of the presumptive motor cortex of $EphA4^{+/+}$ brains (Figure 3.12A) is consistent with the expression of EphA4 on CST axons. Also at this age, as demonstrated by *in situ* hybridization analysis, ephrinB3 expression was detected not only within the developing motor cortex (Figure 3.12D), but also in the developing spinal cord where it was primarily confined to the midline, extending along the entire dorsoventral axis of the midline grey matter (arrows in Figure 3.12E), as has been described previously (Gale *et al.*, 1996a; Bergemann *et al.*, 1998). In contrast to ephrinB3, no EphA4 is expressed at the midline of the developing $EphA4^{+/+}$ cervical spinal cord (arrows in Figure 3.12B) and expression is primarily confined to the intermediate and ventral zones of the spinal grey matter. These expression patterns are consistent with a role for ephrinB3 in preventing EphA4-bearing CST axons from crossing the midline of the spinal cord during development. Note that the ephrinB3 signal observed throughout the
Figure 3.12
grey matter of the spinal cord (Figure 3.12E) was not significantly above background intensity (Figure 3.12F).

Although EphA4 expression on axons of the CST is consistent with a cell-autonomous role for EphA4 in mediating CST axon repulsion away from the midline of the spinal grey matter, the expression of EphA4 within the intermediate and ventral zones of the spinal grey matter (Figure 3.12B) suggested that EphA4 could also play a non-cell-autonomous role in guiding the development of the CST as well. Immunohistochemical analysis of EphA4 protein expression in the cervical spinal cord of P2 EphA4<sup>+/+</sup> animals revealed that, just prior to the time of the first CST axons entering the grey matter of the spinal cord (Gianino <i>et al.</i>, 1999), high levels of EphA4 protein existed within the intermediate zone of the grey matter (Figure 3.13A). As determined earlier in this investigation (Figure 3.9), the intermediate zone is where many CST axons project to during the initial stages of spinal grey matter innervation. Furthermore, high levels of EphA4 protein, not detected by <i>in situ</i> hybridization, were also present within the dorsal funiculus of the spinal cord. The CST in the section shown in Figure 3.13A was also labeled with DiI to reveal the distribution of CST axons (Figure 3.13B). In all cases examined (n = 3) considerable numbers of corticospinal axons were observed to be localized within the strongly EphA4 immunolabeled dorsal funiculus. Given that CST axons in the spinal cord of EphA4<sup>-/-</sup> animals project normally through the dorsal funiculus (section 3.3.3) it is likely that EphA4 expressed within the dorsal funiculus of the spinal cord is required for other aspects of dorsal funicular development (see section 3.4.2 of the discussion for this chapter).

### 3.3.5 Incorrect reorganization of CST terminations within the spinal cord of EphA4<sup>-/-</sup> animals

Following the initial projection of CST axons into the dorsolateral and ventromedial intermediate zone of the cervical spinal grey matter, the growth and refinement of terminal arborizations over the subsequent days leads to the final mature pattern of CST termination. In the mouse, maturation of CST terminations has essentially occurred by the end of the second postnatal week (Gianino <i>et al.</i>, 1999). By P14, CST axons anterogradely labeled with BDA in the cervical spinal cord of EphA4<sup>+/+</sup> animals
Figure 3.13
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

exhibited a mature termination pattern within the grey matter of the spinal cord. The majority of CST axons terminated within the dorsal horn, contralateral to their cells of origin, with a small number of terminations also present within the intermediate zone (Figure 3.14A,B). In contrast to the termination pattern observed in the spinal cords of EphA4+/+ animals, the majority of CST terminations in the spinal cord of P14 EphA4−/− animals remained ventrally displaced, with most axons terminating within the intermediate zone (Figure 3.14C), including those that have aberrantly recrossed the spinal midline (arrowheads in Figure 3.14D).

As described above, the reorganization of CST terminations that leads to the formation of the mature termination pattern within the grey matter of the spinal cord occurs between P7 and P14. During the period in which CST axons begin to assume their final termination pattern, examination of EphA4 expression in the spinal cord of P7 (Figure 3.15B) and P14 (data not shown) EphA4+/+ animals revealed an expression pattern similar to that observed in the P2 spinal cord (Figure 3.13A). High levels of EphA4 receptor protein continued to be detected throughout the intermediate zone of the spinal grey matter. Additionally, there were areas of weak EphA4 expression persisting within the grey matter directly adjacent to the CST in the dorsal funiculus and also within the dorsal horns of the spinal cord (region between arrows in Figure 3.15B) that corresponded to the eventual zone of CST axon projection and termination in the spinal cord of EphA4+/+ animals (Figure 3.15A; region between arrows). In the absence of any EphA4 expression within the intermediate zone, many CST axons within the spinal cord of EphA4−/− animals failed to remain confined to the dorsal horn and consequently were observed to terminate inappropriately within the intermediate zone of the spinal grey matter (Figure 3.15C). Given that ephrinB3 is expressed within the cortical plate and therefore probably on CST axons (Figure 3.12D), these data are consistent with a role for EphA4 expressed within the intermediate zone in guiding the correct reorganization of mature CST terminations within the dorsal horn of the spinal cord. In this instance, in contrast to the guidance of CST axons at the spinal midline where EphA4 is expressed on the CST axons themselves and functions cell-autonomously, EphA4 appears to mediate the reorganization of maturing CST terminations in a non-cell-autonomous manner.
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Figure 3.14
Figure 3.15
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Similar to those CST projections that aberrantly recross the midline in the spinal cord of EphA4\(^{-/-}\) animals (Figure 3.11), aberrant CST terminations within the intermediate and ventral zones of the spinal cord of EphA4\(^{-/-}\) animals are also maintained throughout maturity. As expected, BDA-labeled CST terminations within the spinal cord of mature EphA4\(^{+/+}\) animals are confined to the dorsal horn of the spinal grey matter contralateral to their cells of origin (Figure 3.16A,B). In contrast, the majority of BDA-labeled CST terminations in the spinal cord of mature EphA4\(^{-/-}\) animals are ventrally displaced, with most axons terminating within the intermediate zone (Figure 3.16C), including those that have aberrantly recrossed the spinal midline (arrowheads in Figure 3.16D). Consequently, aberrant CST terminations within the intermediate and ventral zones of the spinal cord of EphA4\(^{-/-}\) animals, first observed at P14, are maintained well after spinal cord maturation is complete.
Figure 3.16
3.4 DISCUSSION

The development of the corticospinal projection occurs over an extended pre- and postnatal period. For the CST to accurately extend toward and innervate its targets, a number of guidance cues are required, many of which we know little about. The investigation presented in this chapter has revealed that EphA4 plays important roles in not only guiding CST axons at the midline as they initially project through the grey matter of the developing spinal cord, but also in confining reorganizing CST terminations to the dorsal horn of the spinal grey matter later in maturation.

3.4.1 The early embryonic development of the corticofugal tract occurs independently of EphA4 mediated guidance

Evidence from this investigation strongly suggests that the early development of the corticofugal tract is not dependent upon the EphA4 receptor. At all the prenatal ages examined, corticofugal axons emerging from the cortex of EphA4\(^{-/-}\) brains appeared to be present in a similar location and quantity as those observed in the EphA4\(^{+/+}\) brains. Given that this investigation has demonstrated that EphA4 and a potential in vivo ligand, ephrinB3, are both expressed in the cortex during the initial development of subcortical axons (Figure 3.12A,D) it was perhaps surprising that EphA4\(^{-/-}\) animals did not exhibit aberrant guidance of corticofugal axons within the cortex. It has been proposed that much of the initial development of axons within the cortex is guided by a scaffold generated by transient populations of neurons in the subplate (Allendoerfer and Shatz, 1994; McConnell et al., 1994; Metin and Godement, 1996; Richards et al., 1997; Molnar and Cordery, 1999). However, while various Eph receptors and ephrins are expressed in the cortex (Mori et al., 1995; Castellani et al., 1998; Gao et al., 1998; Mackarehtschian et al., 1999; Rogers et al., 1999b), their role in the initial development of the subplate structure and subcortical projections still remains to be determined.
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

3.4.2 EphA4 does not play a major role in guiding the developing CST through the medulla and into dorsal funiculus of the spinal cord

The expression of EphA4 protein on axons that comprise the pyramidal decussation suggested that it may play an active role in CST guidance in this area (Figure 2.7A in chapter 2 of this thesis). However, the apparent normal development of decussating CST axons within the medulla of EphA4−/− animals described in this investigation suggests that EphA4 is not critical for this event to occur. It is likely that other molecular interactions, such as that recently described to occur between L1 and its ligand CD24 (Cohen et al., 1998), represent the major mechanisms that guide CST axons through the pyramidal decussation in the medulla.

A previous report described the existence of aberrant terminations of the CST within the medulla of mature EphA4−/− animals (Dottori et al., 1998). As described above, no developmental abnormalities of the medullary decussation of EphA4−/− animals were observed in this investigation, even when the CST decussation in the mature brain was examined. Phenotypic variations in animals due to altered genetic backgrounds is a well characterized occurrence in animals with a targeted gene disruption (Gerlai, 1996; Holschneider and Shih, 2000) and it may be that extensive backcrossing of EphA4−/− animals initially generated on a mixed C57BL/6 × 129/Sv background onto a homogenous C57BL/6 genetic background may have partially altered the phenotype of these animals described in earlier reports.

In this investigation, significant levels of EphA4 protein were detected within the dorsal funiculus during the arrival of CST axons in the cervical spinal cord of EphA4+/+ animals (Figure 3.13A). Furthermore, in the investigation presented in chapter 2 of this thesis, EphA4 protein was also detected in the dorsal funiculus of the mature spinal cord (Figure 2.8A). EphA4 is clearly not critical for the guidance of CST axons within the dorsal funiculus as the CST was observed to develop normally within the confines of the dorsal funiculus in the spinal cord of EphA4−/− animals. Furthermore, EphA4 expression is not confined solely to CST axons within the dorsal funiculus of the developing and mature spinal cord (Figures 2.8A and 3.13A). EphA4 expression in areas of the dorsal funiculus other than those occupied by CST axons suggest it could be expressed on other populations of axons or, alternatively, on other non-neuronal cells. Indeed, given the multiple roles that the Eph receptors and ephrins play in regulating cell migration and patterning (reviewed in Wilkinson, 2000a, 2001) and the
reduced dorsal funiculus present in the spinal cord of *EphA4*\(^{-/-}\) animals, the demonstration of widespread expression of EphA4 within the dorsal funiculus in this investigation may indicate a vital role for EphA4 in controlling the correct structural development of the dorsal funiculus in the embryonic spinal cord.

### 3.4.3 EphA4 functions cell-autonomously to prevent CST axons from aberrantly recrossing the spinal midline

The midline of the developing spinal cord is an important source of molecular information controlling the midline guidance of a number of axonal projections (reviewed in Tessier-Lavigne and Goodman, 1996; Kaprielian *et al.*, 2001) and consequently could be the location of ligands that prevent CST axons from aberrantly recrossing the spinal midline during development. In the spinal cord of *EphA4*\(^{-/-}\) animals, once CST axons began to project from the dorsal funiculus into the adjacent grey matter, severe midline guidance defects were observed. In the absence of EphA4, many axons were observed to inappropriately recross the midline as if their responsiveness to a particular midline repellent had been eliminated. Consistent with this hypothesis is the expression of ephrinB3, a known ligand for EphA4, at the developing midline of the spinal cord. EphrinB3 can bind to, and activate, the EphA4 receptor (Gale *et al.*, 1996a; Bergemann *et al.*, 1998) and could therefore mediate corticospinal axon guidance by contact repulsive mechanisms since it has previously been shown to induce the *in vitro* collapse of commissural axon growth cones (Imondi *et al.*, 2000). In the investigation presented in this chapter, EphA4 mRNA was detected within the motor cortex of the developing brain (Figure 3.12A). In the investigation presented in chapter 2 of thesis EphA4 protein was localized to axons comprising the pyramidal decussation (Figure 2.7A). These data, in combination with the demonstration of EphA4 expression on the CST of EphA4 “gene-trap” animals (Leighton *et al.*, 2001), strongly suggests that EphA4 is expressed on axons that comprise the CST. The repulsive interaction between EphA4-bearing CST axons and ephrinB3 expressed at the spinal midline appears to be the major mechanism that confines corticospinal projections to one side of the spinal cord during development. Furthermore, the importance of EphA4/ephrinB3 interactions in controlling the guidance of developing CST axons at the midline of the spinal cord was recently confirmed by the generation of ephrinB3 deficient animals which phenocopy the EphA4
Chapter 3: Regulation of corticospinal tract termination patterns by EphA4

deficient animals used in this investigation (Kullander et al., 2001a; Yokoyama et al., 2001).

The role of EphA4 in regulating the correct midline development of other axonal tracts in the nervous system is unclear. However, given that EphA4 is important for guiding the midline development of CST axons (this chapter) and anterior commissure axons (Dottori et al., 1998; Kullander et al., 2001b) it is not unreasonable to assume that it may also be required for the midline guidance of other axonal tracts such as RGC axons in the optic chiasm and commissural axons in the spinal cord. The possibility that EphA4 is involved in guiding the development of these tracts will be investigated in the future.

3.4.4 EphA4 functions non-cell-autonomously to confine CST terminations to the dorsal horn of the spinal grey matter during maturation

The investigation presented in this chapter has shown that, in EphA4+/+ animals, CST innervation of the grey matter is initially directed into areas of the spinal cord that express high levels of EphA4 protein (Figure 3.13A). These CST projections are directed ventromedially as well as dorsolaterally and then reorganize during the second postnatal week to a final termination pattern within the dorsal horn of the spinal grey matter that coincides with areas of low levels of EphA4 protein evident at the time this process is occurring. In the spinal cord of EphA4−/− animals, CST projections fail to reorganize within the dorsal horn and the majority of axons terminate within the intermediate and ventral zones of the spinal cord. The findings of this investigation suggest that, independent of the role of EphA4 in preventing CST axons from recrossing the spinal midline, the specific expression of EphA4 within the intermediate zone of the spinal cord may be important for determining the final termination pattern of CST axons within the dorsal horn of the spinal cord grey matter.

Eph receptors and ephrin ligands are well characterized as key regulators of the termination pattern in projections of the visual (Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996; Frisen et al., 1998; Feldheim et al., 2000), hippocamposeptal (Gao et al., 1996; Zhang et al., 1996), peripheral (Donoghue et al., 1996) and cortical systems (Castellani et al., 1998; Vanderhaeghen et al., 2000). Eph receptors and their ligands have also previously been shown to play a role in the development of hippocampal termination patterns by stimulating axonal arborization
and pruning misdirected axons (Gao et al., 1999). EphA4 may play a similar role during the development of CST termination patterns. Given the EphA4 expression pattern within the intermediate zone of the spinal cord during the time that corticospinal axons are assuming their final termination pattern, EphA4 appears to repel corticospinal axons away from areas were it is expressed at high levels (i.e. intermediate zone) to areas where it is expressed at relatively lower levels (i.e. dorsal horn). Consistent with this mechanism is the detection of mRNA for EphA4 ligands, such as ephrinB3, in the developing (present study) and mature (Gale et al., 1996a) motor cortex that could allow the transduction of repulsive signals to axons of the CST.

3.4.5 A model to explain the functions of EphA4 in guiding CST axons within the grey matter of the spinal cord

How may one explain the observations that CST axons in the developing spinal cord are first repelled from areas expressing ephrinB3 at the midline and subsequently from areas that express EphA4 within the intermediate zone? The co-expression of guidance receptors and their ligands has been previously described for neuropilin and semaphorin3A (Luo et al., 1993; Messersmith et al., 1995; Takagi et al., 1995). More recently, the co-expression of EphA receptors and ephrinA ligands on RGC axons has been shown to be important for the development of correct patterns of termination in the retinotectal projection (Hornberger et al., 1999). Moreover, the dynamic regulation of guidance receptors on the axonal surface has been shown to alter the responsiveness of developing axonal populations to guidance cues present in their environment (Dodd et al., 1988; Kidd et al., 1998a; Zou et al., 2000). One possible mechanism to explain the observations of this study is that initial CST axons projecting into the grey matter of the spinal cord express EphA4 to prevent midline crossing. However, following this initial projection into the spinal grey, the relative amount of ephrinB3 (or some other EphA4 ligand) on CST axons could be upregulated, thus allowing these axons to become responsive to EphA4 expression in the surrounding tissue of the intermediate zone and the subsequent confinement of terminating CST axons to the dorsal horn of the spinal cord (Figure 3.17A). Alternatively, EphA4 and ephrinB3 may be expressed simultaneously on CST axons at the time they project into the spinal grey matter and one or the other may be "silenced" at certain times by mechanisms akin to that recently
shown to occur with the silencing of the DCC receptor by slit (Stein and Tessier-Lavigne, 2001). In the absence of EphA4, CST axons fail to respond to ephrinB3 expression and hence aberrantly recross the spinal midline to terminate ipsilateral to their cells of origin (Figure 3.17B). Furthermore, CST axons also fail to rearrange to the dorsal horn of the spinal grey matter during maturation due to the lack of EphA4 expression within the intermediate zone and therefore remain ventrally displaced within the spinal grey matter (Figure 3.17B).

3.4.6 EphA4 and the control of locomotion

The investigation described in this chapter has provided important insights into the molecular mechanisms that underlie the establishment and maintenance of neural circuits that control locomotor activity. Normal locomotion in most mammals is characterized by the coordinated, reciprocal movement of limbs located on opposing sides of the body (Pearson and Gordon, 2000). Control of these characteristic limb movements is thought to arise, in part, from unilateral connections of the CST that allow one side of the cerebral motor cortex to unilaterally modulate certain aspects of limb movement on the contralateral side of the body. The most parsimonious explanation for the abnormal bilateral limb movements exhibited by EphA4\(^{-/-}\) animals is that aberrant bilateral CST projections present within the spinal cord of these animals mediate synchronous bilateral limb movements. Consequently, by maintaining unilateral CST projection patterns in the spinal cord, EphA4 mediated interactions appear to be critical for the correct development of normal locomotor patterns.

Although the CST may be involved in mediating some aspects of limb movement, given that it makes very few direct corticomotoneuronal connections, the exact neural networks that CST projections interact with to control locomotor activity remain unclear. Neural circuits intrinsic to the spinal cord, known as central pattern generators (CPGs), are directly associated with spinal motoneurons and are important regulators of normal patterns of locomotion (reviewed in Kiehn and Kjaerulff, 1998; Grillner, 1999; Marder, 2000; Pearson, 2000; McCrea, 2001). Little is known about the anatomical structure of mammalian CPGs and the other regions of the nervous system with which
Figure 3.17
they interact (Burke et al., 2001). It is intriguing to postulate that the CST may indirectly regulate aspects of locomotion by synapsing with CPG networks. Thus, the abnormal bilateral limb movements exhibited by EphA4<sup>−/−</sup> animals may be a direct result of abnormal CPG modulation by aberrant bilateral CST projections. Also, given the severe disruptions to multiple axonal tracts within the nervous system of EphA4<sup>−/−</sup> animals, aberrant projections of axons within the spinal CPG networks themselves could also contribute to the severe locomotor abnormality exhibited by these animals.

The elucidation of the exact mechanisms that underlie the abnormal regulation of locomotor patterns by the CST in EphA4<sup>−/−</sup> animals will require further investigation. Transecting the CST within the spinal cord of EphA4<sup>−/−</sup> animals, therefore eliminating supraspinal control of locomotion, and examining the resultant limb locomotor patterns, will at least enable the dissection of the relative contributions that supraspinal (i.e. CST) and intraspinal (i.e. CPG) projections make to the overall control of the abnormal locomotor patterns exhibited by EphA4<sup>−/−</sup> animals.

The functional consequence of a ventrally displaced CST termination pattern within the spinal cord of EphA4<sup>−/−</sup> animals is also unknown. Since the neural network(s) that the rodent CST interacts with remains unknown, it is unclear whether or not ventrally displaced CST tract terminations could still make connections with their appropriate targets in the spinal cord. However, given the dramatic behavioural effects observed in EphA4<sup>−/−</sup> animals that result from aberrant bilateral CST terminations, it would not be unreasonable to assume that a ventrally displaced CST termination pattern might also lead to motor abnormalities as well.

In summary, it is clear that a number of attractive and repulsive guidance cues are required for the accurate projection of the CST to its targets within the spinal cord. The investigation presented in this chapter has shown that differing localization of the EphA4 receptor, either to developing CST axons, or to the surrounding grey matter, may provide some of the guidance signals necessary to facilitate the correct development and termination of CST axons within the grey matter of the spinal cord.
CHAPTER 4: EphA4 REGULATES THE POSITION OF A MOTONEURON POOL WITHIN THE SPINAL CORD
Chapter 4: Regulation of motoneuron pool position by EphA4

4.1 INTRODUCTION

The molecular mechanisms that underlie the development of the topographical arrangement of somatic motoneurons within the spinal cord are not yet well understood. As described in chapter 1 of this thesis (section 1.3.2), the columnar identity of somatic motoneurons is characterized by the combinatorial expression of various members belonging to the LIM transcription factor family. Additionally, other transcription factors belonging to the Hox, ETS and winged helix families, also described in chapter 1 of this thesis (section 1.3.2), are similarly involved in the mechanisms that underlie the further segregation of somatic motoneurons within motor columns into distinct motoneuron pools. However, much of what is known about the factors that control the positioning of somatic motoneurons within the spinal cord is derived from studies on transcription factors that are confined to the intracellular environment. Comparatively less is known about what cell-surface molecules are involved in regulating the position of somatic motoneurons within the spinal cord. Also, it is not known how transcription factors, such as those belonging to the families described above, could regulate the expression of cell-surface molecules that are involved in mediating the correct topographic arrangement of somatic spinal motoneurons during development.

The Eph receptors and ephrins are important regulators of topographic map formation in a number of regions of the nervous system (section 1.2.1.7 of chapter 1 of this thesis). In particular, ephrinA5 has previously been demonstrated to be important for regulating the topographic arrangement of somatic motoneurons within the cervical region of the spinal cord (Feng et al., 2000). In the investigation presented in this chapter, the role of EphA4 in the development of specific topographic connections between motoneurons within the spinal cord and muscles in the peripheral limb has been examined. Animals deficient in EphA4 expression have been previously described in chapter 1 of this thesis (section 1.6). These animals exhibit gross locomotor abnormalities (i.e. a hopping gait) and also incorrect guidance of both CST and hindlimb innervating axons during development. In this study, examination of spinal motoneuron topography in EphA4 deficient animals reveals a novel role for EphA4 in the correct rostrocaudal positioning of a specific motoneuron pool within the spinal cord.
4.2 MATERIALS AND METHODS

4.2.1 Animals

The animals used in the investigation reported in this chapter are described in chapter 3 of this thesis (section 3.2.1).

4.2.2 Retrograde labeling

Motoneurons in the spinal cord of mature animals were retrogradely labeled by either direct application of neuronal tracer to the nerve at the periphery or by intra-muscular injection of neuronal tracer into the target muscle. Prior to the commencement of all operative procedures mice were anaesthetized with a solution (10 ml/kg administered into the intraperitoneal space) of ketamine (10 mg/ml; Delvet, NSW, Australia) and xylazine (1.3 mg/ml; Bayer, NSW, Australia).

For experiments requiring the total pool of motoneurons supplying the sciatic nerve to be labeled, the sciatic nerve was first exposed at mid-thigh level. A small piece of gauze soaked with PBS was then inserted between the nerve and the underlying muscle to prevent tracer uptake by tissue other than the sciatic nerve. The sciatic nerve was transected and tetramethylrhodamine dextran crystals (TMRD; Molecular Probes, OR, USA) were applied directly to the cut end of the proximal stump (Sangster et al., 1999). Following tracer application, the cut ends of the nerve were left unopposed and the incision in the overlying muscle and skin was sutured closed.

For experiments requiring the labeling of motoneuron pools supplying specific muscles, neuronal tracers were applied by intra-muscular injection. Muscles were first exposed by incision of the overlying skin and injections of neuronal tracers were carried out using a 30G hypodermic needle attached to a Hamilton syringe. Attempts were made to infiltrate the entire muscle with neuronal tracer during injection in order to label as
much of the projecting motoneuron pool as possible. This often required multiple
injections and great care was taken to ensure that the volume of neuronal tracer injected
into individual muscles was consistent between each animal so that comparisons
between labeling obtained from identical muscles in different animals were valid. A
detailed summary of intra-muscular injections used to investigate specific motoneuron
pools in this investigation is shown in Table 2. Following injection, sites of intra-
muscular injection were cleaned to remove any tracer that may have leaked from the
muscle and the incision overlying the muscle was sutured closed. Animals that
underwent the operative procedures described above were then allowed to recover from
anaesthesia on a heating pad.

Table 2. Summary of intra-muscular injections used to retrogradely label specific
motoneuron pools in the spinal cord.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Neuronal Tracer</th>
<th>Tracer Concentration (% w/v aqueous solution)</th>
<th>Volume of Injected Tracer (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triceps brachii</td>
<td>Fast blue$^1$</td>
<td>2</td>
<td>3.0</td>
</tr>
<tr>
<td>Biceps brachii</td>
<td>TMRD$^2$</td>
<td>10</td>
<td>3.0</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>Fast blue$^1$</td>
<td>2</td>
<td>5.0</td>
</tr>
<tr>
<td>Tibialis anterior</td>
<td>Diamidino yellow$^1$</td>
<td>2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

$^1$Fast blue and diamidino yellow were obtained from EMS-POLYLOY GmBH, Groß-
Umstadt, Germany.

$^2$TMRD was obtained from Molecular Probes, OR, USA.

Subsequent to operative procedures, animals were maintained for a post-operative
survival period of 7 days. Animals were then anaesthetized with ketamine/xylazine and
transcardially perfused with saline followed by 4% paraformaldehyde in PBS. A
complete laminectomy was carried out to allow the removal of the entire spinal cord
with dorsal and ventral roots attached. Spinal cords were then positioned onto a wax
strip and each spinal segment along the entire rostrocaudal axis was identified by the vertical insertion of a small stainless steel pin into the midline of the spinal cord. An individual spinal segment was defined as the point that lies midway above and below the exit of a pair of ventral roots from the surface of the spinal cord. Following identification of spinal segments, spinal cords were cryoprotected in 30% sucrose in fixative for 8-12 hours. Blocks of spinal cord corresponding to the cervical (C1-C8) and lumbar (L1-L6) enlargement were then removed from each spinal cord and longitudinal serial sections 50 µm thick were cut on a freezing microtome. The sections were mounted immediately on slides coated with 1% (w/v) gelatin (Sigma, MO, USA), dried overnight and coverslipped in DPX (BDH Laboratory Supplies, Poole, UK) prior to analysis. For experiments requiring the analysis of retrogradely labeled axons within spinal ventral roots, 5 mm long segments of ventral roots were dissected from the spinal cord prior to cryoprotection. Each ventral root segment was then mounted whole onto a microscope slide, coverslipped in fluorescent mounting medium (DAKO, CA, USA) and immediately examined.

Motoneuronal axons and somas retrogradely labeled with fluorescent tracer were observed using a conventional epifluorescent microscope. The topography of motoneuron pools in each section examined was documented by mapping the location of labeled motoneurons using a computer-linked digitizing system (MD3 Microscope Digitizer and MD-Plot software; Minnesota Datametrics Corporation, MN, USA). Maps for each section of a spinal cord were then superimposed on each other and compiled to provide a complete topographical record of the longitudinal organization of the motoneuron pools examined in this investigation.

4.2.3 Stereological analysis

The total number of motoneurons in the spinal cord contributing to the sciatic nerve was determined stereologically for each animal investigated. A 60 × oil immersion objective attached to an epifluorescent microscope was used to count motoneurons in every second section that contained labeled cells. Using a digital microcator (Heidenhaim ND221 Measured Value Display Unit, Traunreut, Germany) attached to the microscope
stage and Stage 2.10 computer software (Leading Edge, Adelaide, Australia), the entire labeled sciatic pool of selected sections was systematically sampled. The number of labeled neurons per $\mu$m$^3$ ($N_v$) for the motoneuron pool was then estimated using the optical disector method (Sterio, 1984; Gundersen et al., 1988a; Messina et al., 2000). Subsequent to being sampled, the actual thickness of each section was determined using the microcator to allow the average section thickness for each spinal cord to be calculated. The total volume of tissue examined per spinal cord ($V_{\text{cord}}$) containing labeled motoneurons was then calculated using the Cavalieri principle (Gundersen et al., 1988b). This volume of tissue, multiplied by the number of labeled neurons per $\mu$m$^3$, gave an unbiased estimate of the total number ($N$) of neurons in the sciatic motoneuron pool of the spinal cord i.e. $N = N_v \times V_{\text{cord}}$. Quantitative estimations of labeled motoneuron numbers are expressed in the text as mean value $\pm$ SEM and statistical probability (P) was determined with an unpaired Student’s $t$-test.

4.2.4 Immunohistochemistry

A complete description of the generation of the anti-EphA4 polyclonal antiserum used to detect EphA4 in this investigation (64EC and 65EC) is provided in chapter 2 of this thesis. The preparation and immunohistochemical staining of embryonic tissue in this investigation using this antiserum has also been previously described in chapter 2 of this thesis (section 2.2.7).

4.2.5 Histochemistry

Embryonic and mature tissues were fixed by transcardial perfusion of saline followed by 4% paraformaldehyde in PBS. Tissue was then immediately post-fixed in fixative for a further 2-4 hours before being processed for paraffin embedding (Troyer, 1980). Serial coronal/transverse sections (10 $\mu$m) were obtained from paraffin-embedded tissue and stained with cresyl violet or haematoxylin & eosin (H&E) using standard histological techniques.
4.2.6 Image presentation

Images presented in this chapter were prepared as described in chapter 2 of this thesis (section 2.2.8).
4.3 RESULTS

4.3.1 EphA4 is expressed by subpopulations of motoneurons within the LMC of the developing spinal cord

Immunohistochemical analysis of EphA4 expression in transverse sections of the EphA4+/+ spinal cord at E14 revealed strong immunoreactivity of motoneurons positioned within the LMC. Consistent with previous reports of EphA4 protein expression in the spinal cord (Ohta et al., 1996; Iwamasa et al., 1999; Eberhart et al., 2000) EphA4 was detected on LMC motoneurons within both the lumbar (Figure 4.1A-E) and cervical (Figure 4.1F) enlargements. Detailed examination of EphA4 immunoreactivity throughout the entire longitudinal extent of the lumbar enlargement revealed that in sections regularly spaced from L₁ (Figure 4.1A) to L₆ (Figure 4.1E), only subpopulations of LMC motoneurons were EphA4 immunopositive when compared to all LMC motoneurons visualized in adjacent H&E stained sections (Figure 4.1G-K). In sections stained with EphA4 or H&E, the dashed line encompasses the location of the entire LMC population. The specificity of the EphA4 immunohistochemical reactions was confirmed by the absence of specific staining observed in sections incubated with anti-EphA4 antiserum that has been pre-absorbed with synthetic peptide antigen (Figure 4.1L). The specific expression of EphA4 on some motoneurons within the LMC suggested that EphA4 could have a role in mediating the correct development of particular populations of LMC motoneurons in the developing spinal cord.

4.3.2 Motoneurons comprising the LMC are present in the appropriate ventrolateral position in the spinal cord of EphA4−/− animals

Examination of transverse sections of the EphA4−/− spinal cord revealed that, when compared to the EphA4+/+ spinal cord, motoneurons comprising the LMC of the cervical and lumbar enlargement were ventrolaterally positioned in the spinal grey matter.
Figure 4.1
appropriately (Figure 4.2). At E18 in the EphA4<sup>+/−</sup> spinal cord, motoneurons comprising the LMC of both the cervical (Figure 4.2C) and lumbar (Figure 4.2D) enlargement appeared to be similar in morphology and position to those motoneurons observed within the LMC of the E18 EphA4<sup>+/−</sup> spinal cord (Figure 4.2A,B). Furthermore, the similarities observed in motoneurons comprising the LMC at E18 in the EphA4<sup>+/−</sup> and EphA4<sup>−/−</sup> spinal cord were retained well after maturation of the spinal cord was complete. Motoneurons comprising the LMC of both the cervical (Figure 4.2G) and lumbar (Figure 4.2H) enlargement of the mature EphA4<sup>−/−</sup> spinal cord appeared grossly normal in appearance and position when compared to the motoneurons that comprise the LMC of the mature EphA4<sup>+/−</sup> spinal cord (Figure 4.2E,F).

4.3.3 Motoneuron pools innervating specific muscles of the forelimb are positioned appropriately within the spinal cord of EphA4<sup>−/−</sup> animals

To examine the topographical arrangement of individual motoneuron pools within the spinal cord, retrograde analysis of motoneurons innervating specific muscles was carried out. In the forelimb, injections of fluorescent neuronal tracers into the biceps brachii (TMRD) and triceps brachii (fast blue) muscles retrogradely labeled motoneurons within the LMC of the cervical spinal cord (Figure 4.3). In longitudinal sections of both the EphA4<sup>+/−</sup> and EphA4<sup>−/−</sup> cervical spinal cord, motoneuron pools innervating the biceps brachii (Figure 4.3A,C) and triceps brachii (Figure 4.3B,D) muscles were located in discrete, longitudinal columns. Furthermore, examination of the motoneurons innervating forelimb muscles within the EphA4<sup>−/−</sup> spinal cord revealed no gross morphological abnormalities (Figure 4.3C,D) when compared to those motoneurons labeled within the EphA4<sup>+/−</sup> spinal cord (Figure 4.3A,B).

Longitudinal reconstruction of the location of labeled cells within the motoneuron pool innervating each of the biceps brachii and triceps brachii muscles allowed their topographical arrangement to be examined. As was observed in the EphA4<sup>+/−</sup> spinal cord, the motoneuron pools of the biceps brachii and triceps brachii muscles in the EphA4<sup>−/−</sup> spinal cord were positioned in longitudinal columns of the cervical
Figure 4.2
Figure 4.3
enlargement (Figure 4.4A; each labeled motoneuron represented by a circle). Additionally, in both the EphA4+/+ and EphA4−/− spinal cord, the motoneuron pool innervating the triceps brachii muscle was located caudal to the motoneuron pool innervating the biceps brachii muscle.

Further analysis of the segmental arrangement of the motoneuron pools innervating the biceps brachii and triceps brachii muscles revealed similar distributions in the EphA4+/+ and EphA4−/− spinal cord which were consistent with distributions of these pools reported previously (Tada et al., 1979; Ryan et al., 1998; McKenna et al., 2000). In both the EphA4+/+ (n = 9) and EphA4−/− (n = 11) spinal cord, the distribution of the motoneuron pool innervating the biceps brachii muscle was not significantly different, extending along several segments from C2-C6 (Figure 4.4B; each bar represents the motoneuron pool distribution for one single animal). Similarly, the distribution of the motoneuron pool innervating the triceps brachii muscle was also comparable in the EphA4+/+ (n = 7) and EphA4−/− (n = 7) spinal cord, extending from C4-C8 (Figure 4.4B).

4.3.4 Motoneurons comprising the sciatic motoneuron pool within the spinal cord of EphA4−/− animals are present in a longitudinal column and in the correct number

An investigation of the topography of motoneuron pools within the lumbar enlargement of the EphA4−/− spinal cord was also undertaken. In both EphA4+/+ and EphA4−/− animals, direct application of TMRD to the proximal stump of the transected sciatic nerve retrogradely labeled motoneurons comprising the sciatic motoneuron pool within the LMC of the lumbar spinal cord (Figure 4.5). In longitudinal sections of the EphA4−/− lumbar spinal cord, labeled motoneurons of the sciatic motoneuron pool were positioned correctly in longitudinal columns within the LMC of the spinal cord (Figure 4.5C) and appeared morphologically normal (Figure 4.5D) when compared to labeled motoneurons of the sciatic motoneuron pool within the EphA4+/+ spinal cord (Figure 4.5A,B). Furthermore, stereological quantification of the number of labeled
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Figure 4.4
Figure 4.5
motoneurons comprising the sciatic motoneuron pool of the EphA4\textsuperscript{+/c} spinal cord revealed a number of motoneurons (776 ± 80; \(n = 6\)) that was not significantly different to that counted in the sciatic motoneuron pool of the EphA4\textsuperscript{+/-} spinal cord (781 ± 46; \(n = 8\); \(P > 0.05\); unpaired Student’s t-test; Figure 4.5E).

4.3.5 Altered distribution of motoneurons comprising the sciatic motoneuron pool within the spinal cord of EphA4\textsuperscript{+/c} animals

Although motoneurons of the sciatic motoneuron pool in the EphA4\textsuperscript{+/c} spinal cord were positioned in longitudinal columns within the LMC as expected, longitudinal reconstruction of the location of labeled cells within the sciatic motoneuron pool of the EphA4\textsuperscript{+/c} spinal cord revealed that the rostrocaudal distribution of this motoneuron pool was abnormal. In longitudinal reconstructions of the sciatic motoneuron pool in the EphA4\textsuperscript{+/c} spinal cord, labeled motoneurons extended across 4 vertebral segments from L\(_3\)-L\(_6\) of the lumbar enlargement (Figure 4.6A) as has been described in previous reports (Janjua and Leong, 1984; Swett \textit{et al}., 1986). However, in the EphA4\textsuperscript{+/-} spinal cord, although positioned in a discrete longitudinal column, labeled cells of the sciatic motoneuron pool extended across only 3 vertebral segments, from L\(_4\)-L\(_6\) (Figure 4.6A). Examination of the segmental distribution of the sciatic motoneuron pool within the spinal cord of a number of EphA4\textsuperscript{+/c} (\(n = 6\)) animals revealed that the L\(_3\) component was consistently missing when compared to the sciatic motoneuron pool labeled in the spinal cord of EphA4\textsuperscript{+/-} (\(n = 8\)) animals (Figure 4.6B; each bar represents the motoneuron pool distribution for one single animal). Given the similarity between the numbers of motoneurons that comprise the sciatic motoneuron pool in the spinal cords of EphA4\textsuperscript{+/c} and EphA4\textsuperscript{+/-} animals (Figure 4.5E), it would appear that the altered distribution of this motoneuron pool in the EphA4\textsuperscript{+/-} spinal cord is not due to aberrant cell death.
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Figure 4.6
4.3.6 Altered topographic position of the motoneuron pool innervating the \textit{tibialis anterior} muscle within the spinal cord of EphA4\textsuperscript{+/-} animals

Although retrograde labeling of the complete sciatic motoneuron pool revealed an altered topographical arrangement of these motoneurons in the EphA4\textsuperscript{+/-} spinal cord that was not due to aberrant cell death, it still remained unclear which specific motoneuron pool was affected. To further define which motoneuron pool was affected within the EphA4\textsuperscript{+/-} lumbar spinal cord, retrograde analysis of motoneurons innervating specific muscles was carried out. In the hindlimb, injections of fluorescent neuronal tracers into the \textit{gastrocnemius} (fast blue) and \textit{tibialis anterior} (diamidino yellow) muscles retrogradely labeled motoneurons within the LMC of the lumbar spinal cord (Figure 4.7). In longitudinal sections of both the EphA4\textsuperscript{+/-} and EphA4\textsuperscript{+/-} spinal cord, motoneuron pools innervating the \textit{gastrocnemius} (Figure 4.7A,C) and \textit{tibialis anterior} (Figure 4.7B,D) muscles were located in discrete, longitudinal columns as predicted by the labeling pattern obtained for the complete sciatic motoneuron pool. Additionally, motoneurons labeled within the EphA4\textsuperscript{+/-} spinal cord (Figure 4.7C,D) appeared morphologically similar to those neurons labeled in the EphA4\textsuperscript{+/-} spinal cord (Figure 4.7A,B). However, unlike the EphA4\textsuperscript{+/-} spinal cord where there was little overlap between the location of the \textit{gastrocnemius} and \textit{tibialis anterior} motoneuron pools (Figure 4.7A,B), the location of the \textit{gastrocnemius} and \textit{tibialis anterior} motoneuron pools within the EphA4\textsuperscript{+/-} spinal cord appeared to overlap significantly [note overlapping motoneurons (arrows) in Figure 4.7C,D].

Longitudinal reconstruction of the location of labeled cells in the motoneuron pool innervating each of the \textit{gastrocnemius} and \textit{tibialis anterior} muscles of EphA4\textsuperscript{+/-} and EphA4\textsuperscript{+/-} animals revealed a major displacement of the \textit{tibialis anterior} motoneuron pool within the EphA4\textsuperscript{+/-} spinal cord. Although the motoneuron pool innervating the \textit{gastrocnemius} was located in an identical position in both the EphA4\textsuperscript{+/-} and EphA4\textsuperscript{+/-} spinal cord, the motoneuron pool innervating the \textit{tibialis anterior} appeared to be displaced caudally when compared to the homologous motoneuron pool within the EphA4\textsuperscript{+/-} spinal cord (Figure 4.8A). Further investigation of the segmental arrangement of the \textit{gastrocnemius} and \textit{tibialis anterior} motoneuron pools revealed the displacement
Figure 4.7
Chapter 4: Regulation of motoneuron pool position by EphA4

Figure 4.8
of the *tibialis anterior* motoneuron pool within the *EphA4*<sup>+/−</sup> spinal cord to be uniform among almost all animals investigated. Consistent with previous reports describing motoneuron pool topography in the mouse (McHanwell and Biscoe, 1981), in the spinal cord of *EphA4*<sup>+/+</sup> animals, the *tibialis anterior* motoneuron pool (*n* = 9) extended across one vertebral segment from L<sub>3</sub>-L<sub>4</sub> and was positioned rostral to the *gastrocnemius* motoneuron pool (*n* = 13), which extended from L<sub>4</sub>-L<sub>5</sub>, with little overlap existing between the 2 pools of motoneurons (Figure 4.8B; each bar represents the motoneuron pool distribution for one single animal). However, in the *EphA4*<sup>−/−</sup> spinal cord, although the position of the motoneuron pool innervating the *gastrocnemius* remained unchanged (*n* = 11), the motoneuron pool of the *tibialis anterior* was shifted caudally (*n* = 12) and now extended from L<sub>4</sub>-L<sub>5</sub> to overlap the *gastrocnemius* motoneuron pool almost completely (Figure 4.8B).

### 4.3.7 Motoneurons within the spinal cord lumbar enlargement of *EphA4*<sup>−/−</sup> animals project through the appropriate ventral spinal roots to form an anatomically correct sciatic plexus and nerve

Gross examination of the spinal cord of *EphA4*<sup>−/−</sup> animals revealed that, identical to that observed in the spinal cord of all *EphA4*<sup>+/+</sup> animals examined (Figure 4.9A; *n* = 5), the lumbar enlargement consisted of 6 spinal segments in all animals examined (*n* = 6), with each segment projecting a pair of ventral roots to the periphery (Figure 4.9B). In the lumbar enlargement of a normal mouse, the 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> ventral roots converge at the sciatic plexus to form the sciatic nerve (Hebel and Stromberg, 1986). As observed in the spinal cord of *EphA4*<sup>+/−</sup> animals (Fig 4.9A), the sciatic nerve of all *EphA4*<sup>−/−</sup> animals examined (Figure 4.9B) received contributions from the 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> ventral roots that converged normally at the sciatic plexus to form the complete sciatic nerve.

Application of fluorescent tracer (TMRD) to either the sciatic nerve or the *tibialis anterior* muscle retrogradely labeled motoneuronal projections within the ventral roots of the spinal cord (Figure 4.10). In the spinal cord of both the *EphA4*<sup>+/+</sup> (Figure 4.10A-C; *n* = 5) and *EphA4*<sup>−/−</sup> (Figure 4.10D-F; *n* = 6) animals, axons comprising the sciatic nerve were found to exit the lumbar enlargement of the spinal cord in the 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup>
Figure 4.9
Chapter 4: Regulation of motoneuron pool position by EphA4

Figure 4.10
ventral roots as expected. Additional examination of the axons projecting to the *tibialis anterior* in the hindlimb of *EphA4*+/+ animals revealed that, consistent with previous reports (Peyronnard and Charron, 1980), motor axons of the *tibialis anterior* motoneuron pool exited the lumbar enlargement of the spinal cord in only the 4th and 5th ventral roots (Figure 4.10G-I; n = 3). In the spinal cord of *EphA4*−/− animals, despite an altered position within the spinal cord, motoneurons projecting to the *tibialis anterior* still did so correctly through the 4th and 5th ventral roots only of the lumbar enlargement (Figure 4.10J-L; n = 3).

### 4.3.8 All muscles and nerves within the hindlimbs of *EphA4*−/− animals develop normally despite altered topographic positioning of the *tibialis anterior* motoneuron pool within the spinal cord

Given the altered topographical position of the motoneuron pool innervating the *tibialis anterior* muscle in the spinal cord of *EphA4*−/− animals an investigation of hindlimb morphology in mature *EphA4*−/− animals was carried out. Histological examination of hindlimb musculature anatomy in *EphA4*−/− animals revealed no major abnormalities when compared to hindlimbs of *EphA4*+/+ animals (Figure 4.11). In transverse sections stained with H&E, all muscles of the posterior, peroneal and anterior compartments of the hindlimb in *EphA4*−/− animals developed normally and were present in their expected size and location (Figure 4.11D,E) when compared with those of the *EphA4*+/+ animal (Figure 4.11A,B). Furthermore, the 3 major nerves innervating the muscles of the hindlimb, the superficial and deep peroneal nerves and the tibial nerve, were all present within the *EphA4*−/− hindlimb (Figure 4.11D,F) and exhibited no gross histological abnormality when compared to homologous nerves observed in the *EphA4*+/+ hindlimb (Figure 4.11A,C).
Figure 4.11
Chapter 4: Regulation of motoneuron pool position by EphA4

4.4 DISCUSSION

In the investigation presented in this chapter, analysis of the motoneurons that innervate limb muscles within the spinal cord of EphA4<sup>−/−</sup> animals revealed an altered topographical arrangement of the motoneuron pool that specifically innervates the *tibialis anterior* muscle of the hindlimb. The displacement of this motoneuron pool suggests that EphA4 plays an important role in determining the correct rostrocaudal position of a specific motoneuron population within the spinal cord.

4.4.1 EphA4 participates in the control of motoneuron positioning within the LMC of the lumbar spinal cord

Although motoneurons of the *tibialis anterior* motoneuron pool innervated the *tibialis anterior* correctly in EphA4<sup>−/−</sup> animals, their rostrocaudal position within the lumbar spinal cord was caudally displaced by approximately one vertebral segment when compared to the *tibialis anterior* motoneuron pool in the lumbar spinal cord of EphA4<sup>+/+</sup> animals. In contrast to the defect observed in the lumbar spinal cord of EphA4<sup>−/−</sup> animals, the topographical arrangement of those motoneuron pools examined within the cervical spinal cord of EphA4<sup>−/−</sup> animals appeared normal. Thus, given that EphA4 is expressed on a subpopulation of motoneurons comprising the LMC in both the cervical and lumbar spinal cord (Figure 4.1), it appears that EphA4 may only be necessary for the correct positioning of specific motoneuron populations in the lumbar spinal cord. It may be that EphA4 plays no role in determining the topographic arrangement of those motoneuron pools in cervical spinal cord examined in this investigation, or alternatively, its function in the cervical spinal cord may be redundant. An alteration in motoneuron pool distribution similar to that described in this investigation has been recently described in the spinal cord of ephrinA5<sup>−/−</sup> animals. In these animals the motoneuron pool innervating the *acromiotorapezius* muscle of the scapula is elongated and shifted caudally when compared to the homologous motoneuron pool observed in
the spinal cord of ephrinA5\textsuperscript{+/+} animals (Feng et al., 2000). Taken together, the findings of this investigation and those of Feng et al. (2000) suggest an important role for Eph/ephrin interactions in defining the correct topographic arrangement of specific motoneuron pools in the spinal cord.

Alterations of motoneuron topographical arrangement have also been described to occur in the spinal cord of animals with disruptions in genes that encode various members of the LIM and Hox families of transcription factors (Carpenter et al., 1997; Tiret et al., 1998; Kania et al., 2000; Sharma et al., 2000). Indeed, it has been proposed that transcriptional regulators, such as those belonging to the LIM and Hox families, control the expression of cell-surface receptors and ligands that mediate positional identity in spinal motoneurons (reviewed in Jessell, 2000). Given the recent finding that certain Hox members can regulate EphA2 expression within the hindbrain (Studer et al., 1998), and the findings of this investigation and those of Feng et al. (2000) that particular Eph receptors and ephrins are important mediators of spinal motoneuron positioning, it is indeed likely that the expression of certain Eph receptors and ephrins in specific populations of spinal motoneurons is mediated by transcriptional regulators such as those described above.

4.4.2 EphA4 appears to have no role in mediating developmentally regulated apoptosis of spinal motoneurons

In the LMC of the mouse spinal cord approximately two thirds of all motoneurons initially generated have undergone developmentally regulated cell death by the onset of maturity (Lance-Jones, 1982). Several reports have proposed that Eph/ephrin signaling may mediate apoptotic events in motoneurons of the developing spinal cord (Magal et al., 1996; Yue et al., 1999). Indeed, using EphA4\textsuperscript{−/−} animals generated independently of those used in the present investigation, a recent report has suggested a role for EphA4 in promoting motoneuron survival by showing a significant decrease in motoneuron numbers within the L3-L4 and L5 segments of the lumbar spinal cord of EphA4\textsuperscript{−/−} mice (Helmbacher et al., 2000). In the present study, the number of motoneurons counted
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within the sciatic motoneuron pool of the EphA4+/− spinal cord did not differ significantly from those counted in the EphA4+/+ spinal cord. In both the EphA4+/+ and EphA4−/− spinal cord, each sciatic motoneuron pool was found to consist of approximately 800 motoneurons, which is concordant with the numbers of motoneurons determined to be present in the sciatic motoneuron pool within the mouse spinal cord by previous stereological investigations (Baulac and Meininger, 1983). Consequently, the observations of this investigation suggest that EphA4 is not involved in mediating motoneuron survival within the LMC of the lumbar spinal cord. What is the reason behind the discrepancy between the findings of this investigation and those of Helmbacher and colleagues? The suggestion by Helmbacher et al. (2000) that there are reduced numbers of motoneurons in L3-L4 spinal segments of the EphA4−/− spinal cord is readily explained by the finding of this investigation that motoneurons in this area are caudally displaced in the spinal cords of EphA4−/− animals. However, as noted earlier in this discussion, the study by Helmbacher et al. (2000) also revealed that there are reduced numbers of motoneurons in the L5 spinal segments of the EphA4+/− spinal cord. This, in addition to the fact that these animals also exhibit hindlimb deformities associated with aberrant peripheral nerve projections, is not consistent with the findings described in the present investigation. The hindlimbs of the EphA4+/− animals described in the present investigation are normal. Other groups that have also generated EphA4−/− animals do not report their animals to exhibit any hindlimb deformities either (Kullander et al., 2001b; Leighton et al., 2001). Therefore, it may be that genetic modifiers present within the genome of the EphA4+/− animals generated by Helmbacher et al. (2000) may serve to increase the severity of abnormalities arising from disrupting the gene that encodes EphA4 (Nadeau, 2001).

4.4.3 Models to explain the role of EphA4 in mediating the position of the tibialis anterior motoneuron pool in the spinal cord

How could the expression of EphA4 on pools of spinal motoneurons determine their correct topographical position within the spinal cord? One possible mechanism is by restricting motoneuron intermingling at the interface of adjacent motoneuronal pools, as has been shown to occur in the vertebrate hindbrain (Xu et al., 1995; Mellitzer et al.,
Chapter 4: Regulation of motoneuron pool position by EphA4

1999; Xu et al., 1999). Given that two ligands for EphA4, ephrinA2 and ephrinA5, have previously been shown to be expressed within the LMC (Eberhart et al., 2000), interactions between motoneurons of the tibialis anterior motoneuron pool expressing EphA4 and other motoneurons expressing either or both ephrinA2 and ephrinA5 could result in the segregation of the tibialis anterior motoneuron pool to its correct position within the spinal cord (Figure 4.12A). Additionally, given that EphA4 could have an important role in mediating the correct migration of branchial neural crest cells (Smith et al., 1997), it may also serve a similar function in the spinal cord by controlling the directed rostral migration of the tibialis anterior motoneuron pool during early development (Figure 4.12B). An alternative hypothesis to those that implicate EphA4 in the regulation of cell movement is one that implicates EphA4 in the control of cell specification. It may be that EphA4 could mediate the correct specification of motoneuron pools in the spinal cord via lateral-signalling mechanisms akin to those described to occur as a result of Delta-Notch interactions (reviewed in Lewis, 1998; Artavanis-Tsakonas et al., 1999). In this hypothesis, a motoneuron pool that normally lies adjacent to the gastrocnemius motoneuron pool in the spinal cord of EphA4+/− animals may be re-specified in the spinal cord of EphA4−/− animals to project to the tibialis anterior muscle (Figure 4.12C).

4.4.4 The effects of displaced tibialis anterior motoneuron pools in the spinal cord of EphA4−/− animals on central pattern generation and locomotion

The direct effect(s) of aberrantly located tibialis anterior motor pools in the spinal cords of EphA4−/− animals on central pattern generation and locomotion is unclear. Examining the effect of these displaced motor pools on the locomotor patterns of EphA4−/− animals in vivo is complicated by the locomotor defects caused by aberrant CST terminations within the spinal cords of these animals (see chapter 3 of this thesis). However, in vitro spinal cord preparations have been used previously to examine the properties of CPGs and their control of locomotion (Nishimaru and Kudo, 2000) and could be employed to examine the effect(s) of aberrantly located tibialis anterior motor pools on CPG networks and locomotion in EphA4−/− animals.
Figure 4.12
In conclusion, the correct development of neural circuits that control movement requires a complex array of signals mediated by both genetic and epigenetic mechanisms. In addition to its role as a mediator of axon guidance that is described in chapter 3 of this thesis, the investigation presented in this chapter has demonstrated a novel role for EphA4 in mediating the position of a specific motoneuron pool within the spinal cord. This observation has provided further insight into the complex events that control the correct positioning of motoneuron pools within the central nervous system.
CHAPTER 5: REGULATION OF NEUROBLAST PROLIFERATION AND GUIDANCE IN THE MATURE CENTRAL NERVOUS SYSTEM BY EphA4
5.1 INTRODUCTION

In addition to those areas of the mature central nervous system, such as the SVZ of the lateral ventricles and SGZ of the hippocampus, where the generation of neuroblast populations is well characterized, neuroblast production and differentiation can also occur in the mature spinal cord (Weiss et al., 1996; Shihabuddin et al., 2000) and mature neocortex (Magavi et al., 2000) under certain conditions. Thus, the fact that neural progenitor cells (or neuroblasts) are continually produced in a number of discrete areas of the mature central nervous system offers exciting possibilities for the development of therapies that can direct, by suppressing or re-activating particular guidance cues, certain neural progenitor populations (including populations of cells that express therapeutic agents) to injured areas for treatments aimed at repairing the affects of acute neurotrauma and neurodegenerative disease.

Presently, little is known about the mechanisms that underlie the guidance of migrating neuroblast populations to their destinations within the mature central nervous system. As described in chapter 1 of this thesis (section 1.4.2.1), PSA-NCAM interactions are important for neuroblast migration within the lateral ventricle SVZ and RMS, but rather than actively directing neuroblast guidance, PSA-NCAM interactions appear to provide a permissive environment for neuroblast migration. Furthermore, interactions between the secreted guidance cue, slit, and its receptor, robo, also appear to be important for the directed guidance of neuroblasts within the RMS in vivo (Wu et al., 1999).

A recent study has shown that Eph/ephrin interactions are also important regulators of the in vivo guidance and proliferation of neuroblasts migrating within the lateral ventricle SVZ, along the RMS, to the olfactory bulb (Conover et al., 2000). In the study by Conover et al. (2000), artificially clustered ephrinB1 and ephrinB2 ectodomain fusion proteins were infused into the lateral ventricle of experimental animals. This resulted in ephrinB2, but not ephrinB1, fusion proteins not only causing a dramatic increase in the proliferation of neuroblasts within the SVZ, but also causing severe disruption to the chains of neuroblasts migrating within the SVZ as well (Conover et al., 2000). Given that ephrinB2, but not ephrinB1, activates EphA4 receptors (Gale and
Yancopoulos, 1997; Mellitzer et al., 1999), and that EphA4 protein is expressed within areas associated with neuroblast migration in the SVZ (Conover et al., 2000), it is possible that EphA4 could play a role in regulating the proliferation and guidance of migrating chains of neuroblasts within lateral ventricle SVZ and RMS of the mature central nervous system. However, since the study by Conover et al. (2000) used ephrin ectodomain fusion proteins that non-specifically inhibited multiple Eph/ephrin interactions, what role, if any, the EphA4 receptor had in regulating neuroblast proliferation and migration within the lateral ventricle SVZ and RMS of the mature central nervous system could not be specifically determined.

Given the uncertainty of the involvement of EphA4 in mechanisms that regulate neuroblast proliferation and migration in certain areas of the mature central nervous system, this study has sought to identify a precise role for EphA4 in these mechanisms. Populations of cells containing neuroblasts can be isolated from the rostral regions of the lateral wall of the lateral ventricle of the mature brain and maintained as cellular aggregates called neurospheres (Reynolds and Weiss, 1992). Neurospheres comprise a self-propagating, relatively undifferentiated, cell population that contain neural progenitor cells capable of differentiating into the two broad neural cell lineages i.e. neurons and glia (Reynolds and Weiss, 1992). In the investigation presented in this chapter, neurospheres derived from the mature brain have been used as a source of potential neuroblasts. This study has demonstrated the presence of EphA4 in neurosphere populations. Moreover, it has shown that the EphA4 receptor does not appear to be important for regulating the proliferation and differentiation potential of neurosphere populations in vitro and also does not appear to be required for regulating the proliferation and guidance of neuroblasts migrating within the RMS in vivo.
5.2 MATERIALS AND METHODS

5.2.1 Isolation and culture of neurosphere producing cells

The animals used in the investigation reported in this chapter are described in chapter 3 of this thesis (section 3.2.1). Mature EphA4+/+ and EphA4-/- animals (2-6 months old) were killed by cervical dislocation. Their brains were then removed and placed into HEPES-buffered Eagle’s medium (HEM; Gibco, NY, USA) prior to further dissection. Tissue from each brain was dissected from the rostral region of lateral wall of the lateral ventricles and cut into small pieces (≈ 1 mm³). The tissue was then dissociated by transferring it to 3 ml of Mg²⁺/Ca²⁺-free Hank’s balanced salt solution (HBSS; Gibco, NY, USA) containing {10 mM HEPES (w/v); 0.2 mg/ml EDTA; 0.5 mM trypsin (w/v); 0.0001% (w/v) DNase; pH 7.6} for 10 minutes at 37°C. Following this, 3 ml of trypsin inhibitor was added to the tissue suspension {0.14 mg/ml trypsin inhibitor and 0.001% (w/v) DNase in HEM} and the tissue was collected by centrifugation for 7 minutes at 700 rpm. The supernatant was removed and the tissue pellet was then triturated in 200 µl of PBS to produce a single cell suspension of neurosphere producing cells that was subsequently filtered through a 70 µm cell strainer (Falcon, NJ, USA) to remove large cell debris. Eosin exclusion was used to quantify the concentration of viable cells in the filtered single cell suspension. These cells were then cultured in 30 mm tissue culture wells at an initial seeding density of 3.5 × 10⁴ viable cells/well in NS-A basal serum free media (Euroclone; West York, UK) containing 10 ng/ml basic FGF (Boehringer-Mannheim, Mannheim, Germany); 20 ng/ml epidermal growth factor (EGF; BD Biosciences, MA, USA); 2 mM (v/v) L-glutamine; 0.6% (v/v) glucose; 60 µM putrescine; 20 nM progesterone; 30 nM sodium selenite, 25 µg/ml insulin and 100 µg/ml apo-transferrin (Sigma, MO, USA) and maintained at 37°C with 5% CO₂. The ratio of the number of neurospheres formed after 7 days in vitro to the number of cells initially plated is the neurosphere frequency. Quantitative estimations of cell numbers are expressed in the text as mean ± SEM and statistical probability (P) was determined with an unpaired Student’s t-test. Neurospheres were passaged approximately once every 7 days, and propagated in bulk culture at 5, 000-10, 000 cells/cm².
5.2.2 RNA isolation and Northern blotting

Neurosphere cells were harvested from culture, washed once in PBS, and collected by centrifugation for 7 minutes at 700 rpm. Whole embryonic mouse tissue was harvested from timed-pregnant females that had been killed by cervical dislocation. Total RNA was isolated from neurosphere and whole mouse embryonic tissue using the TRizol total RNA isolation reagent (Gibco, NY, USA) according to the manufacturer’s instructions. Poly(A)+ RNA was then prepared from total RNA using oligo(dT) cellulose (Boehringer-Mannheim, Mannheim, Germany) as previously described (Aviv and Leder, 1972). Between 2-4 µg of Poly(A)+ RNA was added to 20 µl of RNA sample buffer {10% (v/v) 10×MOPS buffer (1×MOPS=20 mM MOPS; 1 mM EDTA; 5 mM sodium acetate); 50% (v/v) deionized formamide; 2.2M formaldehyde; 1.2% (v/v) RNA loading dye (50% (v/v) glycerol; 1 mM EDTA; 0.25% bromophenol blue; 0.25% xylene cyanol)}, denatured for 5 minutes at 65°C, and separated on a 1% denaturing RNA formaldehyde agarose gel. Poly(A)+ RNA was then transferred from the agarose gel onto GeneScreenPlus nylon membrane (NEN-DuPont, MA, USA). Following transfer, the membrane was stained with methylene blue {0.02% (w/v) methylene blue dissolved in 0.5 M sodium acetate} to record the positions of 28S, 18S and 5S RNA bands. It was then de-stained for 10-20 minutes in solutions of 20% ethanol followed by 0.2 × standard saline citrate (SSC; 0.15 M sodium chloride; 0.015 M sodium citrate)/1% (v/v) SDS, dried, and pre-hybridized in Northern hybridization buffer {50% (v/v) deionized formamide; 1 M NaCl; 1% SDS; 10% dextran sulphate} for 1 hour at 42°C. Specific [α-32P] dATP labeled cDNA probes were prepared using the Megaprime DNA labeling kit (Amersham, NJ, USA) according to the manufacturer’s instructions. Following denaturation, the probe was added to hybridization buffer, and incubated with the membrane overnight at 42°C. The EphA4 probe corresponded to nucleotides 0-1554 of the EphA4 receptor (Gilardi-Hebenstreit et al., 1992). Following hybridization, the membrane was washed twice with 0.2 × SSC for 5 minutes at room temperature and once with 1 × SSC/1% SDS for 30 minutes at 65°C to remove any unbound cDNA probe. Membranes were then exposed overnight to PhosphorImager screens and visualized using a PhosphorImager quantitative imaging device (Molecular Dynamics, CA, USA).
5.2.3 Immunohistochemical analysis of differentiated neurospheres

Differentiated neurospheres were immunohistochemically analysed using anti-GFAP (1:500; DAKO, CA, USA) and anti-β-tubulin (1:500, Promega, WI, USA) antibodies according to the procedure described in chapter 2 of this thesis (section 2.2.6).

5.2.4 In vivo immunostaining of migrating neuroblasts

Mature animals were deeply anaesthetized with a solution (10 ml/kg administered into the intraperitoneal space) of ketamine (10 mg/ml; Delvet, NSW, Australia) and xylazine (1.3 mg/ml; Bayer, NSW, Australia) before being transcocrially perfused with saline followed by 4% paraformaldehyde in PBS. The brains were then removed, post-fixed overnight in fixative, and dehydrated by immersion in a graded series of alcohols. Following this, the brains were transferred into molten polyethylene glycol MW 1000 (Sigma, MO, USA) for 3-4 hours at 56°C prior to being maintained in a 7:3 mixture of polyethylene glycol MW 1000 and polyethylene glycol MW 1450 (Sigma, MO, USA) overnight at 56°C. The brains were then embedded in polyethylene glycol and serially cut into 5 µm parasagittal sections. Sections were mounted onto microscope slides coated with 2% (v/v) 3-aminopropyltriethoxysilane (AES; Sigma, MO, USA) and air-dried at room temperature for 2-3 hours. Prior to commencing immunohistochemical staining, sections were first re-hydrated in PBS for 10 minutes. Sections were incubated for 1 hour at room temperature in a 10% normal horse serum/PBS solution to block non-specific antibody/antigen complexes. Sections were then washed in PBS and incubated overnight at 4°C in primary anti-PSA-NCAM antibody diluted in blocking solution (1:500; mAB12E3 kindly provided by T. Seki of Juntendo University, Tokyo, Japan). Following primary incubation, sections were then washed in PBS, and incubated for 1 hour at room temperature with a secondary biotinylated goat-anti-mouse IgG antibody (1:200; Vector, CA, USA). Specifically bound anti-PSA-NCAM/biotinylated IgG complexes were subsequently visualized using standard ABC histochemistry with a nickel enhanced DAB reaction product.

5.2.5 Image presentation

Images presented in this chapter were prepared as described in chapter 2 of this thesis (section 2.2.8).
5.3 RESULTS

5.3.1 Neurospheres derived from the brains of mature EphA4\textsuperscript{+/−} animals are morphologically normal and form at the appropriate frequency

Cells derived from the brains of mature EphA4\textsuperscript{+/−} animals gave rise to a population of numerous aggregations of cells called neurospheres after 7 days in culture (Figure 5.1A). These neurospheres exhibited the ability to self-renew and amplify when maintained \textit{in vitro}. Similarly, tissue derived from the brains of mature EphA4\textsuperscript{+/−} animals also gave rise to neurospheres, which appeared morphologically similar to those arising from EphA4\textsuperscript{+/+} animals (Figure 5.1B). These neurospheres also had the ability to self-renew and amplify \textit{in vitro}. Furthermore, quantification of the number of neurospheres generated from tissue derived from the brains of mature EphA4\textsuperscript{+/+} and EphA4\textsuperscript{+/−} animals (Figure 5.1C) showed that the neurosphere frequency of EphA4\textsuperscript{+/−} tissue (6.9 ± 0.3 neurospheres/1000 viable cells; \(n = 3\)) was not significantly different to the neurosphere frequency of EphA4\textsuperscript{+/+} tissue (7.3 ± 0.3 neurospheres/1000 viable cells; \(n = 3\); \(P > 0.05\); unpaired Student’s \(t\)-test).

5.3.2 Neurospheres express mRNA for the EphA4 receptor

Northern blot analysis of neurosphere mRNA revealed the expression of mRNA transcripts coding for the EphA4 receptor (Figure 5.2). In neurospheres derived from the brains of mature EphA4\textsuperscript{+/+} animals (neurospheres\textsuperscript{+/+}), a single 6.5-7.0 kb EphA4 mRNA transcript is present. Some mRNA transcripts were also detected in neurospheres derived from the brains of mature EphA4\textsuperscript{+/−} animals (neurospheres\textsuperscript{+/−}) but sequencing of these transcripts showed them to encode for nonsense mRNA (M. Dottori, personal communication). Whole tissue known to express the EphA4 receptor (E13.5 embryo) was used to demonstrate the specificity of the EphA4 cDNA probe.
Figure 5.1
5.3.3 Neurospheres derived from the brains of mature EphA4−/− animals are capable of differentiating into neurons and glia

Neurospheres derived from the brains of normal mature animals can differentiate into both neurons and glia (Reynolds and Weiss, 1992). The neurospheres derived from the brains of mature EphA4+/+ animals were no exception to this, and when maintained in differentiating conditions, differentiated into cells exhibiting both neuronal (Figure 5.3A) and glial (Figure 5.3B) phenotypes. Similarly, neurospheres derived from the brains of mature EphA4−/− animals also differentiated into cells exhibiting both neuronal (Figure 5.3C) and glial (Figure 5.3D) phenotypes, when maintained in differentiating conditions. The precise phenotype of differentiated neurosphere cells in this investigation was assessed on the basis of morphology and the expression of well-characterized neuronal and glial markers. Cells that expressed the neuronal marker, β-tubulin, that had distinct small, rounded, cell somas and fine axonal or dendritic like processes were classified as neurons. Cells that expressed the glial marker, GFAP, and were stellate, with large cell somas and multiple, thick processes, were classified as glia.

5.3.4 The RMS in the brains of mature EphA4−/− animals is morphologically normal

Given that EphA4 mRNA is present in undifferentiated neurospheres it may play a role in guiding certain populations of migrating neuroblasts in vivo. Consequently, to determine if EphA4 mediated interactions are important for regulating the proliferation and migration of neuroblast precursors within the SVZ of the lateral wall of the lateral ventricle and RMS, an in vivo analysis of migrating neuroblasts within the brains of mature EphA4−/− animals was carried out. Migrating neuroblasts within the brains of mature EphA4+/+ and EphA4−/− animals were immunohistochemically identified with anti-PSA-NCAM antibodies. PSA-NCAM is expressed at high levels by migrating neuroblasts and anti-PSA-NCAM antibodies reveal that neuroblasts migrate in longitudinally orientated chains from their origins in the SVZ of the lateral wall of the lateral ventricle to their destination in the olfactory bulbs (Doetsch and Alvarez-Buylla, 1996). In parasagittal sections of brains derived from mature EphA4+/+ animals (n = 3),
Chapter 5: Regulation of neuroblast proliferation and migration by EphA4

Figure 5.3
neuroblasts comprising the RMS migrate in distinct, longitudinally orientated chains (Figure 5.4A-C). Examination of neuroblasts comprising the RMS in parasagittal sections of brains derived from mature EphA4\(^{-/-}\) animals \((n = 3)\) revealed a morphologically normal RMS (Figure 5.4D-F). Neuroblasts migrating within the RMS of the brains of EphA4\(^{-/-}\) animals did so normally in longitudinally orientated chains (Figure 5.4D) and appeared to be present in very similar numbers (Figure 5.4E,F) to those observed in the brains of EphA4\(^{+/+}\) animals (Figure 5.4B,C). All sections shown in Figure 5.4 of brains derived from both EphA4\(^{+/+}\) and EphA4\(^{-/-}\) animals are representative of an equivalent anatomical location (Figure 5.4G).
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Figure 5.4
5.4 DISCUSSION

Very little is known about the mechanisms that underlie the guidance of neuroblast populations in the mature central nervous system. The investigation described in this chapter has shown that a population of cells containing neuroblasts, neurospheres, express mRNA transcripts for the EphA4 receptor. This investigation has also shown that the EphA4 receptor does not appear to be essential for the proliferation and differentiation of neurospheres in vitro and also does not appear to be required for the proliferation and guidance of certain neuroblast populations in vivo.

5.4.1 Undifferentiated neurospheres express EphA4

It is now becoming clear that many of the receptor/ligand interactions that underlie the guidance of axons also appear to be important for guiding the migration of neural progenitors in the mature central nervous system. Repulsive/inhibitory interactions mediated by diffusible factors such as slit, and non-diffusible factors such as certain ephrins, appear to play a significant role in guiding the migration of neuroblasts in the mature brain from the SVZ of the lateral wall of the lateral ventricle, along the RMS, to the olfactory bulb (Wu et al., 1999; Conover et al., 2000). However, no investigation that examines the role of guidance receptor/ligand interactions in neuroblast migration has unequivocally demonstrated the presence of any particular guidance receptor or ligand on migrating neuroblasts. This investigation has shown for the first time that neurospheres (a population of cells, which includes neuroblasts, that is derived from the lateral ventricle SVZ of the mature brain) express mRNA for the EphA4 receptor. The fact that this investigation has determined that EphA4 mRNA is expressed by neurospheres suggests that migrating neuroblasts could express EphA4 and is consistent with the studies of Conover et al. (2000), which described EphA4 protein expression in vivo in areas associated with neuroblasts migrating from the lateral ventricle SVZ to the olfactory bulbs.
5.4.2 EphA4 does not appear to be required for neurosphere proliferation and differentiation in vitro

Given that EphA4 mRNA is expressed by neurospheres, an investigation of neurospheres derived from the brains of mature EphA4\(^{-/-}\) animals was carried out. Neurospheres derived from EphA4\(^{+/+}\) animals appeared morphologically normal and could differentiate into cells belonging to both the neuronal and glial lineages. Thus it appears likely that EphA4 is not required for the correct differentiation of neurosphere cells. Furthermore, the fact that neurospheres derived from the brains of mature EphA4\(^{-/-}\) animals formed at the appropriate frequency suggested that EphA4 is not an important regulator of the mechanisms that underlie neurosphere proliferation. Disruptions of Eph/ephrin interactions in vivo have previously been described to dramatically increase neuroblast proliferation (Conover et al., 2000). A number of reasons could explain why this effect was not observed in the neurosphere populations examined in this investigation. Firstly, it may be that EphA4 is not the particular Eph receptor involved in regulating neuroblast proliferation or, other Eph receptors may be able to functionally compensate for EphA4 in its absence. Secondly, given that the proliferation of neuroblast precursors is related to the anatomical position of progenitor cell populations within the SVZ of the lateral wall of the lateral ventricle (reviewed in Alvarez-Buylla et al., 2001), the increase in neuroblast proliferation caused by disrupting Eph/ephrin interactions in vivo may be a secondary consequence of altering the spatial relationships of these progenitor cell populations (Conover et al., 2000). These spatial relationships do not exist between neurospheres maintained in vitro and hence, even if EphA4 does play a role in regulating proliferation of neuroblasts by altering the spatial relationships between progenitor cell populations, this effect would not be detected in the neurospheres examined in this investigation.

5.4.3 EphA4 is not required for the proliferation and guidance of neuroblasts migrating within the RMS in vivo

The role of EphA4 mediated interactions in regulating neuroblast proliferation and guidance in vivo was investigated by examining migrating neuroblasts within the RMS
of mature EphA4<sup>−/−</sup> animals. In brains derived from mature EphA4<sup>−/−</sup> animals, neuroblasts migrating from the SVZ of the lateral wall of the lateral ventricle, along the RMS, to the olfactory bulbs appeared to form morphologically normal chains, and when compared to the neuroblasts observed in EphA4<sup>+/+</sup> animals, also appeared to be present in the appropriate number. Thus it seems that the EphA4 receptor is not essential for the correct proliferation and migration of those neuroblasts in the mature central nervous system that are destined for integration within the olfactory bulb neural network. It has been reported previously that perturbing Eph/ephrin interactions, possibly by interrupting EphA4 mediated signalling, can disrupt the guidance of neuroblasts migrating to the olfactory bulb in the mature brain (Conover <i>et al.</i>, 2000). The results of the <i>in vitro</i> and <i>in vivo</i> investigations presented in this chapter strongly suggest that EphA4 is not essential for the correct migration of neuroblasts to the olfactory bulb in the mature brain. Given that the observations of Conover <i>et al.</i> (2000) resulted from multiple Eph/ephrin interactions being disrupted by infusion of artificially clustered ephrin ectodomains, it is likely that other Eph/ephrin interactions, aside from those mediated by EphA4, are important for guiding the correct migration of neuroblast within the mature brain. Furthermore, given the large amount of functional redundancy that could exist as a result of the promiscuous nature of Eph/ephrin interactions, it is also likely that the function of EphA4 in guiding migrating neuroblasts in the brains derived from EphA4<sup>−/−</sup> animals could be compensated for by other Eph receptors.
CHAPTER 6: CONCLUSION
6.1 SUMMARY OF FINDINGS

The investigations described in this thesis have demonstrated that the EphA4 receptor is an important regulator of mechanisms that underlie the correct formation of certain neural circuits in the nervous system. This thesis has shown that EphA4 expressed on CST axons prevents them from aberrantly recrossing the spinal midline early in development, while soon after, EphA4, also expressed within the intermediate zone of the spinal grey matter, serves to confine CST terminations to the dorsal horn of the grey matter of the spinal cord during the later stages of CST maturation. The work contained in this thesis also demonstrated that EphA4 is expressed on subpopulations of motoneurons within the LMC of the spinal cord during development. It revealed that, rather than being important for guiding spinal motor axons to their destinations in the periphery, EphA4 appears to help control the topographical arrangement of motoneuron pools in the spinal cord by regulating the position of the tibialis anterior motoneuron pool within the spinal lumbar enlargement. Finally, the investigations reported in thesis revealed that, although cells derived from the lateral ventricle SVZ of the mature brain express EphA4, EphA4 does not appear to be required to direct the passage of neuroblasts migrating from the lateral ventricle SVZ to the olfactory bulbs of the mature central nervous system.

6.2 IMPLICATIONS FOR NEURAL DISORDERS, DISEASE AND INJURY

The studies presented in this thesis have provided an insight into the mechanisms that could underlie particular neural disorders in humans. The CST in humans, as in rodents, is important for modulating certain aspects of limb movement (Nolte, 1993; Kingsley, 1996; Krukauser and Ghez, 2000). In humans, mirror movements (involuntary movements on one side of the body that mimic the motor pattern of voluntary movements elicited on the contralateral side of the body) characterize a number of complex neurological disorders that disrupt the normal pattern of CST projections in the spinal cord (Regli et al., 1967; Schott and Wyke, 1981; Farmer et al., 1990; Mayston et
Clinical examination of individuals exhibiting mirror movements revealed that bilateral motor potentials could be evoked by unilateral transcranial magnetic stimulation of the motor cortex, suggesting that it is bilateral CST innervation of the spinal cord that underlies the movement abnormalities observed in mirror movement disorders (Mayston et al., 1997; Balbi et al., 2000). The EphA4 deficient animals described in this thesis, and the ephrinB3 deficient animals described by Yokoyama et al. (2001) and Kullander et al. (2001), all exhibit chronic mirror movements similar to those described to occur in humans. Thus, mutations in the EphA4 and ephrinB3 genes may be responsible for the congenital disorders that result in mirror movements in humans and consequently, animals deficient in EphA4 and ephrinB3 may serve as good models for the pathophysiologic investigation of these mirror movement disorders in the future.

The investigations presented in this thesis have also provided insight into some of the guidance receptor/ligand interactions that may be important for guiding regenerating axons to their correct targets in therapeutic strategies directed at repairing damaged axonal tracts following neural disease and injury. Recently, glial cell bridges, neutralizing antibodies to inhibitory molecules and transplanted cells genetically modified to express various neurotrophic factors have been used to successfully increase the number of regenerating axons growing across areas of nervous system injury (reviewed in Fournier and Strittmatter, 2001; Jones et al., 2001; Raisman, 2001). However, without knowledge of the mechanisms required to guide these regenerating axons to their correct targets, biological repair of damaged areas of the nervous system will still remain a very difficult problem to overcome. This thesis has shown that the EphA4 receptor is very important for guiding the CST to its appropriate targets in the spinal cord. Presumably, EphA4 could also play a similar role in guiding the re-growth of the regenerating CST after injury. Indeed, the expression of a number of Eph receptors and ephrins is upregulated following nervous system injury (Miranda et al., 1999; Moreno-Flores and Wandosell, 1999; Knoll et al., 2001a) and it is likely that, as in development, Eph/ephrin interactions will be very important for guiding axonal regrowth during regeneration in the mature nervous system.
Another possible therapeutic strategy directed at repairing damaged areas in the nervous system following neural disease and injury could involve the directed guidance of endogenous reserves of neuronal progenitor cells within the mature nervous system to areas of damage. The investigations presented in this thesis have shown that populations of neuronal progenitors derived from the mature nervous system express EphA4, but it is clear that additional studies are required to further characterize the complete complement and functional role of guidance receptors and ligands expressed by these cells. Ultimately, knowledge of the mechanisms that underlie the directed migration of neuronal progenitors in the mature nervous system will facilitate the development of therapies that, through activating or repressing certain guidance receptor/ligand interactions, will allow endogenous neuronal progenitor cell populations to be directed to sites of neural injury so that damage caused by neural disease and acute trauma can be repaired.

6.3 CLOSING REMARKS

An interconnected network of complex neural circuits characterizes the mature nervous system. The precise development and maintenance of these neural circuits is required for the mature nervous system to perform all its functions correctly. Although it is clear that a vast array of both activity independent and dependent mechanisms act in concert to direct the correct development and maintenance of neural circuits, many of these mechanisms still remain to be elucidated. Knowledge of the mechanisms that underlie the development and maintenance of neural circuits will not only allow an understanding of basic biological functions, but will also provide insights into the mechanisms that underlie our ability to learn, think, remember, communicate and feel emotion. Ultimately, knowledge of the mechanisms that control connectivity in the nervous system may also lead to the development of novel therapeutic strategies directed at restoring nervous system function that is lost after neural disease and acute injury.
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