The role of the ezrin-radixin-moesin (ERM) proteins in development and post-injury responses of central nervous system neurons

by

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SUMMARY

The ezrin-radixin-moesin (ERM) family of proteins mediate diverse cytoskeletal processes, such as cell adhesion and morphology, as well as cell migration. These functions have primarily been elucidated in non-neuronal cell phenotypes, with only limited data pertaining to a role for ERM proteins in CNS neurons. The few studies examining ERM functions in neurons indicate that they contribute to important aspects of developmental neuron growth and morphogenesis; processes that must be precisely executed for the establishment of appropriate connectivity during brain development. Further alluding to their potential importance in the CNS was the identification of ezrin as a binding partner of the developmentally crucial cell adhesion molecule (CAM) L1.

After completion of brain development, the mature CNS has very little capacity for regeneration and functional recovery following injury, partly because upon maturation neurons lose their intrinsic ability for substantial growth, but mainly because the mature nervous system environment is inhibitory to growth, particularly following traumatic injury. Therefore, a current and widely accepted theory is that recapitulation of aspects of CNS development is required for regeneration to occur in the more mature nervous system. Thus, investigation of developmentally significant proteins may provide clues as to the requirements for more mature CNS neurons to mount a regenerative response.

This thesis investigated ERM protein function in CNS neurons, both during development and regeneration, with investigations focussed on ERM proteins’ binding relationship with L1. ERM proteins were specifically expressed during early neuronal morphogenesis, localised to areas associated with motility and growth, and they co-localised with L1, particularly in the axonal growth cone. Perturbation of ERM proteins function utilising transfection of a dominant negative form of ezrin implicated ERMs in multiple aspects of development, including neurite initiation and outgrowth, growth cone morphology, neurite branching and neuron migration. Investigation of ERM function in models of injury to mature neurons demonstrated a re-expression of ERM proteins, potentially to participate in regenerative attempts, including neurite initiation
and outgrowth, as well as post-injury neuron motility. Interrupting ERM activation using an inhibitor of Rho kinase significantly perturbed many of these developmental and post-injury functions, confirming a role for Rho kinase in ERM activation. These results show that ERM proteins play significant roles in aspects of neuronal growth and morphology in both developing and regenerating CNS neurons. Furthermore, elucidation of the roles of ERM proteins and L1 show that common mechanisms are required for developmental and regenerative nervous system growth, which is important for uncovering potential mechanisms of regenerative growth, and identifying therapeutic targets for encouraging post-injury regeneration.
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<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BrdU</td>
<td>5-Bromo-2′-deoxyUridine</td>
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<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>DCX</td>
<td>doublecortin</td>
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<td>DIV</td>
<td>days in vitro</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>E</td>
<td>embryonic day</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>ERM</td>
<td>ezrin-radixin-moesin</td>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>microtubule associated protein</td>
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<td>NF</td>
<td>neurofilament</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>pERM</td>
<td>phosphorylated ERM</td>
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<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol 4,5 bis-phosphate</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>RKI II</td>
<td>Rho kinase inhibitor II</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>Sema</td>
<td>semaphorin</td>
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<td>TBS</td>
<td>tris buffered saline</td>
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<td>TTBS</td>
<td>tris buffered saline with tween</td>
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1 INTRODUCTION

1.1 THE ERM PROTEINS

The ezrin-radixin-moesin (ERM) family of proteins have previously been considered as general membrane-cytoskeleton linker proteins, abundant in dynamic structures such as microvilli, membrane ruffles and filopodia (Bretscher et al., 1983), in many cell types throughout the body. Their localisation reflects their function in facilitating cellular processes such as cell-cell and cell-substrate contacts, maintenance of cell shape, cell motility and membrane trafficking (Louvet-Vallée, 2000). More recently, they have been considered integral to signal transduction pathways and organisation of the cell cytoskeleton during processes of growth and motility. As recently described by Bretscher (1999), the ‘ERM proteins have emerged from relative obscurity to a prominence where they are proposed to play structural and regulatory roles in numerous plasma membrane based processes’. Furthermore, they are now becoming recognised for their importance in the CNS, particularly in developmental processes.

1.1.1 The cell cytoskeleton

The cell cytoskeleton lies directly beneath the plasma membrane and is required for establishment of polarity, determination of cell morphology, as well as attachment to other cells and the extracellular matrix via adhesion molecules (Bretscher, 1999). It functions in membrane trafficking, cell division, migration, growth regulation and differentiation (Bretscher, 1999). Its numerous and varied functions are testament to the fact that, rather than simply providing the cell with its structural framework, the cytoskeleton is a highly dynamic structure that constantly changes in response to the cells surrounding environment.

The main constituents of the cell cytoskeleton are microfilaments, microtubules and intermediate filaments. Microfilaments consist of filamentous actin (F-actin) strands, which are made up of polymerised globular actin (G-actin) monomers (Bear, 2001). Actin filaments are densely localised to areas of high motility and growth, such as the
filopodia and lamellipodia of neuronal growth cones (section 1.2.2). They determine the shape of the cell and are required for whole-cell motility (Alberts et al., 2002). Microtubules are largely composed of tubulin molecules that consist of dimers of α- and β-tubulin proteins. They provide structural support during growth and in neurons are imperative to fast axonal transport. Microtubule associated proteins (MAPs, such as MAP2 and tau) coat the surface of microtubules and provide a link between microtubules and other cellular structures. Finally, there is a great diversity of intermediate filaments in different cell types, and in neurons they are more commonly referred to as neurofilaments (NFs), and include the neurofilament triplet and α-internexin. In all cells they are responsible for providing a structural framework for the cell. They are mechanically very strong, and less dynamic than microfilaments and microtubules (Bear, 2001).

In living cells, all three types of cytoskeletal filaments undergo continual remodelling through the assembly and disassembly of their subunits, with the assistance of accessory proteins, to grow or alter the cells shape or position in response to the changing environment. The ERM proteins link plasma membrane proteins to both actin and microtubule cytoskeletal components (Birgbauer and Solomon, 1989; Algrain et al., 1993), and are likely to play important roles in cytoskeletal remodelling.

1.1.2 ERM protein structure

Ezrin, radixin and moesin are three proteins of approximately 82, 80 and 75kDa, respectively, that make up the ERM family of cytoskeletal-linker proteins (Bretscher et al., 1983; Lankes et al., 1988; Tsukita et al., 1989). They are grouped together due to their high degree of sequence homology, sharing between 70-85% amino acid identity. The ERM proteins also share a high level of homology (60%) with the protein merlin (moesin/ezrin/radixin-like). The ERMs and merlin belong to the band 4.1 superfamily, because they have 85% amino acid identity to the N-terminal half of human erythroid band 4.1 protein. The N-terminal domain of proteins belonging to the erythroid band 4.1 superfamily is highly conserved, and has been named the FERM (four.1 protein, ezrin,
radixin, moesin) domain (Chishti et al., 1998). The FERM domain is common to many proteins that link proteins to the plasma membrane (Bretscher et al, 2002).

Structurally, the proteins can be divided into three domains 1) a highly conserved N-terminal FERM domain 2) a central alpha-helical domain that is predicted to form coiled-coils, and 3) a C-terminal domain (Figure 1.1 A). The N-terminal half of ezrin binds the cytosolic tails of plasma membrane proteins, via the FERM domain, which consists of a 298 amino acid domain that forms three structural modules that together form a compact clover-shaped structure (Pearson et al., 2000). The C-terminal half has an extended structure consisting of 107 amino acids, the last 34 of which bind to F-actin of the cell cytoskeleton (Algrain et al., 1993). Both the N- and C-terminal domains of the ERM proteins have protein-binding capacity, which indicated a cytoskeletal-linker function (Algrain et al., 1993).

Like many cytoskeletal proteins, the ERM proteins' activity is regulated by conformation. In their inactive conformation, ERM N- and C-terminals self-associate (Figure 1.1 B), which conceals both the plasma membrane and F-actin binding sites (reviewed in Bretscher et al., 2002), and is commonly referred to as "head-to-tail" association. More specifically, 296 (1-296) amino terminal residues at the N-terminal domain known as N-ERMAD (N-ERM associated domain) interact with the 107 (479-585) residue C-terminal area known as C-ERMAD (C-ERM associated domain) (Bretscher et al., 1995; Gary and Bretscher, 1995). When ezrin is inactive, both the membrane binding FERM domain and the C-terminal actin binding sites are masked. In vivo, the proteins have also been shown to form intermolecular structures; dimers and oligomers, but their molecular mechanism and physiological relevance is still unknown (Tsukita et al., 1999).

1.1.3 Regulation of activity

While the abundance and biological activity of many proteins is controlled at the DNA or RNA level, ERM activity is controlled at the protein level. Approximately half of the
Figure 1.1 ERM structure and activation. A. Structurally, ERM proteins can be divided into three domains 1) a highly conserved N-terminal FERM domain 2) a central alpha-helical domain that is predicted to form coiled-coils, and 3) a C-terminal domain. The N-terminal binds plasma membrane proteins, via the FERM domain, which forms three structural modules that together form a compact clover-shaped structure (Pearson et al., 2000). The last 34 amino acids of the C-terminal bind to F-actin (Algrain et al., 1993). B. In their inactive conformation, ERM N- and C-terminals self-associate, which conceals both the plasma membrane and F-actin binding sites, and is commonly referred to as “head-to-tail” association. More specifically, 296 (1-296) amino terminal residues at the N-terminal domain known as N-ERMAD (N-ERM associated domain) interact with the 107 (479-585) residue C-terminal area known as C-ERMAD (C-ERM associated domain) (Bretscher et al., 1995; Gary and Bretscher, 1995). When ezrin is inactive, both the membrane binding FERM domain and the C-terminal actin binding sites are masked. In vivo, the proteins have also been shown to form intermolecular structures; dimers and oligomers, but their molecular mechanism and physiological relevance is still unknown (Tsukita et al., 1999). Phosphatidylinositol 4,5 bis-phosphate (PIP₂) binding to the FERM domain, combined with phosphorylation of a specific C-terminal threonine (T558 is moesin, T567 in ezrin and T564 in radixin) are involved in the activation of ERM proteins. The resultant open conformation allows binding to plasma membrane proteins and the cytoskeleton to occur. Figure adapted from Ivetic and Ridley, 2004.
cellular ERM proteins exist in a soluble, inactive state in the cytoplasm, while the other half exist in their activated, insoluble conformation, associated with the cell cytoskeleton (Kondo et al., 1997). The inactive conformation is most likely alleviated by phosphatidylinositol 4,5 bis-phosphate (PIP$_2$) binding to the FERM domain, combined with phosphorylation of a specific C-terminal threonine (T558 in moesin, T567 in ezrin and T564 in radixin) (Figure 1.1 B). However, the specific order of events, species requirements, the differences between cell types and activation mechanisms \textit{in vitro} and \textit{in vivo} are so far unresolved.

Evidence suggests that PIP$_2$ binding to the N-terminal region is initially required, followed by phosphorylation of the C-terminal threonine (Fievet et al., 2004). The requirement for PIP$_2$ in ERM activation was demonstrated when ERM mediated microvillar collapse occurred in L cells when PIP$_2$ was diminished (Yonemura et al., 2002). However, the physiological site of PIP$_2$ binding remains unclear (Bretscher, 2002). It is hypothesised that the binding site is in the FERM domain, but this seems controversial since much of the FERM domain is obscured in the proteins' inactive conformation.

Phosphorylation at the C-terminal threonine site is thought to stabilise the active, open conformation by preventing interaction of the N- and C-terminal domains (Tsukita et al., 1999). Several kinases have been shown to phosphorylate the C-terminal threonine of ERM proteins, including PIP$_2$ (Matsui et al., 1999; Fievet et al., 2004), protein kinase C (PKC$_\alpha$ and PKC$_\beta$) (Ng et al., 2001) and the Rho effector Rho kinase (also known as ROCK) (Matsui et al., 1998). One of the first experiments to show Rho kinase can phosphorylate ERM proteins was performed in Swiss 3T3 fibroblasts, where activation of RhoA led to increased ERM phosphorylation and the formation of cell surface structures (Shaw et al., 1998). In a hippocampal progenitor cell line, Jeon (et al., 2002) demonstrated that glutamate stimulates RhoA and Rho kinase dependent phosphorylation of moesin. However, whether Rho kinase phosphorylates ERM proteins in many cell types \textit{in vivo} is not clear (Matsui et al., 1998; Yonemura et al., 2002). Finally, a relative of Rho kinase, Myotonic dystrophy kinase-related Cdc42-
binding kinase (MRCK), has also been shown to phosphorylate moesin, initially in a cell-free system, and supported by dominant negative MRCK transfection experiments in NIH 3T3 cells (Nakamura et al., 2000). Louvet-Vallée (2000) reviewed ERM phosphorylation and concluded that moesin is phosphorylated by PKC-θ, and ezrin/radixin by Rho kinase, however it is likely that the kinase(s) involved in ERM phosphorylation may be different depending on the cell type involved and the biological context.

1.1.4 ERM localisation

All three ERM proteins are localised in cells throughout the body (Bretscher et al., 1983; Franck et al., 1993), in actin rich surface structures such as microvilli, membrane ruffles and filopodia (Bretscher et al., 1983). Demonstrating the widespread tissue expression of the ERM proteins is that ezrin was first isolated from intestinal brush borders (Bretscher et al., 1983), radixin from rat liver (Tsukita et al., 1989), and moesin from smooth muscle cells of bovine uterus (Lankes et al., 1988).

Melendez-Vasquez et al., (2001) examined the expression and distribution of the ERM proteins in Schwann cells and neurons of the PNS, in vitro and in vivo. Schwann cells expressed high levels of all three ERM proteins in the membrane, whereas neurons expressed lower levels of radixin and moesin. In vivo, the ERM proteins were concentrated at nodal processes of myelinating Schwann cells. A more recent study found ERM proteins localised in microvillar tips of Schwann cells during node formation (Gatto et al., 2003). Both studies implicate ERMs in the myelination process.

Relatively few studies have examined the expression of ERM proteins in the CNS, but some studies suggest that ezrin, radixin and moesin are widely expressed in the adult human brain. Moesin is localised to areas such as the hippocampus, frontal cortex and white matter, cerebellum, spinal cord and leptomeninges (Stemmer-Rachamimov et al., 1997), while ezrin is expressed in adult human cerebral cortex, basal ganglia, hippocampus, hypophysis and optic nerve (Gronholm et al., 2005).
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Indicating a primarily developmental function for the ERM family proteins, peak expression of all three proteins occurs in late embryonic and early postnatal developing rat brain, according to Western blot studies (Paglini et al., 1998), and ezrin expression in mouse whole brain lysates disappears after one month of age (Gronholm et al., 2005). Mintz and colleagues (2003) examined ezrin expression in neonatal rat brain by immunoblotting at time-points between embryonic day 15 (E15) and post-natal day 0 (P0), finding that ezrin was at peak levels between E17 and E19. Immunohistochemical analyses have demonstrated an abundance of ERM immunolabelling localised to the intermediate zone (Johnson et al., 2002; Mintz et al., 2003), a region present only in the developing brain composed of migrating cortical neurons and growing axons, which suggests a role for ERM proteins in these developmental processes.

Recently, a study of ezrin localisation in developing and adult human, rat and mouse CNS, utilising both in vitro and in vivo analyses, reported that ezrin is predominantly expressed in astrocytes, with expression in neurons in more restricted locations in the brain (Gronholm et al., 2005). In neurosphere cultures ezrin was present in astrocytes, while expression was absent in neurons, and interestingly, ezrin was not present in primary hippocampal neuron cultures either (Gronholm et al., 2005). Another immunohistochemical study of human foetus did not reveal ezrin staining in neurons of the cortex (Johnson et al., 2002). Moesin is expressed by glia, such as oligodendrocytes, astrocytes (Stemmer-Rachamimov et al., 1997) and microglia, but not neurons (Johnson et al., 2002). In contrast, a number of in vitro studies have demonstrated expression of one or all three ERM proteins in various neuronal phenotypes, including dorsal root ganglion (Castelo and Jay, 1999), chick sympathetic neurons (Gonzalez-Agosti and Solomon, 1996), cerebellar granule cells (Cheng et al., 2005) and hippocampal neurons (Paglini et al., 1998; Dickson et al., 2002).

At the sub-cellular level, localisation in neurons is of a membranous or diffuse cytoplasmic pattern (Stemmer-Rachamimov et al., 1997), and mostly confined to areas of high motility and growth, such as growth cone lamellipodia and filopodia (Paglini et
al., 1998; Castelo and Jay 1999; Dickson et al., 2002;), which strongly supports a role in neurite outgrowth and the cytoskeletal remodelling associated with growth.

1.1.5 ERM link the cytoskeleton to plasma membrane proteins

Experiments by Algrain et al., (1993) showed that when the individual N- and C-terminal halves of ezrin were transfected into fibroblasts the C-terminal targets actin-filament bundles, while the amino terminal targets the plasma membrane. These experiments identified the ERM proteins as cytoskeletal-linker proteins.

1.1.5.1 Cytosolic proteins

A 34 amino acid residue site on the C-terminal of ezrin was identified as an F-actin binding site (Algrain et al., 1993; Turunen et al., 1994), a site that is conserved in radixin and moesin (Pestonjamasp et al., 1995). This is the major actin-binding site, but one study has shown the capability of another sequence at the C-terminal of ezrin to bind G-actin as well (Roy et al., 1997). Also, Birgbauer and Solomon (1989) demonstrated that ezrin almost completely co-localised with tubulin in the marginal band of erythrocytes, indicating that microtubules are another cytoskeletal binding partner of the ERM proteins. Recent data also suggests that ezrin may, at least transiently, associate with intermediate filaments in epithelial cells (Wald et al., 2005).

1.1.5.2 Plasma membrane proteins

ERM proteins can bind to plasma membrane proteins by either direct or indirect mechanisms (Louvet-Vallée, 2000). The first membrane-binding partner to be identified was CD44, as they directly co-localise in epithelial cells and fibroblasts, and further immunoprecipitation experiments showed a direct interaction between the ERM proteins and the cytoplasmic domain of CD44 (Tsukita et al., 1994). Other membrane protein-binding partners include CD43, ICAM-1-3, syndecan-2 (a heparan sulfate proteoglycan) and NHE-1. These membrane proteins do not share a universal cytoplasmic sequence recognised by the N-terminal FERM domain of the ERM, their juxtamembrane
positively charged amino acid clusters are probably responsible for ERM binding (reviewed by Bretscher, 2000).

Indirect attachments occur when proteins that span the membrane many times are attached via adaptors, such as the first discovered EBP50/NHERF (Reczek and Bretscher, 1998). EBP50/NHERF interacts directly with the N-terminal of ezrin and moesin. Other adaptors have since been identified, including E3KARP (Yun et al., 1998). The indirect linkage appears to be important for regulating the activity of ion transporters, regulating endocytosis of membrane proteins, and localisation of these proteins within the plasma membrane (reviewed by Bretscher, 2002).

1.1.5.2.1 ERM proteins directly link L1 CAM to the actin cytoskeleton

Neural CAM families, including the integrin, cadherin and immunoglobulin families, are vital for normal development of the CNS, but almost certainly retain important functions throughout life. They are involved in many steps of axon guidance including initial outgrowth and growth cone formation, axon fasciculation, de-fasciculation, branching, target acquisition, and synapse formation and maintenance (Wiencken-Barger et al., 2004). Also, CAMs are involved in neuronal migration that takes place during CNS development (section 1.2.3.1). A role for CAMs in the CNS neuronal response to stress (reviewed by Sandi et al., 2004) and trauma (for example, Roonprapunt et al., 2003) have been demonstrated more recently, and expression changes in age related pathologies have been investigated (for example, Yew et al., 1999; Strekalova et al., 2006).

Yeast two-hybrid analysis has recently revealed the CAM L1 as a binding partner of ezrin (Dickson et al., 2002). L1, which is homologous with NILE (Stallcup and Beasley, 1984; Bock et al., 1985) and Ng-CAM (Friedlander et al., 1986), is a well-studied neural CAM belonging to the immunoglobulin superfamily (see reviews by Hortsch, 1996; Walsh and Doherty, 1997; Kamiguchi, 2003). L1 has demonstrated roles in many developmental processes including axon growth and guidance, neuron migration, cell
adhesion, axon pathfinding and axon fasciculation (Hortsch, 1996) and more recently has been implicated in neuronal morphogenesis (Dickson et al., 2002; Cheng et al., 2005). L1 is a 200kDa, phosphorylated glycoprotein that is associated with the plasma membrane, and is comprised of 6 immunoglobulin domains, 5 fibronectin type III repeats, a single transmembrane spanning domain and a short cytoplasmic tail. The cytoplasmic tail may bind directly to ankyrin in the cell cytoskeleton, or may be linked to the cytoskeleton via another protein, such as the ERM proteins. Ezrin binds the cytoplasmic tail of L1, by its C-terminal RSLE sequence and juxtamembrane domain (Cheng et al., 2005).

1.1.6 ERM Function

1.1.6.1 Perturbation of ERM protein expression and activity

One approach to studying the unique functions, expression and localisation of the individual ERM proteins has been the use of knockout mutants. Moesin and radixin knockout mice are both phenotypically normal (Doi et al., 1999; Kikuchi et al., 2002), only the radixin knockout shows some liver abnormalities (Kikuchi et al., 2002). Furthermore, disruption of moesin does not affect ERM-dependent functions, and does not result in increased expression of ezrin or radixin (Doi et al., 1999), indicating that the loss of one of the proteins is not compensated for by the upregulation of others. However, it has been shown that in vivo ERM localisation is tissue specific, which would suggest specific tissue related functions (Berryman et al., 1993).

In vitro, cultured cells co-express the ERM proteins, and antisense oligonucleotide suppression of one of the three proteins does not have any phenotypic effects (Takeuchi et al., 1994). However, the suppression of all three ERM proteins abolishes cell-cell contacts, cell-substrate adhesion and microvilli in mouse epithelial and thymoma cells (Takeuchi et al., 1994). These data, combined with the high amino acid sequence homology of the proteins, may be indicative of redundancy of the individual proteins at the cellular level.
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The variable expression patterns and possible functional redundancy of ERM proteins suggests that the reason for having all three proteins is that they are individually important at a particular stage of development (Johnson et al., 2002). Further investigation into this hypothesis may finally solve the puzzle surrounding the roles of individual ERM proteins.

1.1.6.2 ERM proteins and intracellular signal transduction

ERM proteins were previously considered general cross-linkers between the plasma membrane and actin cytoskeleton. However, more recently their role as intracellular signal transducers has been revealed. There is mounting evidence that the ERM proteins have multiple roles in the signal transduction pathways of the Rho family of GTPases.

1.1.6.2.1 The Rho family of small GTPases

The Rho family of small GTPases consists of seven identified proteins; Rho (A, B and C isoforms), Rac (1 and 2 isoforms), Cdc42 (Cdc42Hs and G25K isoforms), RhoD, RhoG, RhoE and TC10 (Mackay and Hall, 1998). The most widely studied are Rho, Rac and Cdc42 (Luo, 2000), which are known to trigger a variety of cellular responses, including reorganisation of the actin cytoskeleton (Hall, 1998). Given that the Rho GTPases are potent inducers of cellular changes, the activation of GTPases must be temporally and spatially controlled. Switching from the inactive, GDP-bound, to active GTP-bound state, combined with the displacement of RhoGDI, brings about the activation of Rho GTPases. This is facilitated by guanine nucleotide exchange factors (GEFs), or Rho GEFs (see reviews by Mackay and Hall, 1998; Bishop and Hall, 2000; Ivetic and Ridley, 2004). Some Rho GEFs appear to be specific for individual GTPases, whereas others act on all three GTPases, such as Dbl (Rossman et al., 2005), a stimulatory GDP/GTP exchange factor of Rho family members. There are little data to explain the mechanism of activation of Rho GEFs by extracellular agonists. GTPase activating proteins (GAPs) switch Rho GTPases off, by stimulating their intrinsic GTPase activity.
Rho, Rac and Cdc42 have been shown to control the organisation of the actin cytoskeleton in eukaryotic cells, implicating them in processes such as cell movement, axonal guidance, cytokinesis, and morphogenetic processes involving cell shape and polarity (Mackay and Hall, 1998).

1.1.6.2.2 ERMs participate in Rho GTPase signal transduction

Studies by Kotani et al., (1997) indicated that phosphorylation of ERMs during their activation, prior to translocation to the plasma membrane, was a Rho-dependent mechanism, and later Shaw and colleagues (1998) pinpointed RhoA as a requirement. Thus, direct inhibition of RhoA, using the toxin C3 transferase, resulted in inactivation of ERM proteins and a concomitant microvillar collapse (Yonemura et al., 2002). An example from another system is that ERM proteins and RhoA are localised to Schwann cell microvilli during node formation in the myelination process, which may indicate Rho-mediated activation of ERM proteins has a direct role in the process (Gatto et al., 2003).

Data relating to a role for ERM proteins in Rac and Cdc42 signalling are less abundant. However, a review of recent data identified that Rac and ERM proteins are both required for membrane ruffling and lamellipodial extension, and cell migration in phagocytes (Bretscher et al., 2002). Also, Rac regulates PIP$_2$ activation (Tolias et al., 2000), which in turn activates the ERM proteins. Recently, it was shown that Cdc42 and ERM proteins appear to be involved in the formation of filopodia, because transfection of NIH 3T3 cells with constitutively active Cdc42 resulted in increased filopodia formation and phosphorylated ERM proteins at the tips of filopodia (Nakamura et al., 2000).

Louvet-Vallée (2000) reviewed the data and proposed a model for the relationship between Rho GTPases and ERMs. Initially, extracellular signals activate Rho, which activates its targets and leads to an increase in PIP$_2$ levels. PIP$_2$ binds the N-ERMAD, inhibiting the intra-molecular association, and allowing the phosphorylation of the C-terminal threonine, possibly by Rho kinase; thus activating and stabilising the ERM...
molecule to function as a cross-linker between the plasma membrane and actin cytoskeleton. Then, once activated, ERMs can activate Rho, since the N-terminus interacts with Dbl. RhoGDI then displaces Dbl from ERM, and the new interaction leads to activation of Rho by releasing the GDI inhibition \((\text{Takahashi et al., 1997})\), allowing activation of Rho through exchange of GDP to GTP. Contrary to this report, Bretscher (2002) suggested that the radixin/Dbl interaction does not seem to have any activity, and suggests that instead, N-ERMAD binds directly to RhoGDI, which releases Rho, initiating Rho activation (Figure 1.2).

1.1.6.3 Non-nervous system functions

The ERMs participate in numerous processes, including the formation of microvilli, cell-cell contacts, maintenance of cell shape, cell motility, and membrane trafficking (Louvet-Vallée, 2000), in a wide range of cell phenotypes throughout the body.

1.1.6.3.1 Cell morphology

Early experiments implicated ERM proteins in the formation of cell surface structures and regulation of cell shape. For example, antisense oligonucleotide suppression of individual ERM proteins in mouse epithelial and thymoma cell lines resulted in the abolition of microvilli (Takeuchi et al., 1994) and expression of chimeras containing the ERM-binding regions of CD44, CD43 or ICAM-2 in fibroblasts has been shown to result in the elongation of microvilli (Yonemura et al., 1999), indicating some of the ERM relationships involved in cell surface structure formation. Ezrin perturbation by micro-CALI (chromophore assisted laser inactivation) in Fos-transformed and normal Rat-1 fibroblasts blocked membrane ruffling or caused a collapse of the cells leading edge, respectively, identifying roles for ezrin in both pseudopodial extension and maintenance of cell shape (Lamb et al., 1997). Recently, it has also been shown that inactivation of the ERM proteins in lymphocytes causes microvillar collapse, a necessary preceding step to lymphocyte migration (Nijhara et al., 2004).
Figure 1.2 A model for the relationship between Rho GTPases and ERMs. Initially, extracellular signals activate Rho, which activates its targets and leads to an increase in PIP$_2$ levels. PIP$_2$ binds the ERM N-terminal site, inhibiting the intramolecular association, and allowing the phosphorylation of the C-terminal threonine, possibly by Rho kinase (1); allowing the ERM molecule to link plasma membrane proteins to the actin cytoskeleton (2). Then, once activated, ERMs can activate Rho, since the N-terminus interacts with Dbl, a stimulatory GDP/GTP exchange factor of Rho family members. RhoGDI then displaces Dbl from ERM, and the new interaction leads to activation of Rho by releasing the GDI inhibition (Takahashi et al., 1997), allowing activation of Rho through exchange of GDP to GTP. Alternatively, N-ERMAD may bind directly to RhoGDI, which releases Rho, initiating Rho activation (3). Figure adapted from Louvet-Vallée, 2000; Ivetic and Ridley, 2004.
1.1.6.3.2 Localisation of membrane proteins

Yonemura et al., (1993) first demonstrated that CD43 and ERM proteins co-localised in cleavage furrows of dividing cells, but more recent results also suggest that ERM-CD43 binding in lymphocytes results in re-localisation of CD43, excluding the molecules from the immunological synapse, which is required for T cell activation (reviewed by Bretscher et al., 2002; Gautreau et al., 2002). Similarly, natural killer cells need to recruit ICAM-2 to their uropod before they are activated by IL-2. This recruitment is dependent on ezrin, and cells that lack ezrin have uniform distribution of ICAM-2 (Helander et al., 1996, reviewed by Bretscher et al., 2002). ERM proteins also recruit CAMs to the lymphocyte uropod in mechanisms of cell motility. The uropod is a structure that forms at the rear edge of the cell body, and is concentrated with adhesion molecules including CD43, CD44, ICAM-1 and ICAM-3, as well as members of the ERM family (section 1.1.6.3.3).

1.1.6.3.3 Cell motility

Tsukita et al., (1994) demonstrated a direct interaction between ERM proteins and the broadly distributed cell surface glycoprotein CD44, in baby hamster kidney cells. The CD44-ezrin linkage has subsequently been shown to be important for directional cell motility (Legg et al., 2002). Phosphorylated ERM proteins are involved in the migration of lymphocytes, as they are localised to the uropod (reviewed by Lee et al., 2004). In some situations, the uropod is considered the "sticky" part of the cell, which may contain the concentration of surface adhesion molecules that would slow migration if located at the leading edge of the cell (reviewed by Lee et al., 2004), or alternatively, CAMs may be clustered on the uropod to act as a platform for the recruitment of other leucocytes during migration (reviewed by Ivetic and Ridley, 2004). ERM proteins are involved in the localisation of these CAMs to the uropod, as demonstrated by Lee and colleagues, who showed that introduction of a constitutively active form of ezrin results in augmentation of uropod morphology and other processes associated with motility (Lee et al., 2004). In addition, overproduction of wild-type ezrin enhances the motility of epithelial cells (Crepaldi et al., 1997). Finally, a potential role for ERM proteins in
tumour progression has arisen recently, particularly in cell invasion and metastasis, probably due to its roles in cell motility, as well as survival and proliferation (reviewed by Gautreau et al., 2002; Sahai and Marshall, 2003).

### 1.1.6.4 The role of ERM proteins in CNS neurons

There is limited, but compelling, data implicating a functional role for the ERM proteins in neurons, particularly during late embryonic and early post-natal development (Mintz et al., 2003). It was originally demonstrated that the abundance and association of ezrin with the neuronal cytoskeleton increases upon the onset of neuronal differentiation, and was particularly enriched in the growth cone (Birgbauer et al., 1991). Later, Gonzalez-Agosti and Solomon (1996) showed that the withdrawal of nerve growth factor (NGF) from chick sympathetic neurons induced rapid growth cone collapse, with a concomitant decrease in radixin labelling. In contrast, the addition of NGF resulted in rapid growth cone formation and re-localisation of radixin. Castelo and Jay (1999) used the micro-CALI technique to cause localised inactivation of radixin in dorsal root ganglion (DRG) neuronal growth cones. This resulted in a 30% reduction in the area of growth cone lamellipodia, indicating a role for radixin in lamellipodial stability in growth cone motility and neurite outgrowth. In another study of cultured hippocampal neurons, antisense oligonucleotide suppression of radixin and moesin resulted in a reduction of growth cone size, disappearance of radial striations, reduction of growth cone lamellipodia, disorganisation of actin filaments, and failure to produce an axon, thus severely effecting the morphology, motility, and process formation of growth cones (Paglini et al., 1998). Interestingly, the same effects were not observed when ezrin-radixin or ezrin-moesin was inactivated.

Dickson and colleagues (2002) investigated the function of L1-ERM binding in neurons by using neuronal transfection of a dominant negative form of ezrin. Most mammalian ERM function has been examined using the dominant negative ezrin, where the protein is truncated to comprise only the amino-terminal FERM domain (for example, Crepaldi et al., 1997). Hippocampal neurons transfected with the dominant negative form of
ezrin and grown on an L1 substrate for 72 hours exhibited increased axonal branching compared with those grown on a poly-L-lysine or laminin substrate. This result indicated a role for the L1-ERM-actin linkage in the generation of normal neuronal morphogenesis, cytoskeleton stabilisation and appropriate axonal arborisation during early neuronal development. Also, irrespective of growth substrate, the dominant negative ezrin transfection resulted in an abnormal number of fine protrusions on neurite shafts (Dickson et al., 2002). This is in agreement with several studies in non-neuronal cells that have shown that expression of only N-terminal or C-terminal halves of the protein lead to the formation of long surface structures, indicating that the full-length protein has a role in aspects of cell morphology (Henry et al., 1995; Martin et al., 1995; Mackay et al., 1997; Martin et al., 1997). It has subsequently been shown that transfection of cells with a truncated form of L1, therefore interrupting the L1-ERM linkage, causes morphological abnormalities in neurite branching in cultured cerebellar granule cells (Cheng et al., 2005).

Furthermore, Mintz et al., (2003) showed that L1 and ERM co-localise in the embryonic brain, but after birth this transfers to L1 and ankyrin co-localisation, indicating a switch in the predominant cytoskeletal-linker protein used by L1. But interestingly, this study also reported that L1 and ERMs co-localise in the embryonic brain during a period of rapid axon growth and cortical expansion. In particular, they co-localise in the intermediate zone, a developmental structure that is densely packed with migrating neurons and growing axons, further supporting a role for L1 and ERM in neuron growth, but also suggesting a role in neuron migration.

While ERM protein expression and functions within the nervous system have gained little attention so far, investigations strongly support their importance, particularly in the developing nervous system. ERM proteins are likely to be involved in crucial aspects of cortical development, including neuron migration and growth, due to their localisation in the CNS, and previously identified functions in cytoskeletal remodelling and signal transduction in non-neuronal cell types.
1.2 CORTICAL DEVELOPMENT

During evolution the higher vertebrates developed a larger cerebral cortex, with the outermost layer named the neocortex (meaning new cortex). A notable feature of the neocortex is the sorting of neurons in the outer region into six horizontal layers. This layered organisation is conserved over the entire surface of the brain, but can be more distinct in certain areas of the neocortex and can range in thickness (Nolte, 2002). Layer I, at the pial surface is the molecular layer, layer II the external granular layer, layer III the external pyramidal layer, layer IV the internal granular layer, layer V the internal pyramidal layer, layer VI, the multiform layer (Figure 1.3). The names reflect the cell types that predominantly reside in each layer. Perhaps most remarkable is the fact that early neurons must undergo a highly co-ordinated migratory journey from their birthplace in order to take up these specific positions, which is sometimes a considerable distance. For instance, in the primate brain distances required to migrate can reach up to several thousand micrometres (Rakic, 2003). Neurons then develop multiple neurites, the axon and dendrites, which extend and form synaptic connections with other cells, establishing CNS circuitry.

1.2.1 Neuron migration

Neurons destined to form the neocortex are generated at the ventricular lining and periventricular zones. More specifically, cortical pyramidal neurons are generated in the germinal ventricular zones of the dorsal telencephalon, whereas sources of cortical interneurons include the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) of the ventral telencephalon (reviewed by Parnavelas, 2000; Kriegstein and Noctor, 2004). Due to the locations of their generation, cortical pyramidal neurons generally follow a relatively radial path to their position in the neocortex, while interneurons must travel circuitously and a greater distance.

The migratory routes and modes of migration are more complex than previously thought (Kriegstein and Noctor, 2004). Post-mitotic neurons migrate toward the margin of the cerebral wall to form the primordial plexiform layer, or preplate. Subsequently born
Figure 1.3 Laminar structure of the neocortex. Schematic representation of the six layers of the neocortex that form during CNS development. The names of each layer reflect the cell types that predominantly reside in that layer. Early neurons must undergo a highly co-ordinated migratory journey from their birthplace in order to take up these specific positions. Figure adapted from Bear et al., 2001; Nolte, 2002.
neurons migrate into the preplate and as they accumulate form a new series of layers called the cortical plate, which splits the preplate into a superficial marginal zone (layer I) and a deeper layer, the subplate (layers II-VI). The later born post-mitotic neurons that form layers II-VI of the cortical plate are arranged in an ‘inside-out’ sequence, where new cells migrate through the existing cells before stopping at the top of the plate (reviewed by Parnavelas, 2000; Kriegstein and Noctor, 2004). The subplate and ventricular zone are separated by the intermediate zone, which will eventually contain the axons of the cortex (Bielas and Gleeson, 2004) (Figure 1.4).

Recent advances in live imaging techniques have lead to the identification of the characteristics of neuron migration through the cortex at the cellular level. Two modes of migration, radial and tangential migration, have been described (Nadarajah et al., 2001). A third mode of migration, termed ‘multipolar migration’ may also exist (Tabata and Nakajima, 2003).

1.2.1.1 Radial migration

Neurons of the cerebral cortex, such as those destined to become pyramidal neurons, take on a mode of radial migration, moving in a direction perpendicular to the pial surface. However, recent studies have shown that it is not an entirely smooth, directionally focussed process, as neurons pass through transitory phases in which they may alter cellular morphology, direction and speed of movement (Kriegstein and Noctor, 2004). Two characteristic modes of movement during radial migration have been defined, and they have been categorised as translocation and locomotion.

In translocation, cells migrate from the ventricular zone after establishing a long pia-directed process that forms a stable attachment to the pial surface. This indicates that the migratory route is determined before the cell exits the ventricular zone (Nadarajah et al., 2001). The translocating cell moves its nucleus and other contents of the soma radially within this fixed leading process to reach its appropriate position in the cortex. As the soma advances toward the pial surface the leading process becomes progressively
Figure 1.4 A. Radial migration. Neurons of the cerebral cortex arise in the ventricular zone, which lies at the surface of the lateral ventricles, and migrate toward the margin of the cerebral wall to form the preplate. Subsequently born neurons migrate into the preplate and as they accumulate form a new series of layers called the cortical plate, which splits the preplate into the marginal zone (layer I) and a deeper layer, the subplate (layers II-VI). The later born neurons that form layers II-VI of the cortical plate are arranged in an ‘inside-out’ sequence, where new cells migrate through the existing cells before stopping at the top of the plate (represented by darker green). The subplate and ventricular zone are separated by the intermediate zone, which will eventually contain the axons of the cortex.

B. The two major modes of cortical migration. In translocation (left), cells migrate from the ventricular zone after establishing a long pia-directed process that forms a stable attachment to the pial surface. The translocating cell moves its nucleus and other contents of the soma radially within this fixed leading process to reach its appropriate position in the cortex. In locomotion (right), freely migrating cells have a relatively short, free leading process that remains a fairly constant length. Locomoting cells move along radial glia as they receive directional guidance signals. Figure adapted from Bieles et al., 2004.
shorter and thicker, since its terminal remains attached to the pial surface, while the trailing process remains short. Translocating cells do not require glial guides and move with a smooth action, progressing about 60μm/hour (Nadarajah et al., 2001).

Radial glial cells are mitotically active, giving rise to neuronal precursors that utilise a mode of migration termed locomotion (reviewed by Chotard and Salecker, 2004). Locomoting cells are free migrating cells that in contrast with translocating cells have a relatively short, free leading process that remains a fairly constant length, and migrate along radial glial guides. Locomoting cells move along radial glia as they receive guidance signals (Nadarajah et al., 2001). Neurons show a saltatory pattern of short bursts of forward migration (Nadarajah et al., 2003), with an average speed of locomotion of 35μm/hour, slower than translocating cells because their movement is saltatory rather than smooth (Nadarajah et al., 2001).

It has been suggested that translocation is the mode of migration utilised during the earliest stages of neocortical development, and that glial-guided locomotion becomes more common in later stages (Nadarajah et al., 2001; Kriegstein and Noctor, 2004). It is also thought that translocation is the older form of migration, with locomotion arising only when the cortex became larger, and the 'inside-out' pattern of the neocortex developed. Studies of migration disorders lend support to this idea, where in some cases cortical layer formation is disturbed, but migration in the deeper parts of the cortex is unaffected (Nadarajah et al., 2003). For example, in mice lacking the Rho family kinase Cdk5 or its activator, p35, the preplate and early cortical plate form normally, but later-generated cortical neurons collect below the subplate in abnormal positions (Chae et al., 1997; Gilmore et al., 1998).

1.2.1.2 Tangential migration

Not all precursor neurons are able to reach their appropriate destination by radial migration alone and therefore are required to migrate tangentially. An example is early neurons of the rostral migratory stream (RMS), which migrate from the subventricular
zone of the anterior forebrain rostrally, to form interneurons of the olfactory bulb (reviewed by Guan and Rao, 2003). In addition, many cells migrate using a combination of tangential and radial migration. Interestingly, tangentially migrating neurons move at about 50μm/hour, generally more rapidly than those migrating radially (reviewed by Kriegstein and Noctor, 2004).

It is still unclear what signals guide neurons undergoing tangential migration. However, in contrast to radial migration, cells appear to be attached to one another or axons, rather than glial cells, forming chains of migrating neurons (Lois et al., 1996). A number of guidance cues contribute to tangential migration, including those of CAM, neuregulin, semaphorin and slit families (Kriegstein and Noctor, 2004), which are probably strategically placed along migration pathways. Particularly high numbers of tangentially migrating neurons have been observed in the subventricular zone and lower intermediate zone, regions probably rich in signals that influence migration of both interneurons and pyramidal cells (Kriegstein and Noctor, 2004).

1.2.1.3 Multipolar migration
Tabata and Nakajima (2003) reported the presence of a previously undescribed population of multipolar cells in the intermediate zone of the developing brain that did not resemble cells migrating by locomotion or translocation. These cells did not exhibit fixed cell polarity, extending and retracting processes in a dynamic manner and changed direction frequently. Multipolar cells were not highly associated with radial fibers, unlike cells undergoing locomotion. In addition, immunocytochemical studies suggested that the cells were not tangentially migrating neurons, but were early-generated cortical projection neurons (that would normally undergo radial migration). Multipolar cells migrated very slowly toward the pial surface compared with other modes of migration, at a net directional speed of 2.2μm/hour toward the pia. The authors reported that 88% of cells in the intermediate zone exhibited multipolar migration, suggesting that it is a major mode of migration. However, upon leaving the intermediate zone the cells were likely to switch to locomotion.
Nadarajah and colleagues may have previously observed this phenomenon (2001; 2002), describing a population of cells that were bipolar at onset of migration from the ventricular zone, but took on a multipolar form when reaching the intermediate zone. These neurite processes were highly motile, and often formed then retracted branches, which may indicate sensing of directional guidance cues. The cells then continued radial migration or migrated tangentially. Their identity and site of origin remains unclear, but they were likely to be interneurons (reviewed by Nadarajah et al., 2003). It is still unclear whether this is a legitimate form of migration undertaken by neurons in the intermediate zone and the neuronal phenotype involved is also unresolved. However, a recent review by LoTurco and Bai (2006) suggests that this is a true stage of migration, potentially undertaken by all migrating pyramidal neurons, and may be a phenomenon that reflects the competitive interaction between migrating cells for limited radial glial scaffolding to guide them to the cortical plate.

1.2.2 Axon outgrowth

After completing migration a neuron enters a phase of growth, the first stage of which involves developing multiple neurites. One process grows more rapidly than the others, becoming the axon, while the other shorter, highly branched processes become dendrites. The axon elongates, seeking out its path to its appropriate target by integrating information from multiple signals in the environment, in particular molecular guidance cues. The axon is able to sense and respond to this information through its terminal structure known as the growth cone.

Growth cones (Figure 1.5) are highly dynamic structures that sense the surrounding environment and integrate signals to consolidate the direction of axon growth. They are so sensitive that they are capable of detecting a concentration difference as small as one molecule across their spatial extent (Rosoff et al., 2004). Structurally, they consist of long, slender highly dynamic sensory protrusions called filopodia, which rapidly extend and retract while seeking out molecular guidance signals. Filopodia are composed
Figure 1.5 The neuronal growth cone.  

**A.** Scanning electron microscopy image of a hippocampal neuron growth cone, showing distinct filopodia and lamellipodia.  

**B.** Diagrammatic representation of the growth cone. The peripheral (P) domain consists of long slender, highly dynamic filopodial protrusions, which contain F-actin, longitudinally arranged in bundles, but very few microtubules. Lamellipodia are broad, fan shaped structures which consist of crosslinked F-actin, and microtubules. The central (C) domain contains F-actin and microtubules. Figure adapted from Gungabissoon and Bamburg, 2003.
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primarily of F-actin, with very few microtubules present. Lamellipodia are the broad, flat, fan-like structures of the growth cone, which contain F-actin and microtubules, but not intermediate filaments. In combination, the filopodia and lamellipodia form the actin rich peripheral domain of the growth cone. The central domain of the growth cone contains both actin and microtubules (reviewed by Tanaka and Sabry, 1995). In both filopodia and lamellipodia actin filaments are arranged so that their fast growing ends are oriented toward the periphery of the cell (Lewis and Bridgeman, 1992) and actin polymerisation at the tips of filopodia results in extension of the axon (reviewed by Selzer, 2003). The re-arrangements and extension of actin filaments and microtubules in response to extracellular guidance cues result in axon outgrowth.

1.2.3 Guidance cues

In the past two decades, countless CNS molecular guidance cues have been discovered (for example, Table 1.1), and their roles in axon guidance during development have been described. Less is understood about the molecular mechanisms guiding neuronal migration (Wu et al., 1999), however, the same molecular cues that guide axon outgrowth and targeting are also involved in neuronal migration (Song and Poo, 2001). The main disparity is that in axon guidance, the guidance cue acts on the growth cone, whereas in migration it is not yet clear whether molecules only act on the growth cone, or whether they also act on the cytoskeleton of the cell body. This may be an important factor accounting for different responses to the same molecular guidance cues. The following sections describe the effects of individual molecular guidance cues on axon outgrowth and neuron migration.

1.2.3.1 Neural Cell Adhesion Molecules

The ability of a neuron to extend its axon and innervate targets is to a large extent governed by binding of CAMs on the growth cone to CAMs on the surface of other axons and non-neuronal cells (Skaper et al., 2001). In some cases, the binding of CAMs provides strong enough adhesive interactions between the cell and its substrate to create
### Table 1.1 Classification of molecular guidance cues*

<table>
<thead>
<tr>
<th>Family</th>
<th>Class</th>
<th>Members</th>
<th>Receptors</th>
<th>Section(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Adhesion Molecules</td>
<td>Ig Superfamily</td>
<td>L1, NCAM, PSA NCAM</td>
<td>homophilic binding, Integrins</td>
<td>1.2.3.1, 1.3.3.1</td>
</tr>
<tr>
<td>Extracellular Matrix Molecules (ECMs)</td>
<td>Laminins</td>
<td>laminin-1</td>
<td>Integrins</td>
<td>1.2.3.2</td>
</tr>
<tr>
<td></td>
<td>CSPGs</td>
<td>NG2, versican, neurocan, brevican, phosphacan</td>
<td>undetermined</td>
<td>1.3.2.2.1, 1.3.3.1.1</td>
</tr>
<tr>
<td></td>
<td>Slits</td>
<td>Slit-1, Slit-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Netrins</td>
<td></td>
<td>Netrin-1, Netrin-2</td>
<td>DCC, UNC-5</td>
<td>1.2.3.3</td>
</tr>
<tr>
<td>Ephrins</td>
<td></td>
<td>ephrin A5, ephrin B2</td>
<td>Eph A, Eph B</td>
<td>1.2.3.4</td>
</tr>
<tr>
<td>Semaphorins</td>
<td></td>
<td>Sema 3A – 3F, Sema 6A</td>
<td>neuropilins plexins</td>
<td>1.2.3.5, 1.3.3.1.2</td>
</tr>
<tr>
<td>Myelin associated inhibitors</td>
<td>MAG, Omgp, Nogo</td>
<td></td>
<td>Nogo</td>
<td>1.3.2.2.2</td>
</tr>
</tbody>
</table>

* A summary of the guidance cues discussed in this thesis.
enough tension to pull the growth cone forwards, causing axon growth (Lamoureux et al., 1989). CAMs are also important for forming adhesive interactions between cells and the substrate during the migration of cells in multiple systems throughout the body, including neurons during early CNS development.

1.2.3.1.1 L1

Much of the information about L1's role in axon growth and guidance has been provided by the analysis of patients with L1 genetic mutations (for example Fransen et al., 1995; Wong et al., 1995). In such patients, the phenotype can include absence or diminuation of two major axon tracts; the corticospinal tract (CST) and the corpus callosum (Wong et al., 1995). This has also been demonstrated in vitro for numerous neuronal phenotypes, where assays have shown increased outgrowth of axons grown on an L1 substrate (for example Doherty et al., 1995; Kamiguchi and Yoshihara, 2001). Furthermore, L1 function blocking antibodies perturbed neurite outgrowth and caused pathfinding errors in sensory axons (Honig et al., 2002).

Studies of L1 deficient mice have provided examples of cross-talk between guidance cues. L1 was identified as a component of the Sema 3A receptor complex with Neuropilin-1 (NP-1), the receptor complex must be internalised for Sema 3A to have its repulsive action, and therefore cortical axons from L1-deficient mice fail to respond to Sema 3A signals (Castellani et al., 2000; 2004). Similarly, growth cone collapse stimulated by Ephrin B was abolished when retinal axon growth cones were co-stimulated with L1, showing that axon guidance molecules impinge on each other to modulate growth cone responses (Suh et al., 2004).

Several lines of evidence support a role for L1 in neuron migration, which was originally identified when L1 antibodies disrupted the migration of cerebellar granule cells, in vitro (Persohn and Schachner, 1987; Asou et al., 1992a; Miura et al., 1992). Additionally, rat cerebellar granule cells co-cultured on a substrate of L1-expressing astrocytes showed increased migration compared with neurons cultured with control astrocytes (Yazaki et
al., 1996) and an L1 substrate increased the speed of migration of cerebellar neurons (Asou et al., 1992b). Immunohistochemistry has shown that *in vivo* L1 is expressed by migrating neurons in the developing neocortex (for example, Fushiki and Schachner, 1986; Godfraind et al., 1988; Mintz et al., 2003).

L1 knockout mice have also demonstrated the role of L1 in the migration and organisation of dopaminergic neurons in brain development (Demyanenko et al., 2001), while cortical neurons and cortical lamination appeared normal in L1-minus mice (Demyanenko et al., 2001). NCAM, and its polysialated form PSA-NCAM are close relatives of L1, and both have been implicated in neuron migration, because NCAM and PSA-NCAM mutant mice show reduction in size of the olfactory bulb, due to the decreased migration of neuronal precursors which accumulate in the RMS, never reaching the bulb (reviewed by Sobeih and Corfas, 2002).

1.2.3.2 The Extracellular Matrix

The extracellular matrix (ECM) is a complex network of proteins and polysaccharides that fills the intercellular space. Key components of the ECM include laminins, chondroitin sulphate proteoglycans (CSPGs), slits, collagens, tenascins, fibronectin and reelin. Cells produce all components of the ECM, and are in turn affected by them (Sobeih and Corfas, 2002). The ECM influences development of the nervous system, including axonal guidance and neuron migration. The ECM may 1) contribute to the formation of gradients of attractants or repellents 2) allow for the combined presentation of some regulatory signals 3) regulate the formation of brain structures necessary for migration, such as axon tracts 4) modulate the signalling of receptors for guidance or migration modulating cues (Sobeih and Corfas, 2002).

1.2.3.2.1 Laminins

Laminins are considered amongst the most potent, well-studied, but least understood neurite-outgrowth promoting families (Grimpe and Silver, 2002). Although it is not commonly specified, most studies have investigated the functions of Laminin-1.
Knockouts of one subunit of laminin are not viable, with the failure to assemble the earliest basement membranes of the endoderm (Smyth et al., 1999). In the nervous system, laminin is transiently expressed during development, localised to the outer pial surface where pioneering axon tracts commonly form (Hopker et al., 1999), primarily in extracellular locations, associated with Schwann and astroglial cells. Whether neurons express laminin remains controversial (Grimpe and Silver, 2002). Laminin is virtually undetectable in the adult brain, except for the hippocampus (reviewed by Colognato et al., 2005). They have long been recognised for their involvement in developmental processes including neuronal differentiation (Tate et al., 2004), cell polarisation (Matsuzawa et al., 1996; Yurchenko and Wadsworth, 2004) and neurite outgrowth (Kearns et al., 2003; Turney and Bridgeman, 2005), and it has been widely demonstrated that laminin, or specific laminin peptides, support neurite outgrowth, in vitro (for example Bixby et al., 1989; Freire et al., 2002; Liu et al., 2002; Adams et al., 2005; Turney and Bridgeman, 2005).

Laminins are also involved in neuron migration, localised to radial glial fibers along the routes of migrating neurons and axons (reviewed by Liesi, 1990). Mutations in basement membrane laminin in the developing CNS lead to abnormal neuron migration and lissencephaly. Neural progenitor cells from the ganglionic eminence grown on a number of substrates show significantly enhanced migration on laminin (Tate et al., 2004) and laminin also promotes significantly increased cell migration velocity (Kearns et al., 2003). Laminin, present in the developing cerebellum, acts as a permissive migratory substrate for granule cell precursors to migrate from the external granule cell layer into the internal granule cell layer (Pons et al., 2001), and function blocking antibodies inhibit cerebellar granule cell migration in vitro (Liesi et al., 1995).

1.2.3.2.2 Chondroitin Sulfate Proteoglycans (CSPGs)

CSPGs are components of the ECM, generally classified as growth inhibitory molecules that function by restricting axon growth to inappropriate targets (Treloar et al., 1996; Laabs et al., 2005) and serving as choice points for growing axons. For example, in the
developing CNS, retinal ganglion cells (RGCs) grown on laminin stop and turn to avoid a CSPG substrate (Snow et al., 1991). In addition, the presence of CSPGs causes reduced cell attachment, neurite outgrowth, neurite initiation and rates of growth cone migration (Snow et al., 1996). In the more mature CNS, CSPGs are associated with plasticity, and are inhibitory to regeneration, being a major component of the glial scar that inhibits axon regeneration (section 1.3.2).

The effect of CSPGs on neuron migration has not been widely studied. Neural crest cells avoid regions containing CSPGs at several points in their pathway, and inhibition of CSPG synthesis causes them to make aberrant trajectories (Kubota et al., 1999). The CSPG versican has also been shown to inhibit migration of neural crest cells (Landolt et al., 1995).

1.2.3.2.3 Slits

Slits are bifunctional ECM molecules, which bind to Roundabout (Robo) transmembrane receptors expressed on target neurons (Kidd et al., 1999). Early slit mutant studies showed disorganisation of axons at the midline, which was consistent with a role in either the development of midline cells or axon guidance (Rothberg et al., 1990). Slits are now known to be involved in multiple pathways of axon projection (including hippocampal, retinal, olfactory, spinal motor and commissural axons) and neuron migration (including the RMS and neocortex) (Patel et al., 2001; Yanamoto et al., 2002). They may also regulate axonal branching (Wang et al., 1999).

Demonstrating their bifunctional nature and complexity of action is that slits can repel axons from the spinal cord of *Xenopus*, as well as modestly promoting their outgrowth, *in vitro* (Stein and Tessier-Lavigne, 2001). Furthermore, the spatial and temporal regulation of expression of netrin- deleted in colorectal cancer (DCC) and slit-robo systems prevents commissural growth cones that have already crossed the midline from re-crossing (Stein and Tessier-Lavigne, 2001; reviewed by Chotard andSelecker, 2004).
Slit proteins can repel various classes of migrating neuronal cells in vertebrates (Hu, 1999; Wu et al., 1999; Zhu et al., 1999). They are repellent for the migration of both interneuron precursors from the anterior subventricular zone in the telencephalon to the olfactory bulb, and GABA-containing neurons from an extracortical region to the neocortex (Zhu et al., 1999; Song and Poo, 2001). Experimentally, extracellular gradients of Slit-2 cause a reversal in the direction of migration without affecting migration speed (Xu et al., 2004). Uniform application of slit to growth medium of subventricular zone explants causes a significant reduction in the number of neurons migrating out of the explants (Mason et al., 2001), which indicated that slit was inhibitory to migration, but later experiments by Ward et al., (2003) showed that slit was a repellent in neuron migration rather than inhibitory. Slits are repulsive for anterior subventricular zone cells, since in vitro assays showed that cells migrated away from the source of slit (Wu et al., 1999) and application of slit expressing cells to the top of the RMS in brain slices results in complete abolition of migration to the olfactory bulb (Wu et al., 1999).

1.2.3.3 Netrins

The first family of guidance cues to be identified in the vertebrate nervous system was the netrins. Serafini et al., (1994) purified Netrin-1 and Netrin-2, secreted molecules related to laminin, from embryonic chick brain and demonstrated their ability to promote growth of commissural axons. Later, the importance of netrins was demonstrated in Netrin-1 deficient mice, which lack the main forebrain commissures; the corpus callosum, hippocampal commissure and the anterior commissure (Serafini et al., 1996). In the developing brain, Netrin-1, the most studied of the family, is expressed in the ventral region of the spinal cord, and the midline and ventral ventricular zones (reviewed by Barallobre et al., 2005).

Netrins have since been shown to be bifunctional guidance molecules; attractive to some axons but repulsive to others. For example, netrin cues help attract commissural axons to the ventral midline but repel motor axons from the same region (Chisolm and Tessier-
Lavigne, 1999). Two different classes of membrane receptors are involved in mediating netrin attractive/repulsive actions; DCC, which mediate the chemoattractive functions and the UNC-5 family of receptors, which mediate repulsive functions.

More recent experiments investigating netrin function have illustrated the complexity of axon guidance in the developing and adult brain. Firstly, in *Xenopus* young retinal axons are attracted to the optic nerve by Netrin-1, whereas later in development it repels the same axons (reviewed by Barallobre et al., 2005). Furthermore, although Netrin-1 is attractive to axons, in the presence of Laminin-1 this attraction is converted to repulsion (Hopker et al., 1999).

There is evidence for a role for netrins in neuron migration, in several areas of the brain where migration is observed, particularly for dopaminergic neurons of the midbrain, cerebellar granule cells and in the RMS, both in development and throughout life (reviewed by Barallobre et al., 2005). In addition, netrins direct the migration of basilar pontine neurons from their origin to the ventral midline (Yee et al., 1999) and repel neurons that migrate from the anterior forebrain to the olfactory bulb (Wu et al., 1999; Liu and Rao, 2003). They also play a role in the laminar organisation of the cerebellum (reviewed by Hedgecock and Norris, 1997).

1.2.3.4 Ephrins

Ephrins are membrane bound, chemorepellent guidance molecules that have been implicated in axon pathfinding, causing collapse and repulsion of growth cones, as well as neuronal migration (reviewed by Sobeih and Corfas, 2002). In the adult nervous system ephrins have a role in synaptic plasticity, partly through the regulation of actin dynamics during dendritic spine morphogenesis (Penzes et al., 2003, Murai et al., 2003). Ephrin A’s are linked to the membrane by a GPI linkage, whereas ephrin B’s are transmembrane proteins. Ephrin receptors are Eph A and Eph B. Generally, ephrin A’s show an affinity toward Eph A receptors, while ephrin B’s have an affinity for Eph B
receptors (reviewed by Klein, 2004). The same set of receptors are involved in both
growth cone pathfinding and neuron migration (Song and Poo, 2001).

In vitro, ephrins repel RGC axons (Nakamoto et al., 1996; Monschau et al., 1997) and
are involved in the formation of the retinotectal map. Ephrin A5 acts as a repulsive
signal for neurons in the upper cortical layers, but has no axon guidance function for
cells in the deeper cortical layers, instead promoting axon branching in some cortical
neurons (Castellani et al., 1998). Ephrins may cause growth cone collapse in stripe
assays, but in other instances promote cell outgrowth or cell migration (reviewed by
Chisolm and Tessier-Lavigne, 1999), which provides another example of the complexity
of molecular guidance cue action.

The ephrins and their receptors appear to be important for providing short-range
repulsive cues in guiding the migration of neural crest cells, as well as differentiated
neurons such as cerebellar granule cells (Sobeih and Corfas, 2002; Klein, 2004). For
example, ephrin B2 and its receptor EphB2 are expressed in the external granule layer of
the cerebellum specifically at the onset of migration of post-mitotic cerebellar granule
cells toward the internal granule layer, which combined with the loss of responsiveness
to the chemoattractive chemokine SDF-1α (Lu et al., 2001), induces granule cell
migration.

1.2.3 Semaphorins

The semaphorins are predominantly chemorepellent membrane-associated or secreted
proteins, and fibroblasts are their main source of expression in the nervous system
(Grimpe and Silver, 2002). Sema 3A was the first member of the family to be
discovered, and was originally called Collapsin-1, due to its ability to induce growth
cone collapse and paralysis (Luo et al., 1993). Their receptor classes include neuropilins
(NP’s) and plexins.
Polleux et al., (1998) demonstrated a number of properties of semaphorin action, including 1) that cortical neurons respond to a gradient of Sema 3, 2) that Sema 3 can act as a diffusible chemorepellent to cortical axons, and 3) that endogenous Sema 3 contributes to directed cell growth of cortical axons. Finally, they showed that in vivo, approximately half of the neurons in the Sema 3 null mice display aberrantly oriented apical dendrites and axons, indicating Sema 3 is required for appropriate morphological development.

However, semaphorins and their receptors do not always have repellent actions. For example, recently it was shown that Sema 3B is involved in the formation of two pathways of the anterior commissure, actually by attracting some axons, which was in contrast to the role of Sema 3F in the same system (Julien et al., 2005). In many cell types, including sensory ganglion cells, Sema 3A causes growth cone collapse and axon repulsion, but retinal ganglion cell growth cones are unresponsive. In addition, Sema D is chemorepellent to cortical axons, whereas Sema E acts as an attractive guidance cue to cortical axons (Bagnard et al., 1998). These data demonstrate that semaphorin interactions in the developing CNS cannot always be categorised as repellent.

More recently, semaphorins have been implicated in neuron migration. Tamamaki et al., (2003) showed that placing COS1 cells expressing Sema 3A or Sema 3F on neocortical slices reduced, but not completely blocked, the migration of GABA neurons from the ganglionic eminence. However, cell migration was almost completely blocked when cell clusters expressing both Sema 3A and Sema 3F were applied. Similarly, cortical interneurons do not enter the striatum in part due to Sema 3A and Sema 3F expression (Marin et al., 2001). In other experiments, mice deficient of the transmembrane semaphorin, Sema 6A, were shown to have mostly normal cerebellar development, with the exception of aspects of granule cell migration (Kerjan et al., 2005). In combination, their results suggested a role for Sema 6A specifically in the control of initiation of cerebellar granule cell radial migration. Semaphorins also promote cell movement in other systems, such as endothelial cells during remodelling of the vasculature (reviewed by Kruger et al., 2005).
1.2.4 The intracellular response to guidance cues

Amongst other intracellular signalling molecules, the Rho family of small GTPases regulate cell motility. In general, Rho activation is inhibitory to neurite growth, while Rac and Cdc42 activation promote neurite outgrowth. There is mounting evidence from in vitro studies that Rho GTPases are involved in cell migration via their action on the actin cytoskeleton (Bar-Sagi and Hall, 2000). Migrating cortical neurons transfected with dominant negative and constitutively active Cdc42 and Rac1 show significantly inhibited radial migration (Konno et al., 2005).

It is becoming clear that Rho GTPase activity in neuron growth and migration is modulated by guidance cues. For example, it has been shown that Rho GTPases play a role in Sema 3A induced growth cone collapse, as introduction of dominant negative Rac1 into DRG neurons attenuates their response to Sema 3A (Jin and Strittmatter, 1997). Similarly, Kuhn et al., (1999) demonstrated that Sema 3A induced collapse of motor neuron growth cones is mediated by Rac1. Ephrin A5 induced growth cone collapse occurs through the activation of Rho and Rho kinase (Wahl et al., 2000). Rho GTPases are involved in Netrin-1 dependent neuronal migration (Causeret et al., 2004), and brain derived neurotrophic factor (BDNF) attraction is mediated by Cdc42 activation in developing neurons. This is a relatively new area of research and these data represent the beginnings of our knowledge about the relationship between molecular guidance cues and intracellular signalling.

1.2.5 CNS development and regeneration may share common mechanisms

The importance of understanding CNS developmental mechanisms has taken on a new perspective in recent years. It is now thought that for neuronal regrowth to occur following injury a similar set of events must occur. Consequently, there is now a focus toward understanding the mechanisms of axon growth and pathfinding, as well as neuron migration, and the attractive and inhibitory factors that would influence the ability of the mature nervous system to undergo functional regeneration. Furthermore,
whether mature neurons respond to molecular guidance cues the same way as they do during development is uncertain, and is therefore the subject of recent investigation.
1.3 CNS INJURY AND THE REGENERATIVE RESPONSE

Without manipulation, the adult mammalian CNS has a very limited capacity for regeneration after injury, when compared with the embryonic CNS, the PNS, and the CNS of lower vertebrates. If CNS neurons do survive injury, sprouting axons extend only for very short distances, about one millimetre, before they cease growing and degenerate at the lesion border (reviewed by Stichel and Müller, 1998). A number of factors account for the inability of the CNS to regenerate, including the susceptibility of neurons to death after injury, a reduced capacity for post-mitotic neuron growth, and the absence of appropriate neurotrophic factors and permissive guidance cues. The glial or astrocytic scar acts as a physical barrier to regrowth. Also, the mature CNS environment contains chemical inhibitory factors, such as components of mammalian oligodendrocytes, CNS myelin and repulsive guidance cues.

It is now thought that in order to regenerate, particular environmental and cellular aspects of the developmental nervous system must be recapitulated (see recent review by Harel and Strittmatter, 2006). Identification of important cytoskeletal proteins and growth promoting or inhibitory factors during development and within the post-injury milieu will facilitate investigation into possible manipulation of the cellular response to injury, with the ultimate aim being to induce functionally significant regeneration in the injured nervous system.

1.3.1 The intrinsic capacity for mature CNS growth and regeneration

In the past there was a firm belief that the mature nervous system was remarkably unadaptable compared with the developing nervous system, and axons and dendrites retained very little potential for plasticity and could not undergo growth. However, over two decades ago, David and Aguayo (1985) reported significant evidence supporting that mature neurons retain the ability to grow, but instead inhibition was due to their environment. When they provided the injured CNS with a peripheral nerve graft, CNS neurons were able to regenerate. More recent studies using varied approaches to aid regeneration have supported this theory (reviewed by Fawcett and Geller, 1998).
Indeed, there are a number of examples demonstrating the plasticity of the mature CNS, including the ability of neurons derived from stem cells to integrate into existing neuronal circuitry, that axons can sprout into denervated regions and collateral sprouting can occur following injury, as well as the non-injury related dynamic nature of dendritic spines and synapses in the mature CNS (reviewed by Chuckowree et al., 2004).

1.3.1.1 Expression of developmentally significant cytoskeletal proteins

For the mature nervous system to mount a regenerative response to injury, the expression of components required for the assembly of new cytoskeletal structures would be required. Indeed, some investigations, particularly in in vitro models of axonal injury, have demonstrated experimental application, re-expression and re-localisation of growth-associated cytoskeletal proteins, including GAP-43 (for example, Christman et al., 1997; Avwenagha et al., 2003; Zhang et al., 2005; Ikegami et al., 2005), CAP-23, MAP1B (Emery et al., 2000; reviewed by Bulsara et al., 2002), tau (Chuckowree and Vickers, 2003), α-internexin (Dickson et al., 2005), β-III-tubulin (McKerracher et al., 1993; Avwenagha et al., 2003; Chuckowree and Vickers, 2003), F-actin (Avwenagha et al., 2003; Chuckowree and Vickers, 2003) and phosphorylated neurofilament (Chuckowree and Vickers, 2003). Furthermore, when the microtubule stabilising and destabilising agents, taxol and nocodazole, respectively, were applied to transected cortical axons in vitro, the result in both cases was a substantial negative effect on the post-injury sprouting response (Chuckowree and Vickers, 2003), indicating the importance of cytoskeletal components in regenerative growth.

1.3.2 Inhibition of regeneration in the mature CNS

1.3.2.1 The glial scar

In the hours to weeks after CNS injury takes place, a number of different cell types, all with different roles in the post-injury response, infiltrate the injury site. The main cell types involved include astrocytes, microglia, oligodendrocyte precursors, meningeal cells and stem cells. Cells recruited to the injury site fill the vacant space forming a
zone of reactive gliosis. This reactive gliosis is characterised by a dense web of interlacing glial processes that form within a lesioned area (Stichel and Müller, 1998) forming a physical barrier to regeneration, as well as having inhibitory biochemical properties that hinder regenerative growth. Glial reactions peak at about two to three weeks post-injury, but may persist for up to two years (reviewed by Stichel and Muller, 1998). The properties of the glial scar, and the growth inhibitory properties of oligodendrocytes and CNS myelin are amongst the more completely understood inhibitors of regeneration.

1.3.2.2 Inhibitory molecules of the injured CNS

There are two main sources of inhibitory molecules in the post-injury environment. The first is the cells of the glial scar, which secrete inhibitory guidance molecules, including ECM molecules of the CSPG family. The second source is myelin, the breakdown products of which may be inhibitory to neurite growth, and thus regeneration.

1.3.2.2.1 CSPGs

CSPGs are produced by reactive astrocytes, oligodendrocyte precursor cells and meningeal cells of the glial scar (reviewed by Fawcett and Asher, 1999; Tang, 2003; De Winter et al., 2002) and are thought to be inhibitory to regeneration in the mature nervous system based on at least three interrelated findings; 1) they inhibit axon growth in vitro, 2) there is considerable expression of CSPGs in the injury site and 3) disruption of CSPGs in tissue culture models of the glial scar reduces inhibition of regeneration (reviewed by Morgenstern et al., 2002). Expression of the CSPGs NG2, brevican, neurocan, versican and phosphacan is upregulated following brain injury has been demonstrated in numerous investigations (McKeon et al., 1991; reviewed by Morgenstern et al., 2002; Jones et al., 2003), and in a recent review, Tang (2003) speculated that elevated levels of CSPGs may prove to be the ultimate stumbling block for any neuronal regeneration.
CSPGs interact with a number of molecules on the cell surface and extracellular matrix, with complex and varied effects, compounding the difficulties of determining mechanisms of inhibition (Tang, 2003). To this end, a number of studies using different approaches have successfully inhibited CSPGs to facilitate neurite outgrowth. For example, chondroitin sulfate (CS) degrading enzyme improved axonal regeneration and brought about functional recovery of spinal cord axons when the enzyme was applied to rats subjected to spinal cord injury (Bradbury et al., 2002). Similarly, digestion of CSPG by chondroitinase ABC promoted axonal regeneration in the rat nigrostriatal system (Moon et al., 2001), and suppression of CSPG signalling through particular Rho pathway members is successful in increasing axon regeneration (Jain et al., 2004).

In a glial scar model used by McKeon and colleagues (1991), nitrocellulose filters implanted in the injured rat cerebral cortex were removed and used as a substrate for neurite growth in vitro. The filters contained a large amount of CSPG and were very poor substrates for neurite outgrowth. However, treatment of the filters with a CSPG enzyme increased neurite outgrowth.

Interestingly, one mechanism by which NG2 (a CSPG) inhibits regeneration is by binding laminin and, thereby inhibiting laminin’s neurite growth promoting ability (Burg et al., 1996). Furthermore, the presence of L1 on a mixed substrate of laminin and NG2 abolished the inhibitory effects of NG2 (Dou and Levine, 1994). These interesting data demonstrate the complexity of interaction of molecular guidance in the mature CNS, much like during development. In another example, a fibroblast bridge expressing CSPGs, L1 and laminin was permissive to regeneration, indicating that a balance of inhibitory and permissive cues in favour of permissive cues is required for regeneration and it is perhaps not necessary to completely abolish inhibitory cues (Jones et al., 2003).

1.3.2.2.2 Myelin inhibitors
Myelin derived inhibitors of axonal regeneration include myelin associated glycoprotein (MAG), Nogo and oligodendrocyte myelin glycoprotein (Omgp) (Qiu et al., 2000;
reviewed by Grados-Munro and Fournier, 2003). While all three proteins are quite different, they have been shown to bind to the same receptor, the Nogo receptor (NgR) (reviewed by Watkins and Barres, 2002). This was an exciting discovery for the field of regeneration as blocking the receptor provides an ideal target for regeneration-promoting strategies.

MAG is released when the myelin sheath surrounding injured neurons degenerates, but interestingly, it is not inhibitory to embryonic neurons, in vitro (Tang et al., 2001). Its inhibitory properties in mature brain injury and regeneration have been widely demonstrated using a variety of approaches. For example, when animals are immunised with myelin they extensively regenerate axons, with some functional recovery (Huang et al., 1999). In contrast, others have reported, MAG may not be a major contributing factor to inhibition of regeneration, since in vivo, axons can grow through degenerating myelin (reviewed by Stichel and Muller, 1998), and MAG deficient mice do not show improved regeneration (Bartsch, 1996). However, it is unlikely to be a coincidence that the PNS has a much better regenerative ability than the CNS, and MAG is ten times more abundant in the CNS than PNS (Trapp 1988; 1990, reviewed by Filbin, 2006).

1.3.3 The role of molecular guidance cues in the post-injury regenerative response

Many developmental guidance molecules (section 1.2.3) and their receptors show continued expression in the mature nervous system, suggesting that these molecules play a role in the CNS throughout life. However, there appears to be differences in the distribution patterns of these molecules, and additionally, CNS injury appears to induce changes in the expression of guidance molecules such as CAMs and semaphorins. Thus, a question that is currently being asked is whether the expression and action of these guidance cues can be manipulated to result in a recapitulation of the orderly events that occurred during the development of the CNS in order to encourage regeneration in the mature nervous system (Koerberle and Bahr, 2004).
1.3.3.1 L1

The CAM L1 has been proposed to play a role in repair attempts after injury. In an early study, optic nerve crush in the fish resulted in a 25% increase in L1 immunoreactivity (shown by radioimmunoassay) (Blaugrund et al., 1990). Subsequently, Poltorak and colleagues (1993) showed that frontal cortical lesions promoted an increase in L1 (and N-CAM) expression in the striatum. Similarly, after axotomy of CST axons expression of L1 (and a number of other growth-associated molecules) was up-regulated seven days post-injury (Mason et al., 2003).

In more recent years, a number of studies have examined the effects of applying, or experimentally up-regulating L1 in the injured nervous system. For example, Woolhead and colleagues (1998) showed that when Purkinje cells, which do not normally regenerate into peripheral nerve grafts or express GAP-43 or L1, were engineered to express L1 or GAP-43, they showed an enhanced axonal sprouting response into a peripheral nerve graft. Cells expressing both L1 and GAP-43 showed more extensive sprouting within the weeks after injury and graft implantation (Zhang et al., 2005).

*In vivo* studies of spinal cord injury have also supported a role for L1 in enhancing nerve regeneration. For example, L1 transfected embryonic stem cells injected into the spinal cord after compression injury showed greater survival, and extended longer processes, increased migration and populated the lesion site (Chen et al., 2005). Another interesting study involved intrathecal L1 administration for two weeks after spinal cord contusion, which resulted in significantly increased locomotory recovery in rats, compared with controls. However, regeneration of CST axons was not extensive, and the authors concluded that L1 promoted locomotory recovery via a different mechanism, such as regeneration of non-CST motor-related descending tracts, protection and survival of axons, prevention of apoptosis or other changes in the distal cord, including the locomotor pattern generator, or even myelination of axons (Roonprapunt et al., 2003). This study therefore suggests that L1's role in regeneration may not strictly be due to its axon outgrowth promoting properties. In agreement with this study, antisense morpholino oligonucleotides used to knockout L1.1 (homolog of human L1) after spinal
cord injury in adult zebrafish resulted in impaired functional recovery, regrowth and synapse formation, compared with controls, indicating that L1.1 is an important part of the regenerative response (Becker et al., 2004).

Recently, Adcock and colleagues (2004) demonstrated the importance of L1 in axon guidance during nerve regeneration. At the CNS / PNS interface there is a boundary between astrocytes and Schwann cells. Axons will cross from astrocytes to Schwann cells, but not vice versa. The authors hypothesised that this may be either due to the presence of CSPGs, or because L1 is produced by Schwann cells but not astrocytes. Abolishing CSPGs did not alter the effect. However, manipulation of L1 by transfection into astrocytes increased the proportion of axons able to cross the boundary, indicating that L1 is an important factor in this system. In contrast, a recent report has shown that when comparing E9 and E15 brainstem axons, providing the more mature E15 axons with an L1 substrate after injury did not substantially increase regenerative growth (Blackmore and Letourneau, 2006).

1.3.3.1.1 Laminins
Positive staining for laminin in the adult CNS is rare (Grimpe and Silver, 2002). However, as in the developing nervous system, specific laminin peptides have neurite growth promoting ability, and therefore are being considered as useful agents for facilitating the same functions in the injured CNS. They may be useful for the repair of damaged neurons, such as in tissue engineering strategies, to support directed growth across the CNS lesion and glial scar (reviewed by Meiners and Mercado, 2003).

1.3.3.1.2 Semaphorins
The semaphorin family of molecular guidance molecules are well known for their potent chemorepellent properties during CNS development and recent studies have shown Sema 3A to play a predominantly repulsive guidance role in axon regeneration. High levels of Sema 3A expression in various CNS injury models have been reported. For
example, olfactory bulbectomy, as well as penetrating injuries to the lateral olfactory tract, cortex, perforant pathway and spinal cord induce proliferation and migration of fibroblasts-like cells expressing high levels of Sema 3A mRNA (Pasterkamp et al., 1998a; 1999), preventing regenerating axons from entering the scar. In contrast, after axotomy of the olfactory nerve there is local and transient increased Sema 3A expression, which may induce a molecular boundary for regenerating neuron growth rather than inhibiting growth altogether (reviewed by Grimpe and Silver, 2002).

Interestingly, PNS neurons, which have a much better capability for regeneration than CNS neurons, appear to downregulate Sema 3A in response to injury (Pasterkamp et al., 1998b; reviewed by De Winter et al., 2002), a result which may indicate Sema 3A repulsion as a key inhibitor of CNS regeneration.

1.3.4 Strategies to improve post-injury regeneration

A comprehensive approach to the restoration of CNS function following injury is a complex task, as it would need to include: the rescue of neurons from death; replacement of dead and dying neurons; prevention of axonal disruption; promotion of axonal regeneration; remyelination of regenerated axons and the restoration of anatomical connections to recovered function (Selzer, 2003). Therapeutic approaches that restore the extracellular CNS environment of the neuron, characteristic of a more embryonic state, increasing axon regeneration and improving functional recovery after injury are gaining popularity.

Current molecular strategies under investigation include the use of neutralising antibodies against growth inhibitory molecules, as used to inhibit the action of CSPGs (section 1.3.2.2.1), addition of growth promoting factors, such as L1 (section 1.3.3.1), manipulation of the expression of important growth-promoting cytoskeletal components, modification of signalling cascades and preventing or removing the glial scar. Other strategies may include transplantation and cellular approaches, such as the use of peripheral nerve grafts, or transplantation of foetal tissue, stem cells, or ensheathing
cells. Each approach could potentially have a significant contribution and it is commonly recognised that a combination of therapies would be required to encourage functional regeneration.
1.4 PROJECT AIMS

While the mechanisms of nervous system development are gradually being elucidated, much less is understood about how the more mature CNS responds to injury; or what therapeutic approaches may successfully promote regeneration. One way to address this issue is to identify molecules particularly important to the development of the CNS, and their specific mechanisms of action, as they are likely to be highly indicative of the requirements for regenerative growth in the more mature nervous system. Relatively little is known about the functions of the ERM proteins in the CNS, but they appear to mediate many processes that are important for appropriate neuronal development. Further, alluding to their developmental significance, their functions are potentially mediated by interaction with the crucial neural CAM L1. Therefore, this thesis will investigate the role of ERM proteins in nervous system development and the reactive and regenerative response to traumatic injury.

Aim 1

*To investigate the specific expression of ERM proteins, throughout neuronal morphogenesis.*

There has not previously been a comprehensive study that has examined the localisation of ERM proteins in CNS neurons throughout their development and maturation. Therefore, immunocytochemistry will be utilised to determine the localisation of total ERM proteins, as well as the phosphorylated (active) ERM protein pool, particularly in relation to L1. This will be performed in *in vitro* cultures of primary neurons to facilitate observation of their localisation in specific cellular structures and compartments.

Aim 2

*To identify functions of the ERM proteins in neuronal morphogenesis.*

An ideal method for determining specific cellular functions of a protein is to perturb its function. Therefore, a dominant negative form of ezrin will be transfected into neurons. Neuron behaviour will be observed by time-lapse imaging, followed by quantitation of
several aspects of their growth and morphology, compared with neurons not expressing
the dominant negative ezrin.

Aim 3

To investigate the role of ERM proteins and L1 in neurite outgrowth, during
development and in the neuronal response to trauma.

Evidence has implicated both the ERM proteins L1 in neurite outgrowth in developing
CNS neurons. These experiments will seek to confirm these roles, and subsequently
determine whether these proteins are similarly involved in the regenerative neurite
sprouting response that results from injury of more mature neurons. An *in vitro* model
of transection of neuronal processes, combined with the application of L1 protein as a
neuronal growth substrate, will be used.

Aim 4

To identify the characteristics of a motile neuron population in long-term cortical
neuron cultures, particularly in relation to potential ERM involvement.

A population of highly motile neurons, observed utilising the *in vitro* transection injury
model, translocate into the injury site. In these experiments birth-dating and
immunocytochemical techniques will be used to further investigate the specific
phenotype of this neuron population, and to further examine the putative role of ERM
proteins in neuronal migration.

Aim 5

To examine the mechanisms of ERM activation in neurons.

Studies in non-neuronal cell types have indicated that a number of different enzymatic
systems are involved in the activation of ERM proteins, and this may be cell-type
specific as well as dependent on the biological context. Application of an inhibitor of
Rho kinase will be used to examine the role of Rho kinase in ERM activation. The
functional outcomes of perturbing ERM activation will be examined.
Chapter 2 – Materials and Methods

2 MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 Substrate preparation for neuron culture

2.1.1.1 L1-Fc chimera protein

L1-Fc chimera protein was produced according to previously reported methods (De Angelis et al., 1999; Dickson et al., 2002). Briefly, COS-7 cells were maintained in 10mm tissue culture plates in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% FCS (Gibco) and 1% penicillin / streptomycin antibiotic (Gibco). Prior to transfection, cells were subcultured and transferred to 150mm tissue culture plates (IWAKI) and cultured to approximately 70% confluence. To transfect four dishes, a transfection solution containing 60µg L1-Fc DNA (section 2.6), 1mL Dextran (400mg/mL), 1mL Chloroquine (100mM) and 38mL DMEM / 10% FCS medium was prepared. Cultures were washed once with medium and 10mL transfection solution was added to each plate and returned to the incubator (37°C, 5% CO₂). After three hours the transfection solution was replaced with 4mL 10% DMSO / PBS solution at room temperature for two minutes, while gently swirling. This solution was immediately removed and replaced with 20mL DMEM containing 10% low IgG FCS (Gibco). 24 hours later the medium was replaced with 25mL DMEM containing 1% low IgG FCS (Gibco) and returned to the incubator for six days, to allow L1-Fc chimera protein to accumulate.

For L1-Fc protein purification, aliquots containing 0.05g Protein A Sepharose (Amersham Biosciences) in 1mL 0.01M PBS were pre-swelled overnight on a rocker, at 4°C. The conditioned media was centrifuged (7 minutes, 1000rpm, 4°C) and the supernatants were combined. One aliquot of Protein A Sepharose solution was added to each 50mL media, which was then incubated overnight on a rocker, at 4°C. Supernatants were applied to a blank chromatography column (Bio-Rad), followed by two 0.01M PBS washes. The protein was eluted with 2mL 0.1M glycine (pH 3.0), into a tube containing 200µL 1M Tris-HCl (pH 9.0). The presence of L1-Fc protein was
confirmed by Western Blotting (section 2.5) and protein concentration determined by bicinchoninic assay (BCA, Pierce), according to the manufacturers instructions.

2.1.1.2 Substrate Application

Glass coverslips (19mm, Marienfeld) were etched with nitric acid (BDH Chemicals) overnight, followed by tap water and milli-Q™ water washes, dried and heat sterilised prior to application of substrates. Coverslips were placed in 12-well tissue culture trays (IWAKI). A 0.001% Poly-l-lysine (Sigma) solution was applied to coverslips for three hours. Laminin (Sigma, 5μg/mL) was applied after poly-l-lysine, for three hours. For L1 substrates, poly-l-lysine treatment was followed by anti-human IgG Fc antibody (1:10,000, Sigma), which was incubated on coverslips for three hours. Finally, L1-Fc (4μg/mL; Kamiguchi and Yoshihara, 2001) chimera protein was applied and incubated overnight. Semaphorin 3A/Fc substrates (R & D Systems; 4μg/mL) were prepared by the same application sequence as L1-Fc. After substrate application 1mL of ‘initial medium’; Neurobasal™ (Gibco), supplemented with 10% FCS, 0.5mM L-glutamine (Gibco), 25μM glutamate (Gibco), 2% B27 (Gibco) and 1% penicillin / streptomycin antibiotic was applied to tissue culture plate wells. Prepared culture plates containing medium were placed in an incubator (37°C, 5% CO2) overnight, prior to cell culture.

2.1.2 Dissection

Time-mated pregnant Hooded-Wistar rats carrying pups of 18 days gestation (E18) were sacrificed by carbon dioxide exposure, in accordance with the guidelines stipulated by the University of Tasmania Animal Ethics Committee. Rat pups were immediately removed, placed on ice and decapitated. Meninges were carefully cut away using fine scissors, exposing the dorsal surface of the brain. For cortical neuron culture, tissue was dissected from the neocortical region using fine forceps. Hippocampal dissection was performed under microscope guidance. The brain was removed from the skull and the hemispheres separated by cutting away from the Circle of Willis on the ventral surface.
Removal of the meninges and thalamus revealed the hippocampus, which was dissected out using fine scissors.

2.1.3 Tissue processing and culture
Tissue was collected in 5mL 10mM HEPES (Sigma) buffer, at 37°C. Enzymatic digestion of the tissue was facilitated by incubation with 0.125% Trypsin (Sigma), for 15 minutes. Tissue was washed three times with HEPES and mechanically dissociated using a pipette. Staining with trypan blue (Sigma) was used to assess cell viability and concentration. Cells were plated onto coverslips in ‘initial medium’ at 5 x 10^4 cells per coverslip. At 1 day in vitro (DIV), medium was replaced with 2mL ‘subsequent media’, a serum free medium consisting of Neurobasal™ medium supplemented with 0.5mM L-glutamine, 2% B27 and 1% penicillin / streptomycin antibiotics. Every fourth day 1mL of ‘subsequent media’ was replaced with fresh medium.

2.2 TRANSECTION INJURY OF NEURON CULTURE
At 21 DIV neuron cultures consist of a dense meshwork of neuronal processes, and display characteristics of mature neurons, when developmental protein markers are no longer expressed, but markers related to maturity, such as MAP2, synaptic markers and the neurofilament triplet proteins, are (de Lima et al., 1997; Chuckowree and Vickers, 2003; King et al., 2006). At this time, cultures were subjected to transection injury, according to methods established in our laboratory (Dickson et al., 2000). Under microscope guidance a small goniotomy knife (Kaisers) was used to transect neuronal processes in selected regions of the culture. Specifically, injuries were performed in areas of high neuronal process density. Several injuries were performed per coverslip, each approximately one mm in length.

2.3 IMMUNOCYTOCHEMISTRY

2.3.1 Fixation
Cell cultures were fixed using varied methods depending on requirements of primary antibodies. Cells were fixed with either with 4% Paraformaldehyde (PFA, Sigma) for
thirty minutes at room temperature, or ice-cold 10% Trichloroacetic acid (TCA, Sigma) for twenty minutes at -20°C. TCA fixation was required for immunolabelling with an antibody that is specific for phosphorylated ERM proteins (Hayashi et al., 1999). Fixation was followed by three ten minute 0.01M PBS washes. Table 2.1 shows commonly used primary antibodies and the method of fixation required. Fixation was immediately followed by immunocytochemistry.

2.3.2 Indirect fluorescence immunocytochemistry

Following fixation, primary antibodies, which recognise and bind to specific epitopes of cellular proteins, were applied to specimens at appropriate concentrations (Table 2.1), diluted in a 0.3% Triton-X (Fluka) solution, which was used to permeabilise cell membranes. Primary antibodies were incubated on an orbital shaker for two hours at room temperature, then overnight at 4°C, followed by three 0.01M PBS washes. Species and isotype specific Alexa Fluor fluorescent secondary antibodies (Table 2.2) were applied at a dilution of 1:1000 and incubated in the dark, on an orbital shaker for 1.5 hours at room temperature. Specimens were washed three times with 0.01M PBS and mounted onto microscope slides using Permafluor (Beckman Coulter) mounting medium.

2.4 MICROSCOPY AND ANALYSIS

Fixed, fluorescent immunolabelled specimens were examined using a Leica DM LB2 immunofluorescence microscope, equipped with a cooled CCD Magnafire (Optronics) digital camera. Images were acquired using Magnafire (version 1.0) software. Image analysis, such as neurite lengths and distance of cell movement, was performed using Improvision Openlab (version 4.0.2) software. Images were prepared using Adobe Photoshop (version 9.0). Time-lapse microscopy using Normaski Interference Contrast optics was performed using a Leica DMB IRB inverted fluorescence microscope, Hamamatsu ORCA-ER digital camera and Improvision Openlab software. Statistical analysis was performed in Microsoft Excel (Mac Os X); the data was obtained from at
### Table 2.1. Commonly used primary antibodies for immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fixation</th>
<th>Species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-β-tubulin</td>
<td>4% PFA, 4% PFA, 10% TCA</td>
<td>mouse</td>
<td>1:2500</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-β-III-tubulin</td>
<td>4% PFA</td>
<td>mouse</td>
<td>1:10000</td>
<td>Promega</td>
</tr>
<tr>
<td>anti-ezrin-radixin-moesin</td>
<td>4% PFA</td>
<td>mouse IgM</td>
<td>1:5000</td>
<td>Gift from Dr F. Solomon</td>
</tr>
<tr>
<td>anti-microtubule associated protein 2 (MAP2)</td>
<td>4% PFA</td>
<td>mouse</td>
<td>1:1000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>anti-phosphorylated ERM</td>
<td>10% TCA</td>
<td>rabbit</td>
<td>1:500</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-tau</td>
<td>4% PFA</td>
<td>rabbit</td>
<td>1:10000</td>
<td>DAKO</td>
</tr>
</tbody>
</table>
Table 2.2. Secondary antibodies used for immunocytochemistry

<table>
<thead>
<tr>
<th>Emission</th>
<th>Reactivity</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>488</td>
<td>mouse IgG</td>
<td>goat</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>594</td>
<td>mouse IgG</td>
<td>goat</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>488</td>
<td>mouse IgM</td>
<td>goat</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>594</td>
<td>mouse IgM</td>
<td>goat</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>488</td>
<td>rabbit</td>
<td>goat</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>594</td>
<td>rabbit</td>
<td>goat</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>488</td>
<td>guinea pig</td>
<td>goat</td>
<td>Chemicon</td>
</tr>
<tr>
<td>594</td>
<td>rat</td>
<td>goat</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>
least three separate neuronal cultures. Means are reported ± standard error of the mean (SEM). The student’s t-test (Microsoft Excel) was performed in order to determine significance, and p values less than 0.05 (CI 95%) were considered statistically significant. Graphs were prepared in Microsoft Excel or Prism (version 4.0c).

2.5 IMMUNOBLOTTING

Initially, proteins were separated by SDS-PAGE using a Mini-PROTEAN® II (Bio-Rad) system. 7.5% or 12% 0.375M Tris-HCl separating gels were used, depending on the molecular weight range of the proteins to be separated. The separating gel was overlayed with a 4% 0.125M Tris-HCl, pH 6.8 stacking gel. Samples were prepared by dilution in 2x Laemelli sample buffer (1:2), which were then heat treated at 90°C for five minutes and incubated on ice for five minutes. A broad range, pre-stained molecular weight marker (Bio-Rad) was included in each separation to facilitate protein molecular weight determination. The gel was run at 200V for the duration of the separation.

After separation, gels were stained for total protein with Coomassie stain, or used for immunoblotting, utilising a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Prior to transfer, gels were equilibrated in transfer buffer for 15 minutes at room temperature, on an orbital shaker. The gel ‘sandwich’, containing a nitrocellulose membrane (Bio-Rad) and the gel, was inserted into the transfer cell. Protein transfer was performed for 1.5 hours at 4°C, 70V. The nitrocellulose membrane was washed three times with tris buffered saline with tween (TTBS) for five minutes, before blocking with TTBS / 5% skim milk powder / 5% FCS. The length of blocking varied from thirty minutes to 1.5 hours depending on the primary antibodies used. Blocking solution was removed by washing with TTBS. Primary antibodies were diluted in TTBS and incubated with the membrane for 2-16 hours at room temperature or 4°C on an orbital shaker. Membranes were washed three times with TTBS for ten minutes, then incubated with species appropriate horseradish peroxidase (HRP) conjugated secondary antibodies (1:1000 to 1:2000, DAKO), diluted in TTBS, for 1.5 hours at room temperature.
temperature, on a shaker. After this, membranes were washed three times with Tris buffered saline (TBS), for ten minutes.

Protein bands were labelled using an ECL™ chemiluminescence system (Amersham), according to the manufacturers instructions. Bands were developed with Hyperfilm™ (Amersham) using standard darkroom techniques. Briefly, film was exposed to developer (ILFORD) for three minutes, placed in the stop bath (tap water) for thirty seconds, followed by fixative (Kodak) for five minutes and washed in tap water for five minutes. For quantitative analysis, the film was scanned and band intensities analysed using Bio-Rad Quantity One software (version 4.5.0).

2.6 PLASMID DNA PREPARATION
Plasmid DNA encoding L1-Fc was a gift from Assoc Prof Deanna Benson. Plasmid DNA encoding pEGFP-F was purchased from Clontech, and a truncated form of ezrin, encoding only the N-terminus, inserted into the pEGFP-N1 vector, (NEz-GFP) was prepared by Dr Tracey Dickson. The vector maps are illustrated in Figure 2.1.

DNA was transformed using Epicuran coli XL10 Gold Ultracompetent cells (Stratgene), according to manufacturers instructions. Briefly, cells were thawed on ice, and incubated with 4μL β-mercaptoethanol for ten minutes, on ice. 1μL pure DNA was added and incubated for thirty minutes, on ice. The sample was heat pulsed (40-45°C, thirty seconds) and placed on ice for two minutes. 200μL NZY + broth was added and incubated for one hour at 37°C, on a platform shaker at high speed. Following incubation, 50-100μL of the transformation was spread plated onto LB plates containing the appropriate selection antibiotic for the vector (ampicillin or kanamycin; 30μg/mL), dried and allowed to grow overnight at 37°C. Colonies were inoculated into 5mL LB broth containing 5μL of the appropriate antibiotic. Broth cultures were incubated overnight at 37°C on a platform shaker at maximum speed. Qiagen QIAprep® Miniprep purification of DNA was performed according to the manufacturers instructions.
Figure 2.1 Vector information for plasmid DNA. A. The pcDNA3 vector (Invitrogen) containing DNA encoding L1-Fc was a gift from Assoc Prof Deanna Benson, and has been previously described (De Angelis et al., 1999). B. The pEGFP-F vector encoding GFP was purchased from Clontech. C. The pEGFP-N1 vector (Invitrogen) containing DNA encoding the N-terminus of ezrin, has been previously described (Algrain et al., 1993; Dickson et al., 2002). D. pmaxGFP™ was purchased from Amaxa.
Chapter 2 – Materials and Methods

Gel electrophoresis was used to check purified DNA products. Plasmids were either linearised or digested using the appropriate restriction enzymes (New England Biolabs). Plasmids were separated on a 0.8% Agarose gel (Invitrogen) containing Ethidium Bromide (Sigma), in TAE buffer. A 1kb DNA ladder (New England Biolabs) was used for identification of plasmid size. Bands were visualised using the UV transilluminator of an XRS Chemidoc (Bio-Rad). DNA yield was determined using a spectrophotometer (Eppendorf).
3 LOCALISATION AND PERTURBATION OF ERM PROTEINS IN DEVELOPING CNS NEURONS

3.1 INTRODUCTION
The ERM proteins have been implicated in many important cytoskeletal-based mechanisms. However, these functions have predominantly been identified in non-neuronal cell types. For example, they are involved in cell division in NIH-3T3 cells (Henry et al., 1995), are crucial for cell-substrate contacts and microvilli formation in mouse thymoma cells (Takeuchi et al., 1994), and cell motility in lymphocytes (del Pozo et al., 1999; Lee et al., 2004). Initially, ERM proteins were localised to the growth cone in developing neurons (Goslin et al., 1989), which was suggestive of roles in developmentally regulated growth mechanisms. During the last two decades there has been a gradual increase in knowledge of the specific roles of ERM proteins in CNS neurons.

A research group focussed on identifying specific components that control the cell cytoskeleton raised an antibody known as 13H9, which recognised a cytoskeletal component of the marginal band of erythrocytes (Birgbauer and Solomon, 1989). They then used the antibody for immunolabelling in developing cultured hippocampal neurons, another system where the precise control of cellular events by the cytoskeleton was becoming elucidated. The antibody almost exclusively labelled growth cones, in a pattern very similar to phalloidin (F-actin) (Goslin et al., 1989). Therefore, this was amongst the first evidence that ERMs were expressed in the nervous system, as the antibody was later confirmed to be specific for an epitope found in ezrin, radixin and moesin (Winckler et al., 1994). Subsequent investigations of ERM expression, in various neuronal phenotypes, have confirmed this expression pattern (Birgbauer et al., 1991; Gonzalez-Agosti and Solomon, 1996; Castelo and Jay, 1999; Paglini et al., 1998; Dickson et al., 2002; Cheng et al., 2005). Furthermore, in vivo studies have suggested that ERM proteins are specifically expressed during nervous system development, with a rapid decline in their expression coinciding with the final stages of embryonic development (Mintz et al., 2003).
As with many other proteins, much of what is known about ERM function has been learned through the use of genetically modified animals. Essentially, moesin and radixin knockout mice appear to be phenotypically normal (Doi et al., 1999; Kikuchi et al., 2002), which may be due to functional redundancy or compensatory expression of other ERM family members. At the cellular level, a number of approaches have been used to perturb ERM expression or activity, and therefore dissect the functions of the proteins. Perturbation methods used in neurons, in vitro, have included the withdrawal of growth factors (Gonzalez-Agosti and Solomon, 1996), microCALI (Castelo and Jay, 1999), antisense oligonucleotides (Paglini et al., 1998) and dominant negative transfection (Dickson et al., 2002). These studies have identified roles primarily in growth cone morphology and motility, neurite outgrowth and most recently axonal morphogenesis.

The focus of these experiments was to clarify the expression and localisation pattern of ERM proteins in hippocampal neurons, as they are a well-characterised neuronal phenotype, and their development is thought to correlate well with that observed in vivo (Dotti et al., 1988). The developmental expression of total ERM proteins, in relation to the phosphorylated (active) form of ERM proteins, and their recently identified neuronal binding partner L1 (Dickson et al., 2002) was studied. Next, neurons were transfected with DNA encoding either green fluorescent protein (GFP), or a dominant negative form of ezrin linked to GFP (NEz-GFP). Transfection, combined with live cell imaging and quantitation studies was used to determine the effects of ERM perturbation on the development of primary neurons, in vitro.
Chapter 3 - Results

3.2 MATERIALS AND METHODS

3.2.1 Substrate preparation and cell culture
Poly-1-lysine substrates were prepared according to section 2.2.1 (Materials and Methods). Primary hippocampal and cortical neurons were harvested from embryonic rats and cultured according to procedures outlined in sections 2.1.2 and 2.1.3 (Materials and Methods). For developmental (3, 5, 7, 21 DIV) ERM localisation studies, hippocampal neurons were fixed for immunocytochemistry with either 4% PFA or 10% TCA as described in section 2.3.1 (Materials and Methods). Cortical neurons were used rather than hippocampal neurons for ERM perturbation studies utilising transfection techniques, due to the high number of cells required for success of the transfection protocol.

3.2.2 Immunocytochemistry
Neurons were immunolabelled according to the procedures in section 2.3 (Materials and Methods). The primary antibodies used are outlined in Table 2.1. Previous studies have determined the specificity of the phosphorylated ERM antibody (Mintz et al., 2003). An anti-L1 CAM (1:5000) rabbit polyclonal antibody provided by Dr C Lagenaur was used. Alexa Fluor fluorescent secondary antibodies are detailed in Table 2.2.

3.2.3 Transfection studies
Neurons were transfected with pmaxGFP™ (Amaxa), pEGFP-F, or NEz-GFP (a dominant negative form of ezrin encoding the N-terminus of ezrin). pEGFP-F and NEz-GFP were produced according to standard procedures, in section 2.6 (Materials and Methods). Transfection was performed utilising an Amaxa Nucleofector® device and the Rat Neuron Nucleofector® Kit for primary rat hippocampal or cortical neurons. In some experiments, the data obtained from transfection with pmaxGFP™ was combined with pEGFP-F, as there were no significant differences in morphology of the neurons observed using the two different GFP vectors. Thus results from transfection with these vectors are described as GFP, rather than by their specific vector names. After
optimisation of the protocol, including the number of cells required for transfection, the amount of DNA and culture plating density (Table 3.1), neurons were transfected according to manufacturers instructions at the time of harvest (0 DIV).

3.2.4 Live Cell Imaging

Neurons expressing the specific plasmids were transferred to glass imaging dishes containing imaging buffer warmed to 37°C. Neurons were imaged at 1, 2 or 3 DIV, for various periods (most often 10 minutes, but up to 120 minutes) in order to compare behaviour and morphology between NEz-GFP and control GFP transfected neurons. For quantitation studies, the measurements were obtained from the final frame in the imaging sequence.

3.2.5 Microscopy and analysis

Microscopy and quantitation were carried out as described in section 2.4 (Materials and Methods). In transfection studies, aspects of neuronal growth and morphology, including axon length, dendrite length, number of dendrites, percentage of branched axons and dendrites, the maximum number of times axons and dendrites were branched and growth cone area were determined using Improvision Openlab software. Movies were prepared and annotated using Improvision Openlab and Final Cut Express (version 1.0) and QuickTime Pro (version 7.1.3) software, respectively. Eleven GFP control transfected neurons and nine NEz-GFP transfected neurons, from at least three separate neuronal cultures, were included in the analysis.

Table 3.1. Optimum conditions for transfection using Amaxa Nucleofector® products

<table>
<thead>
<tr>
<th>Vector</th>
<th>Number of neurons per reaction</th>
<th>DNA (µg)</th>
<th>Neuron culture density: cells per coverslip</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGFP-F</td>
<td>$6 \times 10^6$</td>
<td>10µg</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>pmxGFP™</td>
<td>$4 \times 10^6$</td>
<td>2µg</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>NEz-GFP</td>
<td>$6 \times 10^6$</td>
<td>10µg</td>
<td>$1 \times 10^5$</td>
</tr>
</tbody>
</table>
3.3 RESULTS

3.3.1 L1 and ERM are expressed during early development, in vitro

It has been established that L1 is predominantly expressed during early neuronal development (Linnemann et al., 1988; Wong et al., 1995; reviewed in Hortsch, 1996; Tsuru et al., 1996). In the current experiments, L1 was localised to neuronal soma, axons and axonal growth cones, but was absent from dendrites (Figure 3.1 C, F). However, L1 immunolabelling was not diminished in long-term cultures at 21 DIV, when cortical neurons are considered relatively mature (Figure 3.1 L). L1 expression persists in mature, unmyelinated axons, in vivo (reviewed by Wong et al., 1995). Notably, L1 labelling specifically co-localised with ERM proteins in axonal growth cones. Labelling for total ERM proteins, independent of their phosphorylation state, was apparent throughout early development (1-7 DIV), and was localised to axonal and dendritic growth cones, as well as more restricted sites along neurites (Figure 3.1 A, D and G). ERM labelling was also dispersed within a proportion of growth cones in relatively mature cultures (Figure 3.1 J, arrowheads indicate ERM labelling), however specific labelling for phosphorylated ERM demonstrated that the proteins were not present in their phosphorylated, or active form (Figure 3.1 K).

Phosphorylated ERM labelling provided more specific information about the expression of active ERM proteins during early development. Between 1 and 5 DIV the pattern of labelling was similar to that of phosphorylation state independent ERM, and therefore concentrated in areas of growth activity, but it was more restricted in its localisation (Figure 3.1 B, E and H). That is, while labelling for total ERM proteins was observed in all growth cones, active ERM was limited to specific areas, often only a subset of filopodia (compare figure 3.1 G and H). At 21 DIV active ERM was not evident within any neurites, filopodia or growth cones (Figure 3.1 K). Cultures were expanded to 28 DIV to determine if the total ERM labelling was maintained in more mature neurons. Immunolabelling showed a complete absence of both phosphorylation state independent and phosphorylated ERM in these neurons at 28 DIV. In contrast to the specific,
Figure 3.1 ERM and L1 localisation during hippocampal development, in vitro. Hippocampal neurons were fixed at 3, 5, 7 and 21 DIV and immunolabelled with antibodies to determine protein localisation during development. A, D, G and J. Immunolabelling with antibodies to phosphorylation state independent ERM (green) and β-tubulin (red). ERM proteins were localised to axonal and dendritic growth cones, as well as occasional filopodia emanating from neurite shafts, which may correspond to sites of new branch formation (arrowheads). B, E, H and K. Phosphorylated, or active ERM (green) and β-tubulin (red) labelling. Phosphorylated ERM was more restricted in its localisation (arrowheads) than phosphorylation state independent ERM. Phosphorylated ERM was completely diminished from cultured hippocampal neurons at 21 DIV (K). C, F, I and L. L1 (red) and phosphorylation state independent ERM (green) labelling. L1 was localised to the axon (C and F, arrows) and soma, and persisted throughout in vitro development. ERM labelling was localised to growth cone and neurite filopodia (arrowheads), and remained localised to some small growth cones at 21 DIV (J, L, arrowheads). ERM and L1 specifically co-localised in the axonal growth cone. Scale bar: A-C, F, G, and J = 40μm; D, E, H, I, K and L = 46μm.
restricted localisation of ERM proteins in neurons, glial cells widely expressed ERM proteins, including phosphorylated ERMs.

In summary, labelling for active ERM proteins in *in vitro* neuronal development peaked at approximately 3 DIV and was diminished by 21 DIV, whereas a pool of inactive ERM remained in neuronal processes and growth cones until it also diminished between 21 and 28 DIV. This immunolabelling pattern is indicative of developmental ERM functions. L1 and ERM proteins specifically co-localised in axonal growth cones, which is suggestive of specific functions resulting from their binding, such as growth cone morphogenesis, axon outgrowth and pathfinding. In addition, they may participate in modulating the neuronal response to molecular guidance cues.

### 3.3.2 Perturbation of ERM function in early development results in alterations in neuron morphology

Initially, neurons transfected with either NEz-GFP or GFP were observed by time-lapse imaging at 1-3 DIV, to identify morphological and behavioural differences in neurons over-expressing the truncated form of ezrin. At 1 DIV (Movie 3.1) GFP transfected neurons were developing normally, with lamellipodia becoming more defined in their localisation, representing the beginning of neurite formation. Lamellipodia and filopodia were very active, extending and retracting as the neuron began to establish neurites. In contrast, neurons transfected with NEz-GFP at 1 DIV (Movie 3.2) appeared to be slightly lagging behind their GFP control transfected culture counterparts, with respect to neurite formation. Many neurons remained rounded, without the presence of large lamellipodia that is characteristic of the initial stages of development in culture. However, it was noted that in NEz-GFP transfected neurons, filopodia emanating from the cell body were highly active, extending and retracting comparably to GFP transfected control neurons.

By 3 DIV GFP transfected neurons had developed multiple dendrites, and a defined axon, which possessed a highly motile distal growth cone (Movie 3.3). Some NEz-GFP
**Movie 3.1 The development of GFP transfected control neurons at 1 DIV.** A primary cortical neuron transfected with the vector encoding GFP at the time of harvest (0 DIV), then time-lapse imaged at 1 DIV. The sequence contains images obtained every 10 seconds for 1 hour. The first image in the sequence was obtained under fluorescence, to demonstrate that the neuron was expressing the plasmid DNA. The neuron was undergoing the early developmental process of neurite initiation, as evidenced by large, active lamellipodia (arrows). These lamellipodia are likely to correspond to the development of neurites within the next day *in vitro*. Scale bar = 25μm.

**Movie 3.2 The development of NEz-GFP transfected neurons at 1 DIV.** A primary cortical neuron transfected with the vector encoding NEz-GFP at 0 DIV, then time-lapse imaged at 1 DIV. The sequence contains images obtained every 10 seconds for 1 hour, and the first image was obtained under fluorescence. Compared with GFP transfected neurons (Movie 3.1), the development of NEz-GFP expressing neurons often appeared to be delayed. While the neuron possessed highly active, long filopodial protrusions (small arrows), it had not established lamellipodia around the cell body, which is a preceding stage to neurite formation. Untransfected neurons in the same culture displayed more characteristic morphology of this stage of development *in vitro*, with established lamellipodia and in some cases, neurites (large arrow). Scale bar = 25μm.

**Movie 3.3 GFP transfected neuron at 3 DIV.** A primary cortical neuron transfected with the vector encoding GFP at the time of harvest (0 DIV), then time-lapse imaged at 3 DIV. The sequence contains images obtained every 5 seconds over a 10 minute period, with the first image obtained under fluorescence. The sequence shows that the transfected neuron had established multiple, short neurites (small arrows) as well as one longer neurite, which is likely to be the axon. The axonal growth cone (large arrow) possessed highly motile filopodia and lamellipodia. Filopodia located along the axon and associated with the new dendrites were also motile. Scale bar = 25μm.
**Movie 3.4 NEz-GFP transfected neuron at 3 DIV.** A primary cortical neuron transfected with a vector encoding NEz-GFP at 0 DIV, then time-lapse imaged at 3 DIV. The movie sequence is comprised of images obtained every 10 seconds for 1 hour, and the first image was obtained under fluorescence. The neuron progressed through development with respect to the formation of a single axon and multiple dendrites (compare with Movie 3.3), however the axonal growth cone (large arrow) was small and lacked lamellipodia. Furthermore, rather than defined dendrites, multiple long filopodia emanated from the cell body (small arrow). In addition, the axon was branched (arrowheads), which was not normally observed in GFP control transfected neurons at this age. Scale bar = 25μm.

**Movie 3.5 NEz-GFP transfected neuron at 3 DIV.** A primary cortical neuron transfected with the vector encoding NEz-GFP at 0 DIV, then time-lapse imaged at 3 DIV. Images were obtained every 5 seconds over a 10 minute imaging period, and the first image was obtained under fluorescence. This neuron displayed further abnormal morphology (compared with Movie 3.4), such as the lack of an axonal growth cone (large arrow). Also neurites appeared slightly flattened, with “blebbing” (arrowhead). Dendrites were short and lacked filopodia and growth cones (small arrows), and filopodia were short and inactive. Scale bar = 25μm.

**Movie 3.6 NEz-GFP transfected neuron at 3 DIV.** Under the same conditions as Movie 3.5, a primary cortical neuron transfected with a vector encoding NEz-GFP at 0 DIV, then time-lapse imaged at 3 DIV. Images were obtained every 5 seconds over a 10 minute imaging period, and the first image was obtained under fluorescence. The neuron displayed many characteristics of poor health including flattening of neurites and “blebbing” (also observed in Movie 3.5). This neuron also lacked an active axonal growth cone (large arrow). Filopodia associated with the branched primary dendrite (arrow) were inactive (arrowhead). Scale bar = 25μm.

Note: Movies supplied in QuickTime format on DVD affixed to the back cover.
transfected neurons displayed relatively similar morphology and behaviour (Movie 3.4). However, a subset of NEz-GFP neurons displayed abnormal morphology, including “blebbing” along the neurites, flattened neurites and small growth cones (Movies 3.5 and 3.6). Due to the robust expression and health of neurons transfected with the two different GFP constructs, it was unlikely that the transfection process caused the abnormal morphology, which suggests that it was specifically the expression of dominant negative ezrin that caused the morphological abnormalities. Interestingly, NEz-GFP transfected neuron filopodia were active (Movie 3.6).

Therefore, live cell imaging studies did not uncover obvious differences in the behaviour, particularly with respect to filopodial and lamellipodial activity, between neurons transfected with GFP or NEz-GFP, at any of the timepoints observed. Therefore, aspects of neuron morphology and outgrowth were quantitatively assessed at 3 DIV (Figure 3.2), a timepoint at which neurite outgrowth is well established and neurons are polarised (de Lima et al., 1997; Dotti et al., 1988). NEz-GFP transfected neurons showed no significant difference in axon length (181μm ± 22) compared with GFP transfected neurons (152μm ± 22) (p = 0.39) (Figure 3.2 A). Similarly, dendrite length in neurons transfected with NEz-GFP (47μm ± 6), compared with GFP transfected controls (37μm ± 3) were not significantly different (p = 0.09) (Figure 3.2 B). Notably, there were tendencies for the NEz-GFP transfected neurons to have longer axons and dendrites, but this did not reach statistical significance.

The number of dendrites was counted in order to examine a role for ERM proteins in neurite initiation (Paglini et al., 1998). NEz-GFP and GFP transfected neurons at 3 DIV both had an average number of three dendrites (NEz-GFP 2.5 ± 0.42; GFP 2.9 ± 0.31, p = 0.46) (Figure 3.2 C). ERM proteins have also been proposed to play a role in axon branching (Dickson et al., 2002), and so the amount of branching in both axons and dendrites was examined. 57% (± 20%) of NEz-GFP transfected neuron axons were branched, while 25% (± 16%) of GFP transfected neurons had branched axons, a difference that was not statistically significant (p = 0.23) (Figure 3.2 D). The results were similar for branched dendrites; 33% (± 13%) of dendrites were branched in
Figure 3.2 Perturbation of ERM function in developing primary cortical neurons. Cortical neurons were transfected with plasmid DNA encoding GFP or NEz-GFP at 0 DIV, using the Nucleofector® system. At 3 DIV, neurons were time-lapse imaged and then aspects of their morphology were quantitated. * represent p values less than 0.05. Error bars are ± SEM.
neurons transfected with NEz-GFP, in comparison with 9% (± 5%) dendrites branched in neurons transfected with GFP, which did not reach statistical significance (p = 0.09) (Figure 3.2 E). Of the axons that were branched, NEz-GFP transfected neuron axons were branched a maximum of two times (± 0.38), compared with GFP transfected neurons, which were branched once (± 0.16; p = 0.08) (Figure 3.2 F). Similarly, dendrites belonging to NEz-GFP transfected neurons that were branched, were branched an average number of three times (± 0.16), compared with once in GFP control transfected neurons (± 0.09; p = 0.13) (Figure 3.2 F).

ERM proteins have been implicated in growth cone morphology, particularly lamellipodial formation (Castelo and Jay, 1999); therefore axonal growth cone area was quantitated. NEz-GFP transfected neuron growth cones were an average area of 49μm² (± 17μm²), compared with GFP transfected neuronal growth cones, which were an average of 130μm² (± 32μm²). While the variation in growth cone size was large, it was not statistically significant (p = 0.08) (Figure 3.2 G).

In summary, compared with control, GFP transfected neurons, NEz-GFP transfected neurons showed a trend towards increased axon and dendrite outgrowth, increased axon and dendrite branching and decreased growth cone size, but none reached statistical significance.
3.4 DISCUSSION

These experiments have shown that the ERM proteins are developmentally expressed in long-term primary neuron cultures. In particular, phosphorylated (active) ERM expression was diminished by 21 DIV, which coincides with the time at which neurons are considered relatively mature (de Lima et al., 1997; Dickson et al., 2000; Chuckowree and Vickers, 2003; King et al., 2006). Furthermore, transfection experiments, in which neurons were stimulated to over-express a truncated, and thus inactive form of ezrin, alluded to many potentially important functions in neuron development.

Initial studies demonstrated that L1 was localised to the soma, axons and axonal growth cones of neurons early in development. This pattern of labelling persisted in relatively mature cultures (21 DIV), although it is well documented that L1 exerts its most vital roles early in development (for review see Hortsch, 2000). L1 and ERM proteins co-localised in axonal growth cones, which might indicate specific roles for the binding relationship in processes such as axon guidance and outgrowth. It was imperative for subsequent studies pertaining to ERM protein function in CNS neurons to precisely characterise their temporal and spatial localisation, in neuronal cultures. The ERM proteins were predominantly localised to axonal and dendritic growth cones during early development, which agrees with previous data (Goslin et al., 1989; Birgbauer et al., 1991), but were also often observed in fine filopodia along the neurites. In contrast with other previous studies, in these experiments neuron cultures were extended to later timepoints, to determine ERM expression patterns in more mature neurons. Phosphorylation state independent ERM labelling persisted in relatively mature cultures (21 DIV), but by 28 DIV was diminished. In contrast, phosphorylated, or active, ERM was more highly restricted in its localisation throughout development, and diminished by the time maturation occurred (21 DIV). These studies therefore confirm that ERM proteins are specifically expressed during development and are thus likely to have important developmental functions.

Perturbation studies also supported the likelihood of developmental functions of the ERM proteins. While no significant differences between morphology in control GFP or
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NEz-GFP treatment were observed, the data showed clear trends towards deviations from normal growth and morphology. Some notable changes included the increase in axon and dendrite length, an increase in axon and dendrite branching, as well as a decrease in growth cone size, all demonstrated in NEz-GFP neurons at 3 DIV.

A pioneering study by Gonzalez-Agosti and Solomon (1996) examined the role of radixin in growth cone morphogenesis. Experiments showed that withdrawal of nerve growth factor (NGF) from chick sympathetic neurons resulted in growth cone collapse, with a concomitant decrease in radixin immunolabelling. When NGF was reapplied, the growth cone was re-established, as was radixin labelling. In the same set of experiments, a less dramatic approach to growth cone perturbation, the application of an electrical field, was used. This resulted in growth cone turning, which showed asymmetrical radixin labelling localised to the side of the growth cone that turned (Gonzalez-Agosti and Solomon, 1996). In another study, abolishing radixin in the growth cone of DRG neurons using microCALI techniques reduced the area of growth cone lamellipodia by 30%, compared with controls (Castelo and Jay, 1999). Antisense oligonucleotide double suppression of radixin and moesin resulted in a number of deleterious effects on growth cones of primary hippocampal neurons, including a reduction in growth cone size and the loss of lamellipodia (Paglini et al., 1998), both of which were also observed in the current transfection experiments. Interestingly, the same study also observed a decrease in the number of filopodia, but the remaining filopodia were increased in length (Paglini et al., 1998).

In these transfection studies increased axon and dendrite branching was observed in NEz-GFP neurons compared with GFP controls. Increased axon branching was previously observed in hippocampal neurons transfected with the same NEz-GFP construct (Dickson et al., 2002). More specifically, the increased branching was observed when the transfected neurons were grown on a substrate containing L1, a membrane binding partner of the ERM proteins in neurons, which strongly suggested that the L1-ERM-actin relationship is involved in mediating appropriate axonal morphogenesis during neuronal development (Dickson et al., 2002). The experiments
presented here now implicate ERMs in dendrite branching, in addition to axon branching. In a recent study, transfection of cerebellar granule neurons with two separate truncated forms of L1, without the RSLE or juxtamembrane regions (which are both sites for ERM binding) disrupted the L1-ERM linkage, resulting in reduced branching (Cheng et al., 2005). The opposite effects on neurite branching, compared to the study by Dickson and colleagues (2002), were attributed to different sites of action (Cheng et al., 2005).

Live imaging studies performed at 1 DIV suggested that neurons transfected with NEz-GFP were lagging behind control neurons with respect to neurite initiation and establishment of polarity. This has been observed in experiments using oligonucleotide suppression of radixin and moesin (Paglini et al., 1998). However, in the current experiments, the more gradual initiation of neurites was followed by increased neurite outgrowth in both the axon and dendrites at 3 DIV. ERM proteins have long been postulated to be involved in neurite outgrowth, however direct evidence is scarce in the literature. Others have shown that suppression of radixin and moesin leads to a decrease in the rate of axonal outgrowth (Paglini et al., 1998). In contrast with these findings, Castelo and Jay (1999) showed that even when perturbation of radixin in growth cones dramatically changed their morphology, neurite extension was not affected. The increased outgrowth observed in these experiments is highly suggestive of a function in regulating neurite growth, perhaps by mediating neurite retraction.

In conclusion, these studies have shown that the localisation of ERM proteins, particularly phosphorylated ERM, declines in more mature neurons in long-term cultures. Phosphorylated ERM is more restricted in its localisation than total ERM proteins, and ERMs co-localise with L1, particularly in axonal growth cones of polarised neurons. Perturbation of ERM function in cultured neurons supports important roles in neurite outgrowth, growth cone morphology and neurite branching.
4 THE ROLE OF ERM PROTEINS AND L1 IN DEVELOPMENTAL AND POST-INJURY REGENERATIVE NEURITE OUTGROWTH

4.1 INTRODUCTION

Encouraging the adult CNS to regenerate following injury remains at the forefront of neurobiology research and is an issue of clinical significance. Regeneration of the nervous system potentially requires the re-expression of developmentally significant molecules, since neuron re-growth is likely to involve cellular mechanisms in common with development. These developmentally important molecules may include cytoskeletal-associated proteins implicated in processes such as neurite outgrowth, which makes the ERM proteins potential candidates.

ERM proteins are expressed during neuronal development in dynamic cellular structures such as neuronal growth cones (for example, Goslin et al., 1989; Birgbauer et al., 1991; Dickson et al., 2002; Mintz et al., 2003; Chapter 3), and are therefore thought to be intricately involved in mediating growth cone morphology and motility (Paglini et al., 1998; Castelo and Jay, 1999), processes related to neurite outgrowth. ERMs function by binding to plasma membrane proteins and the underlying cytoskeleton, providing a linkage between these important components of the cell.

L1 is a nervous system specific plasma membrane binding partner of the ERM proteins (Dickson et al., 2002). Neuronal L1 is predominantly membrane-associated, localised specifically to the axon during early development (Persohn and Schachner, 1987; Linnemann et al., 1988), and participates in key developmental processes including cell adhesion, neurite outgrowth, axon pathfinding and fasciculation (Hortsch, 1996). The vital nature of L1 is demonstrated by mutations to the human L1 gene (Wong et al., 1995; Fransen et al., 1997), which results in distinct neurological deficits, known as CRASH syndrome (corpus callosum agenesis, retardation, adducted thumbs, shuffling gait and hydrocephalus) (Fransen et al., 1995). L1 knockout mice show very similar abnormalities, including increased ventricle size, impaired axon decussation in major tracts, weakness in the hind limbs and reduced survival (Cohen et al., 1997; Dahme et
al., 1997; Demyanenko et al., 1999; reviewed by Kamiguchi et al., 1998). Therefore, L1 is known to be essential for development, but in addition, it has been demonstrated to also promote neuronal regeneration after trauma (see recent examples Roonprapunt et al., 2003; Becker et al., 2005; Chen et al., 2005; Zhang et al., 2005).

Therefore, in the present study the role of ERM proteins and L1 was investigated in developmental growth and regenerative re-growth of mature neurons following trauma. More specifically, whether the mechanism by which L1 aids regeneration involves binding with the ERM proteins was examined. An in vitro experimental model that involves transection of neuronal processes (Dickson et al., 2000; Chuckowree and Vickers, 2003; Chung et al., 2003) was utilised. Neurite transection was performed at 21 DIV when neurons in culture are relatively mature, as defined by the absence of developmental proteins and presence of proteins related to maturity (de Lima et al., 1997; Chuckowree and Vickers, 2003; King et al., 2006). Immunolabelling for ERM proteins was used to investigate whether they are involved in the regenerative response. Furthermore, L1 was supplied as a growth substrate to quantitatively determine whether L1 facilitated the post-injury regenerative neurite sprouting response. In another aspect of this investigation, two different neuronal populations were examined, those derived from the cortex and the hippocampus, to investigate potential variation in their regenerative capacities.
4.2 MATERIALS AND METHODS

4.2.1 Substrate preparation and cell culture

Cell culture substrates containing 0.001% poly-l-lysine or L1-Fc (4μg/mL), and primary dissociated cortical or hippocampal neuron cultures were prepared according to the procedures outlined in sections 2.1.1 to 2.1.3 (Materials and Methods). To determine the neurite outgrowth promoting ability of L1-Fc, neuron cultures grown on L1-Fc substrates were fixed with 4% PFA at 2, 3 and 4 DIV. In vitro injuries were performed at 21 DIV, as described in section 2.2 (Materials and Methods). For post-injury studies, neurons were fixed (at 1, 6, 12, 24, 30 and 72 hours) with 4% PFA for immunocytochemistry, details are provided in section 2.3.1 (Materials and Methods). Scanning Electron Microscopy (SEM) studies required 2.5% glutaraldehyde fixation as described in subsequent sections.

4.2.2 Immunocytochemistry

Immunocytochemistry was performed using standard procedures in section 2.3 (Materials and Methods). For developmental studies of the effect of the L1-Fc substrate on neurite outgrowth, neurons were double immunolabelled with antibodies to MAP2 and tau. Mature injured neurons fixed via the 4% PFA method were labelled with antibodies to phosphorylation state independent ERM and either β-tubulin or tau. Further antibody details are provided in Table 2.1. To visualise antibody localisation, isotype and species appropriate fluorescent secondary antibodies were used (Table 2.2).

4.2.3 Microscopy and analysis

Microscopy and image acquisition was performed according to section 2.4 (Materials and Methods). Improvision Openlab software was used to determine axon length in developmental neurite outgrowth studies, where 75 individual axon lengths were measured at each timepoint of 2, 3 and 4 DIV. For injury studies, the length of the regenerating neurites from the border of the injury site to the tip of the growth cone, as well as growth cone area, were quantitated. One hundred individual neurites were
examined for each experimental condition. The data was obtained from at least three separate neuronal cultures.

4.2.4 Scanning Electron Microscopy

Both early developmental (1, 3, 5 and 7 DIV) and mature hippocampal neurons (21 DIV) at 6 hours post-injury were prepared for SEM as previously described (Chuckowree et al., 2003). Briefly, cells were fixed in 2.5% Glutaraldehyde / 0.1% phosphate buffer (pH 7.4) and post-fixed with 0.1% osmium tetraoxide. Specimens were dehydrated by progression through an ethanol series, critical point dried and sputter coated with gold prior to examination under a Jeol JSM-840 scanning electron microscope. Digital images were captured using ImageSlave software.
4.3 RESULTS

4.3.1 L1 substrate promotes neurite outgrowth during in vitro development

To determine the ability of L1-Fc protein to promote neurite outgrowth, axon lengths of hippocampal neurons grown on L1 and poly-l-lysine at 2, 3 and 4 DIV were compared. By 2 DIV hippocampal neurons had reached Stage 3 of growth, having a defined axon (Dotti et al., 1988), therefore axon lengths could be compared from 2 DIV onwards (Figure 4.1). At each time-point assessed, hippocampal neurons grown on the L1 substrate had longer axons than those grown on poly-l-lysine (Figure 4.1 A). At 2 DIV, the mean axon length on poly-l-lysine was 115µm, compared with 123µm on L1 (p = 0.206). At 3 DIV the average axon length on poly-l-lysine was 120µm, compared with 135µm on L1 (p = 0.052), and at 4 DIV, the mean length on poly-l-lysine was 125µm, versus 143µm on L1 (p = 0.057). As the neurons developed in vitro, the divergence in rate of axon outgrowth on the different substrates became more pronounced. However, the differences did not reach statistical significance, and examination beyond 4 DIV was not possible due to the formation of contacts between neurites of different neurons. This data primarily confirmed that L1 protein, applied as a growth substrate, was biologically active and capable of promoting neuronal growth in culture, in accordance with previous investigations (for example, Esch et al., 1999).

4.3.2 ERM proteins are re-expressed in the neuronal response to trauma

Initially, cultured cortical and hippocampal neurons grown on poly-l-lysine or L1 substrates were injured at 21 DIV, returned to normal incubator conditions, then fixed at varying timepoints post-injury (1, 6, 12, 24, 30 and 72 hours; Figure 4.2) an immunolabelled with antibodies to the MAP tau and the ERM proteins. At 1 hour post-injury there were no changes evident at the injury site. By 6 hours post-injury new regenerative neurite sprouts entered the injury site. At the later timepoints (particularly 24 - 72 hours) neurons had established a robust regenerative response, in which neurites had extended across the injury site. Therefore, later timepoints were not suitable for neurite length quantitation studies, and these experiments determined that the most
Figure 4.1 Hippocampal neuron growth on L1 and poly-l-lysine substrates. Neurons were immunolabelled with an antibody to tau during early in vitro development and axon lengths were measured at 2, 3 and 4 DIV. A. Quantitation demonstrated that at each developmental timepoint axons on the L1 substrate were longer than those grown on poly-l-lysine, however this difference was not statistically significant (p > 0.05). B. A representative hippocampal neuron at 2 DIV. C. A neuron at 3 DIV. D. A neuron at 4 DIV. In B, C and D arrowheads identify the axon. Scale bar: B and C = 20µm; D = 26µm.
Axon Outgrowth on poly-l-lysine and L1 at 2, 3 and 4DIV
Figure 4.2 Time course of regenerative neurite sprouting following transection. Cortical neurons were cultured on poly-l-lysine in vitro to relative maturity (21 DIV), and transection injury was performed. Neuron cultures were fixed at 1, 6, 12, 24, 30 and 72 hours post-injury, and immunocytochemistry was utilised to label for tau and the ERM proteins. Arrows indicate the injury site. At timepoints after 6 hours post-injury regenerative neurite sprouts crossed the injury site, therefore 6 hours post-injury was subsequently used for quantitation of regenerative neurite length and growth cone area. Scale bar: 1, 6, 24 and 30 hours = 48μm; 12 and 72 hours = 70μm.
appropriate timepoint for quantitation of regenerative neurite characteristics was 6 hours post-injury.

Following injury at 21 DIV, neurons were fixed at 6 hours post-injury and labelled with antibodies to the ERM proteins. Previous experiments demonstrated that ERM immunolabelling is more abundant during early development (1-7 DIV), and particularly phosphorylated ERM is diminished at 21 DIV (Chapter 3). In these experiments, ERM labelling was observed specifically localised to the growth cones of regenerative neurites that entered injury sites (Figure 4.3 A and B), demonstrating that injury stimulated the re-expression, or changes in the localisation, of the ERM family of proteins. Phosphorylated ERM was also present in the growth cones of post-injury neuritic sprouts. In contrast, the uninjured areas of the culture displayed no active ERM immunolabelling.

This re-expression of the ERM proteins was observed in neurons grown on both L1 and poly-l-lysine substrates. However, a clear difference in some aspects of regenerative neurite morphology was observed when the substrates were compared.

4.3.3 The neuronal response to injury on L1 substrate results in longer regenerative neurites, compared to poly-l-lysine

In order to establish the role of L1 in the response to trauma, specifically in this neuronal culture model, regenerative neurite growth of injured neurons grown on L1 substrate or poly-l-lysine was compared. L1 has previously been shown to influence neurite outgrowth in vivo (for example, Honig et al., 2002). In the current study regenerative neurite lengths from parallel cultures was compared, with the single variable being growth substrate. Both hippocampal and cortical derived neurons had significantly longer neurites, 6 hours post-injury, when grown on L1 (cortical neurons 58\(\mu\)m ± 5.17; hippocampal neurons 44\(\mu\)m ± 2.84) than when grown on poly-l-lysine alone (cortical neurons 40\(\mu\)m ± 1.92; hippocampal neurons 34\(\mu\)m ± 1.81) (Figure 4.4; p < 0.05 for both neuronal populations).
Figure 4.3 Growth cones of hippocampal neurons grown on L1 and poly-l-lysine substrates. Neurons were grown in culture until 21 DIV, then subjected to transection injury. A. Immunocytochemistry of regenerative neurite sprouts grown on poly-l-lysine, with antibodies to β-tubulin (red) and phosphorylation state independent ERM (green), showed that growth cones were typically small, ‘bell-shaped’ structures with few distinct filopodia (arrows). B. Regenerative neurite growth cones on L1 substrate immunolabelled with tau (red) and phosphorylation state independent ERM (green). Growth cones showed a more filopodial morphology and were larger (arrows). SEM was utilised to further investigate the morphology of growth cones. C. This confirmed the morphology of growth cones on poly-l-lysine (arrow). D. Growth cones on L1 were much larger and filopodial (arrow). E. A hippocampal neuron grown on poly-l-lysine at 3 DIV. F. A hippocampal neuron at 3 DIV on L1. G. A higher magnification of boxed areas shown in E. H. A higher magnification of boxed area shown in F. Growth cone morphology of regenerative neurite sprouts (A-D) reflects that of early development (E-H). Scale bar: A, B, E - H = 10μm; C = 1μm; D = 2μm.
Figure 4.4 *Quantitation of regenerative neurite sprouting.* Cortical and hippocampal neuron cultures were injured at 21 DIV, then fixed 6 hours post-injury for quantitation of characteristics of regenerative neurite sprouts. **A.** Regenerative neurite lengths, which were significantly longer on L1 than poly-l-lysine for both cortical and hippocampal neurons. **B.** Regenerative neurite growth cone areas, which were significantly larger on L1 than poly-l-lysine for hippocampal neurons but not cortical neurons. * represent p values less than 0.05. Error bars are ± SEM.
A

Regenerative neurite length 6h post-injury

B

Regenerative growth cone area 6h post-injury
4.3.4 Regenerative neurite growth cone morphology differs depending on growth substrate

There was a distinct difference in the growth cone morphology of post-injury regenerative neurites on the Li substrate compared with neurons grown on a substrate of poly-l-lysine. Although there was variability in growth cone appearance on each substrate, the Li substrate was more often associated with larger, fan shaped growth cones with obvious lamellipodia and numerous filopodia (Figure 4.3 B and D). In contrast, injury-induced regenerative neurite growth cones from neurons grown on poly-l-lysine were characterised by fewer filopodia, and were smaller, ‘bell-shaped’ structures (Figure 4.3 A and C). This observation was further investigated in two ways, firstly by quantitation of growth cone area, and secondly by closer examination using SEM, as illustrated in Figure 4.3 (C - H).

Quantitation of growth cone area of both hippocampal and cortical regenerative neurites after injury revealed that those on the Li substrate had greater mean area (cortical neurons 155\(\mu\)m\(^2\) ± 7.77; hippocampal neurons 215\(\mu\)m\(^2\) ± 12.44) than those grown on poly-l-lysine (cortical neurons 152\(\mu\)m\(^2\) ± 8.25; hippocampal neurons 117\(\mu\)m\(^2\) ± 8.11) (Figure 4.4 B). This was statistically significant for hippocampal neurons (p < 0.05), but not for cortical neurons (p = 0.827). SEM was used to compare post-injury growth cones with developing axonal growth cones at 3 DIV, grown on poly-l-lysine and Li substrates. Results showed that developing and post-injury neurite growth cones shared similar morphology for both substrates (Figure 4.3 C and D, compared with E-H). Thus, SEM studies confirmed that Li supplied as a growth substrate influenced neuronal growth cone morphology, in development, and similarly in the neuronal response to trauma.
4.3.5 Neurons derived from the cortex show more advanced regenerative neurite growth than those from the hippocampus, at 6 hours post-injury

Comparison of the regenerative abilities of hippocampal and cortical derived neurons revealed that cortical regenerative neurites reached greater lengths by 6 hours post-injury than those from hippocampal neurons grown on both poly-l-lysine (p < 0.05) and L1 (p < 0.05) substrates (Figure 4.4 A). This result indicates that neurons taken from the cortex mount a more robust, or at least more rapid, regenerative sprouting response following *in vitro* injury. Immunocytochemistry did not clearly define a correlation between ERM expression and the differing ability of these neurons to regenerate.

Interestingly, when the growth cone area of neurons originating from the hippocampus and cortex on poly-l-lysine and L1 substrates in the post-injury response was compared, hippocampal growth cones were found to be significantly larger on L1 (p < 0.05), but in contrast, cortical growth cones were significantly larger on poly-l-lysine (p < 0.05; Figure 4.4 B). These data suggest that L1 promotes different morphology of cortical and hippocampal growth cones, which may account for the variation in regenerative responses between the neuronal populations.
4.4 DISCUSSION

The recently characterised binding relationship between the ERM cytoskeletal-linker proteins and L1 is likely to have a significant contribution to neuronal development, based on the previously elucidated roles of the individual proteins. Thus, it was hypothesised that involvement in developmental processes would make the ERM proteins and L1 probable candidates for participation in the regenerative neuronal response to trauma, which was examined using an in vitro model of transection. Data showed that, like during neuron development, providing neurons with a growth substrate containing L1 increased regenerative neurite outgrowth after injury. Furthermore, ERM proteins were re-expressed after injury, localised specifically to regenerative neurite growth cones. These experiments also revealed differences in growth cone morphology of neurons grown on an L1 substrate, compared with poly-l-lysine, and showed that cortical neurons potentially possess a more robust regenerative ability than hippocampal neurons.

The ERM proteins are predominantly localised to axonal and dendritic growth cones during early development. ERM labelling persists in relatively mature cultures (21 DIV), albeit restricted to a few growth cones. In contrast, phosphorylated, or active, ERM is more highly restricted in its localisation throughout development, and is diminished by the time maturation occurs (Chapter 3). Regenerative neurite growth cones of injured cultured neurons at 21 DIV were positively labelled for the ERM proteins. Since ERMs are not expressed by uninjured cultures of this age, it could be concluded that ERM proteins were either re-expressed, or re-localised from the soma or other part of the neuron, to the regenerative neurite growth cone. This suggests that a recapitulation of developmental events involving the ERM proteins is a component of the neuronal response to injury, a role for ERMs that has not previously been reported.

The involvement of the ERM binding partner L1 in the neuronal response to trauma was also assessed. An effective method for studying L1 in in vitro systems involves providing neurons with purified L1 as a growth substrate (Lagenaur and Lemmon, 1987). Substrate-bound or soluble L1 facilitates L1 mediated processes such as neurite
outgrowth (Doherty et al., 1995) when compared with neuron growth on regular culture substrates such as poly-l-lysine. This was confirmed in these experiments, where axon lengths measured during early hippocampal development demonstrated longer axons of neurons grown on the L1 substrate. Furthermore, previous studies suggest that in both *in vitro* and *in vivo* models, upregulation or experimentally applied L1 following neuronal injury may aid the regenerative response, (Poltorak et al., 1993; Aubert et al., 1998; Brook et al., 2000; Becker et al., 2001; Mason et al., 2003) although the exact mechanism is unknown. Indeed, in the current investigation, L1 facilitated the outgrowth of regenerative neurites in injured hippocampal and cortical neuron cultures, as regenerative neurites of neurons from both origins grown on the L1 substrate were significantly longer than those grown on poly-l-lysine alone. Taken together, these results implicate both L1 and the ERM proteins in the post-injury regenerative response.

The present study also demonstrated the influence of growth substrate on growth cone morphology. This has been documented for developing neurons (Payne et al., 1992; Brittis et al., 1995; Burden-Gulley et al., 1995; Burden-Gulley and Lemmon, 1996; Burden-Gulley et al., 1997), where poly-l-lysine, laminin and L1 offered as growth substrates resulted in strikingly varied growth cone morphology. This study has now demonstrated that growth cones of hippocampal and cortical regenerative neurites from relatively mature, injured neurons displayed different morphologies on the L1 substrate when compared with poly-l-lysine. Growth cones on the L1 substrate were larger and appeared to have more filopodia. This indicates that L1 substrate promotes growth cone morphology likely to be linked to neurite outgrowth.

Unexpectedly, post-injury data indicated that relatively mature cultured cortical and hippocampal neurons also differed in their post-injury neurite growth response, since cortical regenerative neurites were significantly longer than hippocampal regenerative neurites at 6 hours post-injury, when grown on both poly-l-lysine and L1 substrates. This suggests the possibility that neurons taken from the cortex and hippocampus have differing abilities to mount a post-injury regenerative sprouting response, and / or that the rate of regenerative sprouting differs between neuron types. The latter is likely given
that by 72 hours post-injury, hippocampal and cortical neurons had comparable patterns of neurite sprouting. Comparisons between the regenerative abilities between different neuronal populations have not previously been widely reported.

Furthermore, hippocampal neurite growth cones were significantly larger than cortical growth cones when the growth substrate was L1, whereas the reverse was true for the poly-l-lysine substrate. In combination, this indicates that hippocampal neurons may be more responsive to the L1 substrate, which resulted in significantly longer sprouting neurites on the L1 substrate than their counterparts grown on poly-l-lysine substrate and significantly larger growth cones than those from cortical regenerative neurites grown on L1. Also, cortical regenerative neurite sprout growth cones shared similar morphology and area on both substrates, suggesting they were less responsive to the L1 substrate. In interpreting these results it must also be considered that the differences in cortical and hippocampal regenerative responses and growth cone morphology may be due to differences in their relative maturity. At E18, when the neurons were harvested for culture, cortical development is more complete than hippocampal development, much of which occurs post-natally.

Therefore, this data illustrates that the ERM proteins, and their binding partner L1, are involved in both developmental processes and the neuronal regenerative response to trauma. Active ERM was localised to areas of re-growth by injured neurons during regenerative attempts, and L1 facilitated this response resulting in a more robust neurite sprouting response. These experiments have shown that growth characteristics after injury are influenced by growth substrate, and suggest that neurons of hippocampal and cortical origin cultured in vitro may differ in their response to injury. These data implicate ERMs, as well as L1, in the adult CNS response to trauma, information that will contribute to avenues of investigation into interventions for improvement of outcomes following many forms of neuronal injury.
5 IDENTIFICATION AND CHARACTERISATION OF A POPULATION OF MOTILE NEURONS WHICH EXPRESS ERM PROTEINS, IN LONG-TERM CORTICAL CULTURE

5.1 INTRODUCTION

During cerebral cortical development, post-mitotic neurons generated in the proliferative ventricular zones migrate towards the pial surface in order to reach their correct position in the neocortex, a distance of several thousand micrometers in some species (reviewed by Tsai and Gleeson, 2005). Neurons cease migration when they receive the appropriate stop signals, and then settle into their position in the horizontal laminar organisation of the neocortex (reviewed by Bielas et al., 2004). They then develop an axon and dendrites, which form synaptic connections with other cells. From this point, neurons of the cortex are considered to become relatively immobile in the environment that is densely packed with neurons, their supporting cells, and the extracellular components. However, some neurons are able to migrate through regions of the more mature brain. For example, neural precursors are continually produced in the subventricular zone (Luskin, 1993) and migrate through the RMS to replace neurons of the olfactory bulb throughout life (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996). There is also evidence suggesting that multiple forms of CNS injury induce neurogenesis in the mature brain (reviewed by Parent, 2003; Kulbatski et al., 2005), and cells can migrate toward cortical lesions (Sundholm-Peters et al., 2005). This has particular implications for brain injury and healing in the mature nervous system, since it has previously been assumed the adult CNS has a limited capacity for plasticity (reviewed by Chuckowree et al., 2004).

Developmental neuron migration is partially influenced by directional guidance cues. These cues may exist in the form of ECM molecules and CAMs, as well as the more classically defined chemo-attractive and -repellent molecular guidance cues. While there has been a great deal of investigation into the roles of guidance cues in axonal outgrowth and pathfinding, much less is understood about how those same molecules influence neuron migration (see reviews by Park et al., 2002; Ward et al., 2003), and
whether they are similarly effective in modulating the behaviour of more mature neurons. Guidance cues interact with their specific receptors on the surface of neurons, which activates intracellular signalling cascades (for review see Song and Poo, 2001), in turn modulating microtubule and actin cytoskeleton components (reviewed by Meyer and Feldman, 2002; Tsai and Gleeson, 2005) and their associated molecules, to bring about the structural changes required for a neuron to undergo translocation. Doublecortin (DCX), a MAP-like protein (Feng and Walsh, 2001), has received increasing attention due to its crucial role in developmental neuron migration, which is evidenced by disorders of migration resulting from mutation in the gene encoding DCX (reviewed by Gleeson, 2000).

The ERM proteins are also potentially important for neuron migration, as they function in mechanisms important for motility, including cell-substrate adhesion, and the localisation of surface adhesion molecules (for example, Lee et al., 2004). They have also been shown to be important for migration in a number of cell types, including lymphocytes, epithelial cells and tumour cells (for example, Sahai and Marshall, 2003; Nijhara et al., 2004; Borm et al., 2005). This, combined with their expression in regions of migration in the developing brain (Mintz et al., 2003), ideally places them as important for neuron migration, although this role has not been fully investigated.

*In vitro* neuron cultures derived from the embryonic neocortex are a heterogeneous population of neuronal phenotypes, which correlate well with *in vivo* neuronal phenotypes (Fletcher and Banker, 1989; Banker and Goslin, 1998). When cortical neurons are maintained in long-term culture, they develop characteristics comparable to mature neurons *in vivo*, including polarisation (de Lima et al., 1997), the loss of developmental markers (Chuckowree and Vickers, 2003), expression of specific protein markers related to maturity (de Lima et al., 1997; Chuckowree and Vickers, 2003) and formation of synapses (Ichikawa et al., 1993; de Lima et al., 1997), as well as exhibiting electrophysiological properties, including the generation of potentials (Dichter, 1978; Brodie et al., 1986). Furthermore, *in vitro* techniques have been used successfully to study neuron migration (for example, Behar et al., 2001; Kholmanskikh et al., 2003),
and have particular merit in facilitating the observation of individual cells (Bellion et al., 2005; Nobes and Hall, 1999).

The current experiments have utilised the previously described in vitro transection model (Chapter 4) to observe the motile capacity of neurons in primary cortical cultures. This model bears similarity to the wound-healing assays used to observe motility in other cell types, such as fibroblasts (Nobes and Hall, 1999). A highly motile population of neurons in long-term culture was observed after transection injury. To establish specific phenotypic characteristics, their cytoskeletal profile was examined. Furthermore, whether ERM proteins play a role in this in vitro translocation, and migration in the embryonic brain, was examined. Cytoskeletal disrupting pharmacological agents were used to determine the role of the microtubule cytoskeleton in mediating the observed motility, and the motile neurons' response to isolated guidance cues including L1, laminin and Sema 3A was observed.
5.2 MATERIALS AND METHODS

5.2.1 Substrate preparation and cell culture
Laminin, L1-Fc protein, poly-l-lysine and Sema 3A/Fc were applied as substrates, described in section 2.1.1 (Materials and Methods). A range of Sema 3A/Fc concentrations were tested, and at 4μg/mL showed inhibitory effects, while supporting adhesion and neuron growth. Laminin was the standard culture substrate in these experiments. Primary dissociated cortical neuron cultures were prepared as described in sections 2.1.2 and 2.1.3 (Materials and Methods).

5.2.2 In vitro neuronal transection injury
Post-injury motility assays were performed on cortical neurons in long-term culture (21 DIV) by subjecting them to transection injury, according to procedures described in section 2.2 (Materials and Methods). Coverslips were photographed to produce a time-lapse imaging sequence (images obtained every 4 hours over a 24 hour period post-injury), then fixed with 4% PFA, or in all other experiments, fixed at 24 hours post-injury with 4% PFA or 10% TCA for immunocytochemistry. For cytoskeletal disruption studies, taxol (Sigma), a microtubule stabilising agent (Schiff and Horwitz, 1980), was initially reconstituted with 20% DMSO / 0.01M PBS, then applied to neuron cultures at a final concentration of 10μg/mL (Chuckowree and Vickers, 2003). Nocodazole (Sigma), a microtubule destabilising agent (Samson et al., 1979), was reconstituted with 50% DMSO / 0.01M PBS, then applied at a final concentration of 1μg/mL (Chuckowree, 2005). Drugs were applied immediately after the injuries were performed. Vehicle control treated cultures were processed concurrently.

5.2.3 5-Bromo-2'-deoxyUridine (BrdU) incorporation
To assay the approximate birth date of neurons in the current in vitro model, cortical neuron cultures were incubated with 5μM or 10μM of the thymidine analogue BrdU (Sigma) at 1, 7 and 17 DIV for 72 hours, or applied at 21 DIV, at the time of transection injury. Injuries were performed on cultures at 21 DIV and fixed 24 hours post-injury.
Dividing cells within the time frames of application were identified by immunolabelling with an antibody with immunoreactivity to BrdU (Table 5.1).

5.2.4 Immunocytochemistry

Cortical neuron cultures were immunolabelled with a range of primary antibodies (details are provided in Tables 2.1 and 5.1) using standard protocols described in section 2.3 (Materials and Methods). Isotype and species appropriate Alexa Fluor secondary antibodies were used to visualise antibody localisation (Table 2.2). To visualise cell nuclei, Nuclear Yellow (0.01%) was applied for ten minutes following completion of secondary antibody incubation.

5.2.5 Immunohistochemistry

Sections from E18 rat cortex were examined to determine the localisation of ERM proteins in vivo, a developmental stage when cortical migration is not yet completed (Ignacio et al., 1995). Brains were removed, immersion fixed with 4% PFA, and cryoprotected with sucrose solutions. 40μm coronal sections were prepared using a cryostat. Sections were immunolabelled with an antibody to DCX (1:2000; Table 5.1), and an antibody to phosphorylated ERM (1:100; Table 2.1), and stained with Nuclear Yellow (0.01%). Isotype and species appropriate Alexa Fluor secondary antibodies (1:500) were used to visualise antibody localisation (Table 2.2).

5.2.6 Microscopy and analysis

A Leica DM IRB inverted microscope equipped with a heated stage (37°C) was used for time-lapse microscopy and immunofluorescence analysis was performed using a Leica DM LB2 microscope, as described in section 2.4 (Materials and Methods). For cytoskeletal disruption studies and assessment of the role of guidance cues in motility, Improvision Openlab software was used to determine the average number of neurons located within lesion sites, per mm of lesion length, was determined and the distance of translocation was measured as the minimum distance from the edge of the lesion to the
Table 5.1 Primary antibodies for immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-BrdU</td>
<td>mouse</td>
<td>1: 1000</td>
<td>BD</td>
</tr>
<tr>
<td>anti-GFAP</td>
<td>mouse</td>
<td>1: 2000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>anti-ferritin</td>
<td>rabbit</td>
<td>1: 2000</td>
<td>DAKO</td>
</tr>
<tr>
<td>anti-GAD 65</td>
<td>mouse</td>
<td>1: 500</td>
<td>Chemicon</td>
</tr>
<tr>
<td>anti-GAD 67</td>
<td>mouse</td>
<td>1: 1000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>anti-calretinin</td>
<td>rabbit</td>
<td>1: 5000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>anti-nestin</td>
<td>mouse</td>
<td>1: 2000</td>
<td>BD</td>
</tr>
<tr>
<td>anti-α-internexin</td>
<td>mouse</td>
<td>1: 1000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>anti-NeuN</td>
<td>mouse</td>
<td>1: 1000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>anti-NF-L</td>
<td>rabbit</td>
<td>1: 500</td>
<td>Novus</td>
</tr>
<tr>
<td>anti-NF-M</td>
<td>rabbit</td>
<td>1: 1000</td>
<td>Serotec</td>
</tr>
<tr>
<td>anti-NF-H</td>
<td>rabbit</td>
<td>1: 1000</td>
<td>Serotec</td>
</tr>
<tr>
<td>anti-NCAM-L1</td>
<td>rat</td>
<td>1: 1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-DCX</td>
<td>guinea pig</td>
<td>1: 10000</td>
<td>Chemicon</td>
</tr>
</tbody>
</table>
neuron cell body. Fifteen to twenty injury sites from three separate neuronal cultures were included in the statistical analysis.
5.3 RESULTS

5.3.1 Motile neurons infiltrate the injury site following transection in primary cortical neuron cultures

The well-established in vitro transection injury model based on neurons in long-term culture (21 DIV; Dickson et al., 2000) results in a typical progression of changes, beginning with retraction of the injury site edges (Chuckowree and Vickers, 2003), and then a cell free injury site (Figure 5.1 A). The model has been successfully used to observe the regenerative neurite sprouting response (for example, Dickson et al., 2000; Chuckowree and Vickers, 2003; Chapter 4). The sprouting response is robust, with injury sites almost completely covered with regenerative neurites by 48 to 72 hours post-injury (Chapter 4). In the current experiments, time-lapse microscopy was performed over the 24 hours following transection injury, and has shown that accompanying regenerative neurite spouting is a phenomenon in which a distinct population of motile neurons enter the injury site (Figure 5.1). This response was initiated within the first 4 hours of injury and by 24 hours was well established (Figure 5.1 A). The cells positively immunolabelled with the neuronal marker β-III-tubulin (Figure 5.1 B), but were not immunolabelled with an antibody to the astrocyte marker GFAP (Figure 5.1 C), the principle glial cell phenotype in cortical neuron cultures.

Particular aspects of the morphological characteristics of the motile neuron population are indicative of their motile ability. They are small, multipolar neurons that, in many cases, did not appear to be polarised (Figure 5.1 B). Furthermore, they had not developed the long neurites characteristic of other neurons at 21 DIV, such as pyramidal neurons, nor did they appear to synapse with other neurons in culture. In combination, these characteristics initially suggested that they might be a neural progenitor population. However, in contrast to this hypothesis, previous studies from our laboratory have shown that at 21 DIV all neurons are at least 14 days of age in cortical neuronal cultures (Chuckowree and Vickers, 2003), due to the inability of post-mitotic neurons to undergo any further divisions. Therefore, further studies were performed to
Figure 5.1 Identification of a distinct population of motile neurons in long-term cortical neuron cultures. Cortical neurons were grown in culture until 21 DIV, when they were subjected to transection injury. A. The response to injury was examined by time-lapse microscopy. Injury sites were photographed from 0 hours (immediately after the injury) to 24 hours post-injury, at 4 hour time intervals. Within 4 hours post-injury, neurons had entered the injury site (arrow, inset is a magnification of the neuron), a phenomenon that increased over the 24 hours post-injury. Immediately after the 24 hour post-injury timepoint the neuron cultures were fixed and immunolabelled, to identify whether the motile cells were neurons (β-III-tubulin labelling) or glial cells (GFAP labelling). B. β-III-tubulin immunolabelling, a magnification of the boxed area in A, demonstrated that the motile cells were neurons. The neurons were small, multipolar neurons, only a subset of which appeared to be polarised (arrows). C. Co-immunolabelling with GFAP demonstrated that none of the cells within the injury site (in B) were a glial phenotype (arrows). Dashed lines outline the injury border. Scale bar: A = 100μm, inset = 50μm; B-C = 50μm.
identify the neurons based on their neurochemical characteristics, with a particular focus on determining their phenotype and stage of maturation.

5.3.2 Motile neurons do not incorporate BrdU, indicating that they are 21 days old

To assay the approximate birth date of neurons in culture, the thymidine analogue BrdU was applied to cultures at a range of time-points, including 1-4 DIV, 7-10 DIV, 17-20 DIV and immediately after injury at 21 DIV, to detect dividing cells within those time-frames. Neuron cultures were then injured at 21 DIV, and immunolabelled 24 hours post-injury to examine when the motile neuron population emerges.

Interestingly, the motile neuron population did not incorporate BrdU at any of the timepoints tested (Figure 5.2 A-D), indicating that these neurons were present at the beginning of the culture period and therefore at least 21 days old. However, this motile ability does not fit with the traditional view of more mature neurons in long-term culture. The possibility that the cells resulted from proliferation in response to the injury was unlikely because BrdU applied at the time of injury was not incorporated into the motile neurons (Figure 5.2 D).

BrdU application to cell cultures at a concentration of 10μM, at 1 DIV (Figure 5.2 A) for 72 hours may have had a toxic effect, therefore resulting in an observed decreased amount of incorporation into cells compared to other timepoints. This possibility was recognised because more proliferating cells at earlier timepoints would have been expected. Therefore, to account for any toxic effect a lower concentration of BrdU, 5μM, was employed as comparison with the 10μM concentration, and all of the representative cultures shown in Figure 5.2 were treated with the lower concentration. The continued efficacy of BrdU incorporation to identify dividing cells at the lower concentration is demonstrated in Figure 5.2, with immunolabelling often observed in the nuclei of cells with glial morphology rather than neurons (for example, Figure 5.2 D).
Figure 5.2 BrdU incorporation in cortical neuron cultures. To determine the approximate birth date of the motile neuron population, cultures were incubated with BrdU at various timepoints throughout the culture period and then injured at 21 DIV. 24 hours post-injury, cultures were fixed and immunolabelled with NF-M, to identify neurons, and an antibody to BrdU, to detect dividing cells. A. BrdU applied at 1 DIV and removed after 72 hours. Very few cells were BrdU labelled at this early timepoint. B. BrdU applied at 7 DIV for 72 hours. C. BrdU applied at 17 DIV for 72 hours. D. BrdU added immediately after injury. The large arrow indicates a cell of glial morphology that incorporated BrdU. A to D show that the motile neuron population (arrows) did not immunolabel with the antibody to BrdU (arrowheads) at any of the timepoints tested. Scale bar = 50 μm.
5.3.3 The cytoskeletal profile of motile neurons indicates they are differentiated, but not mature

In order to determine the specific phenotype and stage of maturation of the motile neuron population after injury, cortical neuron cultures were grown until 21 DIV, subjected to transection injury, fixed at 24 hours post-injury and immunolabelled with a range of antibodies. The results are summarised in Table 5.2 and illustrated in Figure 5.3. The neurons did not positively immunolabel with antibodies with reactivity to GFAP (Figure 5.3 A), a marker of astrocytes, or ferritin (Figure 5.3 A), a marker for microglia, but as previously discussed the cells were immunolabelled with an antibody to the neuron-specific type III β-tubulin (Figure 5.1 B, Figure 5.3 D). β-III-tubulin is a component of microtubules and often considered a marker of immature neurons, however in long-term cultures neurons were observed to continue to uniformly express β-III-tubulin throughout the cell (for example, Figure 5.3 D).

Due to the morphology of motile neurons, it was initially hypothesised that they might be an interneuron phenotype. However, the motile neuron population did not immunolabel with the GABAergic neuron markers GAD 65 and GAD 67 (for example, Ogoshi and Weiss, 2003; Figure 5.3 B and 5.3 C, respectively), nor were they positive for calretinin (Figure 3D), a calcium binding protein localised to a subset of GABAergic neurons (for example, Pappas and Parnavelas, 1998). Therefore, the motile neurons did not display common neurochemical characteristics of interneurons.

The expression of markers related to development and maturity was examined in the motile neuron population. The neurons did not immunolabel with an antibody to nestin (Figure 5.3 E), which is an intermediate filament protein expressed by neural precursors (recently reviewed by Michalczyk and Ziman, 2005), which suggested that they had differentiated into neurons. The motile neurons did, however, express α-internexin (Figure 5.3 F), an intermediate filament protein that starts to be expressed during neuronal differentiation (Shea and Beermann, 1999), and shows compartmentalised
Table 5.2 The cytoskeletal profile of motile neurons

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-GFAP</td>
<td>astrocytes</td>
</tr>
<tr>
<td>anti-ferritin</td>
<td>microglia</td>
</tr>
<tr>
<td>anti-β-III-tubulin</td>
<td>microtubules, neuron specific</td>
</tr>
<tr>
<td>anti-GAD 65</td>
<td>GABAergic interneurons</td>
</tr>
<tr>
<td>anti-GAD 67</td>
<td>GABAergic interneurons</td>
</tr>
<tr>
<td>anti-calretinin</td>
<td>a subset of GABAergic interneurons</td>
</tr>
<tr>
<td>anti-nestin</td>
<td>neural progenitor cells</td>
</tr>
<tr>
<td>anti-α-internexin</td>
<td>neuronal intermediate filament</td>
</tr>
<tr>
<td>anti-NeuN</td>
<td>post-mitotic neurons</td>
</tr>
<tr>
<td>anti-NF-L</td>
<td>neuronal intermediate filament, marker of early neurons</td>
</tr>
<tr>
<td>anti-NF-M</td>
<td>neuronal intermediate filament, marker of early neurons</td>
</tr>
<tr>
<td>anti-NF-H</td>
<td>neuronal intermediate filament, marker of more mature neurons</td>
</tr>
<tr>
<td>anti-MAP2</td>
<td>microtubule associated protein</td>
</tr>
<tr>
<td>anti-tau</td>
<td>microtubule associated protein</td>
</tr>
<tr>
<td>anti-NCAM-L1</td>
<td>polarised neurons</td>
</tr>
<tr>
<td>anti-DCX</td>
<td>migrating neurons</td>
</tr>
</tbody>
</table>
Figure 5.3. Characterisation of the motile neuron population by examination of the cytoskeletal profile. Neurons were grown in culture until 21 DIV, when they were injured. 24 hours post-injury they were fixed and immunolabelled with a range of antibodies to facilitate identification of the phenotype of the motile neuron population. Refer to Table 5.2 for specific immunoreactivity details. A. Neurons within the injury site did not immunolabel with antibodies to either GFAP (green) or ferritin (red). B. Motile neurons were immunopositive for NF-M (red), but not GAD 65 (green). C. Neurons were immunolabelled with the MAP tau (red), but not GAD 67 (green). D. β-III-tubulin (green) is uniformly expressed in cortical neurons at 21 DIV (arrowhead) and was similarly expressed by motile neurons, but they did not label with calretinin (red). E. Tau (red) labelled motile neurons did not immunolabel for nestin (green). F. The motile neurons were labelled with both α-internexin (green) and NF-M (red). G. Tau (red) labelled motile neurons were not immunopositive for NeuN (green), which labelled only a subset of neuronal nuclei in cultures (arrowhead). H. β-III-tubulin (green) co-immunolabelling with NF-L (red). I. β-III-tubulin (green) positive motile neurons were in some cases very weakly labelled with NF-H (red - arrow). J. Tau (red) labelling was more intense in the axon of polarised neurons (arrow), but motile neurons did not immunolabel with MAP2 (green). K. In many areas β-III-tubulin (green) co-localised with L1 (red), but L1 labelling was more notable in the axon of polarised neurons (arrow). L. β-III-tubulin (green) labelled motile neurons were not labelled with DCX (red). Dashed lines in B, C, E and J-L outline the injury border. Scale bar = 50μm.
localisation in the axon and, particularly dendrites (Benson et al., 1996), of a sub-population of neurons in long-term culture (Dickson et al., 2005). In contrast, the neurons did not immunolabel with the post-mitotic neuronal marker NeuN (Figure 5.3 G), but it was noted that this marker only labelled a small population of neurons throughout the cultures. Motile neurons were labelled with NF-L (Figure 5.3 H) and NF-M (Figure 5.3 F), but NF-H was either absent, or alternatively showed very weak immunolabelling confined to the soma (Figure 5.3 I). NFs are neuron-specific cytoskeletal intermediate filament proteins expressed by distinct neuron populations (Vickers and Costa, 1992; Kirkcaldie et al., 2002), predominantly in the axon (Benson et al., 1996). Neuronal labelling with NFs is indicative of their level of maturity, because NF-L and NF-M are expressed earlier than NF-H (Benson et al., 1996; reviewed by Lariviere and Julien, 2004). NF-H expression is often associated with more mature neurons, which might imply that the motile neurons had not reached full maturity. The motile neurons also did not express MAP2 (Figure 5.3 J), which increases in dendrites with maturation (de Lima et al., 1997) and persists in long-term cortical cultures. Thus, these data indicate that the neurons were differentiated, but perhaps not mature.

In contrast to MAP2 immunolabelling, motile neurons did express the MAP tau (Figure 5.3 J). Tau is involved in the establishment of polarity in neurons (Caceres and Kosik, 1990). Tau was localised to the cell body of motile neurons, with weaker localisation in neurites. Occasionally there was more distinct localisation in one neurite, which was likely to be the axon (Figure 5.3 J). The NCAM-L1 antibody immunolabels the axon specific, developmentally expressed, CAM L1 (Persohn and Schachner, 1987; Linnemann et al., 1988). Motile neurons showed weak positive cellular distribution of L1, with more intense labelling in one neurite, the axon, in some motile neurons (Figure 5.3 K). Therefore, most motile neurons had not yet become polarised.

Quite surprisingly, the motile neurons did not immunolabel with DCX (Figure 5.3 L), a marker of migrating immature neurons (Gleeson et al., 1999) that is strongly associated with microtubule function. DCX expression is most often associated with cell bodies and the leading processes of migrating neurons and the axons of axons of differentiating
neurons (Francis et al., 1999; Friocourt et al., 2003). Therefore, the current results suggested that DCX expression is not required for motility per se in the motile neuron population. In contrast, other larger neurons with more highly extended and elaborate processes within the same culture were strongly immunopositive for DCX (Figure 5.4).

5.3.4 **ERM proteins are localised to the motile neuron population, *in vitro* and immature neurons of the embryonic cortex, *in vivo***

As previously described, under normal culture conditions, by 21 DIV phosphorylated ERM is not expressed (Chapter 3). However, immunocytochemical analysis of the motile neurons that had undergone translocation into the injury site revealed a striking expression of phosphorylated ERM localised in neurite filopodia and growth cone-like structures (Figure 5.5 A). While other elements of the cytoskeletal profile suggested that the motile neurons had not reached the same level of maturity as other neurons in culture, examination of uninjured cultures did not reveal any obvious neuron populations that continued to express phosphorylated ERM at 21 DIV (Figure 5.5 B). Thus, it was unlikely that this population had continually expressed phosphorylated ERM throughout the culture period. Therefore, phosphorylated ERM might have been specifically re-expressed for translocation, which strongly supports a role for ERM proteins in migration.

Due to the expression of phosphorylated ERM in the motile neuron population, ERM expression was assessed in a well-characterised stage of migration in the embryonic cortex during brain development to determine whether ERMs are also involved in migration of immature neurons *in vivo*. Analysis of brain sections from E18 rats revealed extensive DCX labelling, which identified immature migrating neurons, in the cortical plate and intermediate zone, regions also known to be strongly immunopositive for the ERM proteins at the same stage of development (Mintz et al., 2003). In agreement with that study, double labelling revealed that axons labelled with DCX were co-labelled for the ERM proteins (Figure 5.6 A-D). In addition, a subset of neurons located within the intermediate zone, at the border of the ventricular zone, also
Figure 5.4 DCX immunolabelling in cortical neuron cultures at 21 DIV.

Primary cortical neurons in culture were injured at 21 DIV, and immunolabelled with antibodies to β-III-tubulin (green) and DCX (red). While the motile neuron population did not express DCX (Figure 5.3), a subset of neurons in the cultures did co-immunolabel with antibodies to β-III-tubulin and DCX (arrows). Scale bar = 30μm.
Figure 5.5 Phosphorylated ERM expression in the motile neuron population. Neurons were grown in culture until 21 DIV, when they were injured. 24 hours post-injury they were fixed and immunolabelled with antibodies to β-tubulin (red) and phosphorylated ERM (green). A. Immunocytochemical analysis of the motile neurons that underwent translocation into the injury site revealed a striking expression of phosphorylated ERM, localised in neurite filopodia and growth cone-like structures (arrows). B. In contrast, examination of uninjured areas of the cultures did not reveal any obvious neuron populations that continued to express phosphorylated ERM at 21 DIV. Neurons in uninjured areas displaying similar morphology to the motile neuron population did not express phosphorylated ERM (arrows), which suggested phosphorylated ERM was expressed specifically by translocating neurons in the response to injury. Scale bar = 50μm.
Figure 5.6 Phosphorylated ERM and DCX immunolabelling in migrating neurons of the embryonic neocortex. The embryonic (E18) cortex was examined by immunohistochemistry to determine whether migrating neurons, identified by DCX immunolabelling (red), also expressed phosphorylated ERM (green). A. The cortical plate of E18 neocortex contained neurites immunopositive for phosphorylated ERM proteins (arrow). B. DCX immunolabelling of the same region as shown in A, the arrow indicates the same neurite was also labelled with DCX. C. A composite image of phosphorylated ERM and DCX immunolabelling demonstrates co-localisation, as indicated by the arrow. D. An increased magnification of A, to show specific ERM labelling (arrowhead). E. An increased magnification of B, to show specific DCX immunolabelling (arrowhead). F. Neuronal cell bodies at the border of the intermediate and subventricular zones were often labelled for phosphorylated ERM (arrow). G. DCX immunolabelling of the same area in E demonstrated labelling with DCX, as indicated by the arrow. H. A composite image of phosphorylated ERM and DCX immunolabelling demonstrates the somal co-localisation (arrow). I. An increased magnification of F, to show specific ERM localisation (arrow). J. An increased magnification of G, to show specific DCX localisation (arrow). Scale bar: A – C, F – H = 20μm; D - E, I - J = 10μm.
expressed both DCX and ERM proteins (Figure 5.6 E-J). These data further implicate the ERM proteins as contributing to the specific mechanisms underpinning neuronal migration.

5.3.5 Microtubule destabilisation induced by nocodazole significantly increases neuron motility

The cytoskeletal altering agents taxol and nocodazole were used to examine the role of the microtubule cytoskeleton in translocation in the motile neuron population. The agents were applied immediately after transection injury, then 24 hours post-injury cultures were fixed and immunolabelled to facilitate observation of changes in motile ability, compared with vehicle controls.

Interestingly, microtubule stabilisation through taxol application resulted in no significant changes in the number of neurons that entered the injury site (3 ± 0.83 neurons/mm injury) compared with the vehicle control treated cultures (7 ± 1.93 neurons/mm injury; p = 0.08; Figure 5.7 A). Similarly, nocodazole treatment, which is used to destabilise microtubules, resulted in no significant changes in the number of neurons that entered the injury site in the treated cultures (5 ± 1.99 neurons/mm injury) compared with vehicle controls (9 ± 2.34 neurons/mm injury; p = 0.08; Figure 5.7 B). Taxol caused no changes in the distance of neuron translocation into the injury site (25 ± 1.69µm), compared with controls (28 ± 1.38µm; p = 0.15; Figure 5.7 C). In contrast, nocodazole treatment resulted in significantly increased distance of translocation into the injury site (33 ± 1.88µm), compared with controls (25 ± 0.96µm; p < 0.05; Figure 5.7 D). It was also observed, particularly in nocodazole treated cultures, that neurons which had entered the injury site were often rounded, with no neurites observed by immunolabelling. Therefore, it is possible that the neurites were lost in response to treatment, although simultaneous labelling of nuclei did not suggest that the health of neurons was compromised by either taxol or nocodazole treatment.
Figure 5.7 The effect of microtubule altering agents on the motile neuron population. Primary cortical neuron cultures were grown until 21 DIV, when they were subjected to transection injury. Immediately after injury they were treated with taxol (10μg/mL) or nocodazole (1μg/mL), or their vehicle controls. 24 hours post-injury cultures were fixed and analysed for the effect of microtubule disrupting agents on the motility of the neuron population. A. The number of neurons per mm of lesion after taxol treatment, compared with vehicle controls. B. The number of neurons per mm of lesion after nocodazole treatment, compared with vehicle controls. C. The mean distance of neuron translocation into the lesion after taxol treatment, compared with vehicle control treated cultures. D. The mean distance of neuron translocation into the lesion after nocodazole treatment, which was significantly greater than vehicle control treated cultures. * represent p values less than 0.05. Error bars are ± SEM.
A. Number of motile neurons - taxol

B. Number of motile neurons - nocodazole

C. Distance of neuron translocation - taxol

D. Distance of neuron translocation - nocodazole
5.3.6 Motile neurons are significantly influenced by substrate bound molecular guidance cues

The effect of molecular guidance cues on neuron motility has not been widely studied, although it is thought that the same molecules important for axon guidance are probably involved in neuron migration (reviewed by Song and Poo, 2001). Therefore, the effects of poly-l-lysine, laminin, L1 and Sema 3A on motility in this neuron population were studied. Neurons were grown on substrates containing each of the guidance cues until 21 DIV, injured and then 24 hours post-injury the number of neurons and their distance of translocation into the injury site were examined.

Culturing cortical neurons on substrates of the different guidance cues resulted in significant changes in aspects of neuron motility. This primarily demonstrates that post-mitotic neurons in long-term culture are responsive to guidance cues. Laminin substrates promoted the greatest neuron motility, with significantly more neurons entering the injury site (17 neurons/mm ± 1.41) than on any other substrate, including poly-l-lysine (11 neurons/mm ± 1.42), L1 (9 neurons/mm ± 0.93) and Sema 3A (5 neurons/mm ± 1.3) (p < 0.05; Figure 5.8 A). Figure 5.8 B shows that a similar pattern was observed with respect to the distance of movement, in response to the guidance cues. Therefore, the laminin substrate promoted the greatest average distance of translocation (37μm ± 0.94), which was significantly greater than poly-l-lysine (34μm ± 1.01), L1 (29μm ± 1.0) and Sema 3A (26μm ± 1.06) (p < 0.05). The poly-l-lysine substrate promoted the next greatest mean distance of translocation, which was significantly greater than the distance of translocation on L1 (p < 0.05) and Sema 3A (p < 0.05). L1 also promoted a significantly higher mean distance of neuronal translocation when compared with Sema 3A (p < 0.05). Therefore, these results confirm that developmental guidance cues potentially influence the motility of neurons after their differentiation.
Figure 5.8 The effect of molecular guidance cues on the motile neuron population. Primary cortical neurons were grown on substrates containing laminin, L1, poly-l-lysine or Sema 3A until 21 DIV, when they were subjected to transection injury. 24 hours post-injury cultures were fixed and analysed for differences in aspects of the motility of the neuron population. A. The number of neurons per mm of lesion when grown on the four different substrates. B. The average distance of neuron translocation in response to substrate bound guidance cues. * represent p values less than 0.05. Error bars are ± SEM.
A

Number of motile neurons

B

Distance of neuron translocation

- Number of motile neurons
- Distance of neuron translocation

Graphs showing the number of motile neurons and the distance of neuron translocation for different treatments.
5.4 DISCUSSION

These experiments have demonstrated the existence of a highly motile population of neurons in long-term primary cortical neuron cultures, identified because they rapidly infiltrate the injury site following transection. They did not incorporate BrdU at any of the timepoints tested throughout the culture period. Immunolabelling with a range of markers indicated that the neurons were differentiated, but had possibly not reached the maturity of other neurons in culture. Motile neurons specifically expressed phosphorylated ERM proteins, which suggested a role in neuron translocation and migration. The role of the microtubule cytoskeleton in motility was examined, namely by application of taxol or nocodazole, which showed that microtubule destabilisation significantly enhanced aspects of neuron motility. The motile neurons were responsive to molecular guidance cues, in a similar manner to that expected of neural precursors or immature neurons, with laminin substrates promoting significantly greater motility than those containing L1 or Sema 3A.

The transection injury model facilitated the novel observation that a population of highly motile neurons infiltrates the injury site. BrdU incorporation studies initially indicated that the neurons were present from the beginning of culture; therefore they were at least 21 days old. Neurons at 21 DIV are considered long-term cultures and most individual neurons displayed morphological and neurochemical characteristics of maturity, but this did not apply to the motile neuron population. Examination of the motile neurons’ cytoskeletal profile suggested that they were not neural precursor cells, because they were not immunolabelled with nestin. Instead, they were post-mitotic and differentiated into a neuronal phenotype, as indicated by their immunoreactivity to markers including β-III-tubulin, α-internexin, NF-L and NF-M. In addition, a subset of the motile neurons was polarised, identified by specific axonal immunolabelling with the markers tau and L1.

Factors that indicated the motile neurons had not matured included their lack of immunolabelling with antibodies to MAP2 and NF-H. Multiple MAP2 isoforms and phosphorylation states (Riederer and Innocenti, 1992) make the exact expression profile
of MAP2 complicated, but it appears that expression is conserved amongst all neuronal phenotypes of the cortex in vivo, except in aging neurons an eventual decrease in MAP2 mRNA and protein expression occurs (Riederer and Innocenti, 1992). Furthermore, while the NF triplet is only expressed in a specific subset of cortical neurons (Vickers and Costa, 1992; Kirkcaldie et al., 2002), the motile neurons expressed NF-L and NF-M, which indicated they would also eventually express NF-H. NF-L is the first NF expressed, followed by NF-M and then NF-H (reviewed by Lariviere and Julien, 2004), a pattern that is also observed in culture (Benson et al., 1996). Therefore, the virtual absence of NF-H immunolabelling was another indicator that the neurons had not reached maturity. However, a very weak expression of NF-H immunolabelling in the cell bodies of some motile neurons was observed, but it was not possible to decipher whether this represented the initial stages of NF-H production in the perikaryon (Lariviere and Julien, 2004).

The motile neuron population did not express DCX, which is proposed to function in microtubule stabilisation. DCX is most predominantly expressed by migrating immature neurons during development (Gleeson et al., 1999), and is thus commonly used as a marker of neuron migration. DCX is also expressed by newly generated neurons in the adult brain (Brown et al., 2003) migrating along the RMS (Gleeson et al., 1999; Koizumi et al., 2006). The lack of DCX expression in the motile neuron population may be related to the fact that these neurons appear to have completed differentiation and in some cases were polarised, which might mean they are more mature than the neurons commonly identified by this marker. It may also be that the actin cytoskeleton might be the predominant system used in this motility, perhaps due to their phenotype or stage of maturity. The expression of DCX by some clearly differentiated neurons in non-injured areas of the culture was unexpected, but confers with an immunohistochemical study by Nacher and colleagues, which identified differentiated cells immunoreactive for DCX in a number of areas of the adult rat brain not related to neuronal migration (Nacher et al., 2001).
The observation that phosphorylated ERM immunolabelling was specific for the motile neuron population in \textit{in vitro} experiments strongly implied a role in neuron migration. Previous immunohistochemical studies support this conclusion. For example, ezrin is localised to migrating neurons of the intermediate zone in the prenatal human cerebrum (Johnson et al., 2002), and in another study, phosphorylated ERM was localised to processes throughout the intermediate zone of the embryonic (E17) rat cortex (Mintz et al., 2003). Therefore, this study sought to extend on this information by specifically examining phosphorylated ERM expression in the embryonic cortex in relation to DCX, which is localised to immature migrating neurons. DCX immunolabelling co-localised with phosphorylated ERM in axons and cell bodies of neurons within migratory zones of the cortex. This provides further evidence that ERMs are not only involved in \textit{in vitro} translocation, but developmental migration, \textit{in vivo}. Their associations with both the actin and microtubule cytoskeleton make them likely candidates for multiple functions in neuron migration.

To further understand the role of the microtubule cytoskeleton in neuron motility the microtubule stabilising agent taxol, and the microtubule destabilising agent nocodazole were used. Both agents have been used extensively to study microtubule dynamics in functions such as neurite initiation (Smith, 1994; Dehmelt et al., 2003), neurite outgrowth (Ruthel and Banker, 1998; Gallo and Letourneau, 1999; Chuckowree and Vickers, 2003), growth cone morphology and behaviour (Mansfield and Gordon-Weeks; 1991; Williamson et al., 1996; Rajnicek and McCaig, 1997; Gallo, 1998; Buck and Zheng, 2002) and axonal transport (Thies and Meller, 2000; Francis et al., 2005), but have only rarely been used in studies of neuron motility and migration (Schaar and McConnell, 2005). Schaar and McConnell (2005) showed that the application of nocodazole (1\textmu M) resulted in no difference in the velocity or the distance of nuclear translocation of neurons isolated from the anterior subventricular zone, \textit{in vitro}, which suggested that another cytoskeletal system controls nuclear movement. Indeed, the actin cytoskeleton is strongly implicated in migration in many cell types. In contrast, in these experiments nocodazole significantly increased the distance of translocation into the injury site. The sensitivity of neurons to nocodazole treatment in this model might be
related to their lack of DCX expression, because Yoshiura et al., (2000) demonstrated that in some cases DCX stabilises microtubules against nocodazole treatment.

In these experiments, the identified motile neuron population was affected by molecular guidance cues, which is promising for reasons related to post-injury regenerative responses in the mature brain. Various forms of CNS insult have been shown to induce neurogenesis in both the subventricular zone and hippocampal dentate gyrus (reviewed by Parent, 2003). There is also an increasing interest in the possibility of implanting stem cells to assist in functional recovery after CNS trauma and in neurological disorders (recently reviewed by Lindvall and Kokaia, 2006). Cell therapies, utilising endogenous or exogenous cells, are potentially feasible approaches, however unless the cells can migrate to areas of the brain where they are needed to replace lost neurons and are able to integrate into the neuronal circuitry they are not useful, and potentially detrimental. One approach to guiding migration of cells to their appropriate positions involves the manipulation of the expression patterns of guidance cues to patterns more like those observed during CNS development. These experiments show that post-mitotic cortical neuron motility is effected by each of the guidance cues tested in a manner similar to their previously determined roles in axon guidance (for example, Bixby et al., 1989; Doherty et al., 1995; Polleux et al., 1998) and neuron migration (for example, Persohn and Schachner, 1987; Kearns et al., 2003; Tamamaki et al., 2003), which indicates that the same developmental cues may be useful for guiding neurons at varying stages of maturity.

Therefore, these analyses suggest that the motile neurons were committed to a neuronal fate, but had not matured at the same rate as other neurons typically observed in cortical neuron cultures. There may be as many as ten thousand different types of neuron (Muotri and Gage, 2006), and the neurochemical profile described here may be a reflection of the great diversity of neuronal phenotypes, rather than a product of culture conditions on developing neurons. Indeed, it is entirely possible that this population may never mature, or perhaps the onset of changes toward maturity was initiated by the injury itself. An interesting recent study has demonstrated that a population of
hippocampal precursor cells, under similar culture conditions, can be induced to
differentiate into neuron-like cells by injury (Tseng et al., 2006). In a similar way, the
motile neuron population in these cultures may be stimulated to mature by factors
related to the injury.

Therefore, these experiments identified a neuron population in long-term cortical
cultures that does not fit the traditional view of mature neurons, with respect to their
expression of cytoskeletal proteins, such as the ERM proteins, morphology and their
motile ability. There are likely to be many yet undescribed neuronal phenotypes that
may be of potential importance in the mature brain, perhaps after traumatic injury. We
may be able to further manipulate their individual characteristics in therapeutic strategies
to assist in functional regeneration in the adult brain.
Chapter 6 - Results

6 RHO KINASE ACTIVATES ERM PROTEINS AND MEDIATES THEIR FUNCTION IN CORTICAL NEURON GROWTH, MORPHOLOGY AND MOTILITY

6.1 INTRODUCTION

ERM proteins exist in the cytosol in an inactive state that, upon activation, undergo a conformational change which allows binding to both plasma membrane proteins and actin filaments of the cytoskeleton (see review by Ramesh, 2004), resulting in a physical, and signal transduction link between the plasma membrane and cytoskeleton. The mechanisms underlying ERM activation and function are so far unresolved, and their stimulation and resulting cellular actions may be cell type specific as well as dependent on biological context.

Like many cytoskeletal proteins, ERM protein activity is regulated by conformation. When inactive, the N- and C-terminal domains of the protein self-associate, masking the actin binding site (Gary and Bretscher, 1995). PIP$_2$ binding to an un-identified site in the N-terminal domain brings about activation (Hirao et al., 1996), while sequential phosphorylation (Fievet et al., 2004) of a specific C-terminal threonine (ezrin Thr 567, radixin Thr 564 and moesin Thr 558) stabilises ERM proteins in their active conformation, probably by preventing interaction of the N- and C-terminal domains (Matsui et al., 1998; Tsukita et al., 1999). Several kinases capable of phosphorylating the ERM C-terminal threonine have been identified, including PIP$_2$ (Matsui et al., 1999; Fievet et al., 2004), PKC (PKC$_\alpha$ and PKC$\theta$) (Ng et al., 2001), MRCK (Nakamura et al., 2000) and Rho kinase (Matsui et al., 1998; Yonemura et al., 1999). Matsui and colleagues (1998) first identified that Rho kinase is capable of phosphorylating the ERM C-terminal threonine, but later the same group demonstrated that Rho kinase inhibition using the inhibitor Y27632 did not suppress Rho-dependent ERM activation (Matsui et al., 1999). However, in the rat hippocampal progenitor cell line, H19-7/IGF-IR, glutamate stimulates RhoA and Rho kinase dependent phosphorylation of moesin (Jeon et al., 2002). These data are indicative of a complex relationship between Rho signal transduction and ERM activation.
In the present study, the specific outcomes of Rho kinase activation of ERM proteins in neurons was investigated, both in previously elucidated ERM functions such as developmental neuronal growth and morphology and post-injury regeneration, as well as examining the potential role in neuronal translocation.
6.2 MATERIALS AND METHODS

6.2.1 Substrate preparation and cell culture
Poly-l-lysine, laminin, L1 and Sema 3A substrates were prepared as described in section 2.1.1 (Materials and Methods). Poly-l-lysine was the standard culture substrate for these experiments. Primary cortical neuron cultures were established according to procedures described in sections 2.1.2 to 2.1.3 (Materials and Methods).

B35 neuroblastoma cells (ATCC®) were maintained in DMEM containing 10% FCS and 1% penicillin/streptomycin. Prior to experimental use, cells were differentiated using a combined serum deprivation and incubation with dibutyryl cyclic AMP (cAMP) approach (Schmid et al., 1999). Cells were grown in DMEM containing 1% penicillin/streptomycin and 1mM dibutyryl cAMP (Sigma), but not FCS, for approximately 16 hours. Following this, the medium was replaced with fresh DMEM / 1% penicillin/streptomycin. For experiments, Rho kinase inhibitor was applied to cells approximately 24 hours following differentiation.

6.2.2 Rho kinase inhibitor
In these experiments, a highly specific inhibitor of Rho kinase, Rho kinase inhibitor II (Calbiochem) was utilised, which is abbreviated RKI II. RKI II shows little activity towards Erk, PKA or PKC, PDGFR or c-Kit/SCFR (IC\textsubscript{50} > 10\mu M to > 30\mu M) (Takami et al., 2004). It was initially reconstituted to 5mg/mL with sterile DMSO and stored at -20°C.

6.2.3 Rho kinase inhibition of phosphorylated ERM in primary cortical neurons
To determine whether the Rho kinase inhibitor would effect the phosphorylation of ERM proteins in primary cortical neurons early in culture (0-2 DIV), preliminary experiments tested a range of concentrations (5 - 250\mu M), time-points post-plating for addition (0 hours to 1 DIV) and time-points for fixation (15 minutes - 24 hours) and immunolabelling, to observe the effects of inhibition (data not shown). Based on this preliminary analysis, the effect of treatment with 'low' (50\mu M) and 'high' (250\mu M)
concentration RKI II was compared in detail, with their respective DMSO vehicle controls. The 'low' concentration lies within the expected specificity range, and is comparable to the concentrations used for other inhibitors of Rho kinase, such as Y27632 (for example, Borisoff et al., 2003; Monnier et al., 2003). Therefore, the 'high' concentration represents the upper limit of this range, and the possibility of reduced specificity at the highest concentrations must be considered. RKI II was diluted 1:10 in Neurobasal™ medium and added to 1mL culture medium at 24 hours post-plating (1 DIV) and then 24 hours after addition (2 DIV) cultures were fixed with 10% TCA, as described in section 2.3.1 (Materials and Methods). Parameters of phosphorylated ERM expression and neuronal development were analysed, including axon length, axon phosphorylated ERM positive filopodia, growth cone phosphorylated ERM positive filopodia, dendrite number, dendrite length and dendrite phosphorylated ERM positive filopodia.

6.2.4 Immunoblot analysis of RKI II activity
To determine the efficacy of RKI II and confirm its effect on the levels of phosphorylated ERM in developing primary cortical neurons in the presence or absence of inhibitor, quantitative immunoblotting was utilised. Differentiated B35 neuroblastoma cells were treated with 250μM RKI II or DMSO vehicle control for 24 hours. For immunocytochemistry, B35 cells were fixed with 10% TCA. For immunoblotting, cells were washed once with ice cold 0.01M PBS and harvested using a lysis buffer containing 1% Triton, 500mM Sodium Fluoride (NaF, Sigma), 1mM Orthovanadate (Na₃VO₄, Sigma), 1mM Phenylmethanesulfonyl fluoride (PMSF, Sigma), 1μM Calyculin A (a serine/threonine phosphatase inhibitor, Sigma) and one complete protease inhibitor tablet per 10mL buffer (Roche). Equal amounts of total protein, determined by BCA assay, were separated by SDS-PAGE, and then transferred to membranes, according to standard procedures detailed in section 2.5 (Materials and Methods). Membranes were probed with a primary antibody specific for phosphorylated ERM (anti-rabbit; 1:1000; Chemicon), and a moesin antibody that recognises total ERM, independent of phosphorylation state (anti-mouse; 1:1000; BD Transduction Laboratories). A GAPDH antibody acted as a loading control (anti-mouse; 1:5000;
Chemicon). HRP conjugated secondary antibody (1:1000-1:2000) incubation was followed by detection using an ECL™ chemiluminescence system.

### 6.2.5 Transection injury of relatively mature neurons for regenerative neurite growth and neuron motility assays

For post-injury regenerative neurite growth and motility assays primary cortical neurons were cultured until relative maturity (21 DIV), when developmental markers are no longer expressed, but those related to maturity, including MAP2 and synaptic markers are (de Lima et al., 1997; Chuckowree and Vickers, 2003; King et al., 2006). Areas of high neuronal density were subjected to transection injury using methods described in section 2.2 (Materials and Methods). To assess regenerative neurite sprouting, cultures were immediately treated with either 50µM or 250µM RKI II or their DMSO vehicle controls, then fixed at 6 hours post-injury, the optimum time-point for quantitation of the regenerative neurite sprouting response as previously determined (Chapter 4). For neuron movement assays, cultures were immediately treated with 250µM RKI II or DMSO vehicle control, then fixed at 24 hours post-injury, when neuron translocation into the injury site was well established (Chapter 5). Cultures were fixed with 10% TCA.

### 6.2.6 Immunocytochemistry

Immunocytochemistry was performed according to standard procedures in section 2.3.2 (Materials and Methods). Cultures were immunolabelled with primary antibodies to β-tubulin and phosphorylated ERM (Table 2.1) and Alexa-fluor fluorescent secondary antibodies were used to visualise antibody localisation (Table 2.2).

### 6.2.7 Microscopy and analysis

All microscopy and analysis was performed as described in section 2.4 (Materials and Methods). For developmental studies, where parameters of phosphorylated ERM expression and neuronal development were analysed, 80 neurons from four neuronal cultures were included in the analysis. Regenerative neurite lengths were measured from the border of the injury site to the growth cone tip, and 100 neurites from four
neuronal cultures were included in the analysis. The number of post-injury regenerative neurites was expressed as neurites/mm of lesion, and twenty lesions from four neuronal cultures were included in the analysis. The net distance of neuron movement was measured as the minimum distance from the edge of the lesion to the centre of the cell body. The number of neurons that had undergone movement into the injury site was expressed as neurons/mm of lesion. For RKI II analysis on poly-l-lysine only, 15 injury sites from three neuronal cultures were included in the analysis. For RKI II inhibition on poly-l-lysine, laminin, L1 and Sema 3A substrates, twenty injury sites from three neuronal cultures were analysed. Quantity One software (Bio-Rad) was used to perform intensity analysis of bands on immunoblots. Individual lanes were selected for comparison. The X axis of the graph is the Rf value and the Y axis is the pixel intensity value at each point along the lane. Background subtraction was not required. The area under the intensity plot curve is a measure of the size of the protein band.
6.3 RESULTS

6.3.1 Rho kinase inhibition reduces the number of phosphorylated ERM positive filopodia and alters cortical neuron growth and morphology during early development, \textit{in vitro}

In developing CNS neurons \textit{in vitro}, phosphorylated ERM proteins are localised to areas of high motility and growth, including growth cones and filopodia emanating from neurite shafts (Chapter 3). Therefore, a number of aspects of phosphorylated ERM localisation and neuronal growth and morphology, in response to RKI II treatment during development, were examined (Figures 6.1 and 6.2). There was a tendency for axons to be slightly longer in neurons treated with the 'low' (50µM) concentration of RKI II compared with controls (vehicle control 117µm ± 4.29, 50µM RKI II 127µm ± 4.80, \(p = 0.17\)), while the 'high' (250µM) concentration of RKI II caused significantly reduced axon lengths (vehicle control 97µm ± 4.25, 250µM RKI II 66µm ± 3.65, \(p < 0.05\)) (Figure 6.1 A-B, Figure 6.2 A). Similarly, dendrite length was significantly increased by the 'low' (50µM) concentration of RKI II (vehicle control 19µm ± 0.63, 50µM RKI II 20µm ± 0.56, \(p < 0.05\)), but significantly decreased when treated with the 'high' (250µM) concentration (vehicle control 18µm ± 0.55, 250µM RKI II 16µm ± 0.48, \(p < 0.05\)) (Figure 6.1 A-B, Figure 6.2 B). Also, the number of dendrites was significantly decreased both at the 'low' (50µM) (vehicle control 2.96 ± 0.09, 50µM RKI II 2.71 ± 0.09, \(p < 0.05\)) and 'high' (250µM) RKI II concentrations (vehicle control 2.9 ± 0.09, 250µM RKI II 2.29 ± 0.08, \(p < 0.05\)) (Figure 6.1 A-B, Figure 6.2 C).

The specific effect of Rho kinase inhibition on cellular phosphorylated ERM was quantitated, by determining the number of phosphorylated ERM positive filopodia, compared with controls. Representative neurons immunolabelled with phosphorylated ERM and β-tubulin treated with the vehicle control or RKI II are shown in Figure 6.1. Observations showed that treatment with 'high' (250µM) RKI II decreased the amount of phosphorylated ERM filopodia, for example, at the distal tips of dendrites (Figure 6.1 A-B), as well as along the axon (Figure 6.1 C-D). Figure 6.1 E and 6.1 F demonstrate the decrease in phosphorylated ERM positive filopodia of the axonal growth cone as a
Figure 6.1 The effect of Rho kinase inhibition on neuronal morphology and phosphorylated ERM localisation. Primary cortical neurons were treated with 'high' (250µM) concentration of RKI II, or vehicle control, from 1 to 2 DIV, then fixed and immunolabelled with primary antibodies to β-tubulin (red) and phosphorylated ERM (green). Large images are composites of the β-tubulin and phosphorylated ERM insets. A. Vehicle control treated neurons exhibited normal developmental morphology; a long axon (large arrow) and multiple dendrites. Filopodia at the distal tips of dendrites were often immunolabelled with phosphorylated ERM (small arrows and pERM inset). B. RKI II treated neurons possessed axons of similar length to vehicle treated control (large arrow), but had significantly reduced phosphorylated ERM labelling, only at the distal tip of the axon in the example shown (large arrow and pERM insert). The number and length of dendrites were significantly decreased (small arrow). C. Vehicle control treated neurons characteristically displayed numerous phosphorylated ERM positive filopodia emanating from the axon and dendrite shafts (arrows and pERM inset). D. RKI II treated neurons showed significantly fewer neurite filopodia immunolabelled with phosphorylated ERM (arrows). E. Axonal growth cones treated with the vehicle control were large, displaying typical lamellipodial morphology (large arrow) and numerous phosphorylated ERM positive filopodia (small arrows and pERM inset). E. In contrast, the axonal growth cones of neurons treated with RKI II were smaller, less likely to have lamellipodial structures and significantly fewer filopodia immunolabelled with phosphorylated ERM (arrows and pERM inset). Scale bar: A-C = 20µm, insets 40µm; D = 13µm, inset 26µm; E = 8µm, inset 16µm; F = 5µm, inset 10µm.
Figure 6.2 Quantitative analysis of the effect of Rho kinase inhibition on neuronal morphology and phosphorylated ERM localisation. Primary cortical neurons were treated with ‘low’ (50μM) or ‘high’ (250μM) concentration RKI II, or their vehicle controls, from 1 to 2 DIV, fixed and immunolabelled with antibodies to β-tubulin and phosphorylated ERM prior to analysis. * represent p values less than 0.05. Error bars are ± SEM.
result of 'high' (250µM) RKI II treatment, compared with controls. Quantitatively, when treated with the 'low' (50µM) concentration of RKI II, the number of phosphorylated ERM positive filopodia of the axon (vehicle control 1.64 ± 0.18, 50µM RKI II 0.61 ± 0.09), dendrites (vehicle control 1.45 ± 0.08, 50µM RKI II 0.93 ± 0.06) and the axonal growth cone (vehicle control 1.19 ± 0.12, 50µM RKI II 0.56 ± 0.1) were all significantly reduced (p <0.05) when compared with vehicle treated controls (Figure 6.2 D-F). In addition, the number of phosphorylated ERM positive filopodia was significantly decreased (p <0.05) in neurons treated with 'high' (250µM) concentration of RKI II, along both the axon (vehicle control 1.08 ± 0.13, 250µM RKI II 0.23 ± 0.07) and dendrites (vehicle control 0.98 ± 0.06, 250µM RKI II 0.45 ± 0.05) and of the axonal growth cone (vehicle control 0.96 ± 0.13, 250µM RKI II 0.59 ± 0.1) (Figure 6.2 D-F). Concomitantly with the reduced number of phosphorylated ERM positive growth cone filopodia were notable alterations in growth cone morphology, often exhibiting a spikier, less lamellipodial morphology (Figure 6.1 E-F).

These data indicate that RKI II treatment has significant negative effects on the number of phosphorylated ERM positive filopodia, as well as aspects of neuronal growth and morphology, including axon and dendrite outgrowth and the number of dendrites formed.

6.3.2 Rho kinase inhibition reduces the amount of phosphorylated ERM protein

Immunoblotting was used to determine the amount of phosphorylated ERM in B35 neuroblastoma cells, in the presence and absence of RKI II, in order to quantitatively correlate protein levels with immunocytochemical observations. The B35 neuroblastoma cell line was used because primary neuron cultures are invariably a mixed population containing glial cells (<5% within the first 7 DIV), which also express phosphorylated ERM proteins more abundantly than neurons (Figure 6.3 A). Thus, using immunoblotting, it would not be possible to separate neuronal and glial phosphorylated ERM expression. Also, interestingly, glial cells in culture appear to be minimally affected by RKI II (Figure 6.3 B). Initially, immunocytochemistry was used
Figure 6.3 Phosphorylated ERM localisation in glial cells, primary cortical neurons and B35 neuroblastoma cells in the presence or absence of ‘high’ (250μM) concentration of RKI II. A. Cultures derived from the embryonic neocortex are a mixed population of cells that also contain glial cells. Vehicle control treated cultures were immunolabelled with β-tubulin (red) and phosphorylated ERM (green), which showed that glial cells widely express phosphorylated ERM (large arrow), compared with neurons (small arrow). B. This phosphorylated ERM (green) localisation was not affected in RKI II treated cultures (arrow), compared with control cultures shown in A. Therefore, rather than primary cortical neuron cultures, a pure population of B35 cells was utilised for immunoblotting analysis. Undifferentiated or cAMP differentiated B35 cells were treated with ‘high’ (250μM) concentration RKI II or vehicle control for 24 hours, comparable to primary cortical neuron treatment regimes. Cells were immunolabelled with primary antibodies to β-tubulin (red) and phosphorylated ERM (green). C. In the absence of RKI II, vehicle treated control cortical neurons showed phosphorylated ERM immunolabelling in numerous filopodia, particularly at the distal tips of neurites (arrows). D. The number of phosphorylated ERM positive filopodia was significantly reduced in ‘high’ (250μM) concentration RKI II treated neurons, as previously discussed. E. In vehicle control treated undifferentiated B35 cells phosphorylated ERM labelling was abundant and localised around the entire cell in spiky filopodial protrusions (arrows). F. Phosphorylated ERM localisation did not appear markedly affected by RKI II treatment in undifferentiated B35 cells (arrows). G. In vehicle treated controls, the morphology and phosphorylated ERM labelling in cAMP differentiated B35 cells was comparable to neuronal localisation patterns, thus more restricted to peripheral filopodial structures and concentrated at growing tips (arrows). H. Phosphorylated ERM localisation was reduced in differentiated B35 cells after RKI II treatment (arrow). Scale bar = 20μm.
to observe the localisation of phosphorylated ERM proteins in undifferentiated and differentiated B35 cells, compared with cortical neurons, in the absence or presence of 'high' (250μM) concentration RKI II (Figure 6.3 C-H). As previously described, application of 'high' (250μM) concentration RKI II markedly reduced phosphorylated ERM labelling in primary cortical neurons (Figure 6.3 C-D). Phosphorylated ERM labelling in undifferentiated B35 cells was very abundant and localised around the entire cell in spiky filopodial protrusions (Figure 6.3 E), and was not markedly affected by 'high' (250μM) concentration RKI II (Figure 6.3 F). In contrast, phosphorylated ERM labelling in cAMP differentiated B35 cells was more comparable to patterns of localisation observed in neurons and was more restricted to peripheral filopodial structures and concentrated at growing tips (Figure 6.3 G), and phosphorylated ERM labelling appeared to be diminished after 'high' (250μM) concentration RKI II treatment (Figure 6.3 H).

Immunolabelling of the proteins harvested from vehicle control treated B35 cells with the phosphorylated ERM antibody resulted in two distinct bands (Figure 6.4 A), which are likely to correspond to ezrin/radixin (approximately 80kDa) and moesin (approximately 75kDa). Treatment with the 'high' 250μM RKI II concentration resulted in a decrease in phosphorylated ERM immunolabelling, particularly diminished immunolabelling was notable in the ezrin/radixin band. This is further demonstrated by the intensity plot shown in Figure 6.4 B. The vehicle control treated intensity plot (red) shows two peaks, representing the ezrin/radixin and moesin bands. The RKI II treated intensity plot (blue) shows one peak of reduced intensity. The single peak in the RKI II treated sample corresponds to the lower molecular weight band (moesin); therefore the higher molecular weight band (ezrin/radixin) was more negatively affected by RKI II treatment. An antibody that detects all three ERM proteins, independent of phosphorylation state, was used to immunolabel the total ERM protein pool, and also resulted in two distinct bands (Figure 6.4 A). However, there was no marked difference between the vehicle control and RKI II treated samples, either in the bands (Figure 6.4 A) or the intensity plot (Figure 6.4 C), indicating that RKI II affected only the amount of phosphorylated ERM and not total ERM. These results were observed in two separate
Figure 6.4 Immunoblotting analysis demonstrating the effect of Rho kinase inhibition on the amount of phosphorylated ERM in B35 neuroblastoma cells.

A. Protein harvested from differentiated B35 cells treated for 24 hours with either vehicle control or 'high' (250μM) concentration RKI II, were separated by SDS-PAGE and probed with a phosphorylation specific ERM antibody, an antibody that recognises the total ERM protein pool, and an antibody to GAPDH. Using the phosphorylated ERM specific antibody, in vehicle control samples two distinct bands were detected, a weaker band of higher molecular weight, and a more strongly labelled one of lower molecular weight, which are likely to correspond to ezrin/radixin and moesin, respectively (red arrows). Blots of the RKI II treated B35 cells probed with the phosphorylated ERM antibody showed a notable decrease in intensity of both bands (blue arrow) compared with the vehicle control. In contrast, labelling with the antibody directed to ERM proteins, irrespective of phosphorylation state, did not show any marked changes in intensity.

B. Intensity plots of phosphorylated ERM immunoblots of vehicle control treated cultures show two peaks of intensity (red arrows), which correspond to the two bands identified in A. In contrast, protein harvested from cultures treated with RKI II show a single peak of immunolabelling with decreased intensity (blue arrow). The higher molecular weight band (ezrin/radixin) was more notably affected by RKI II inhibition, with a single intensity peak remaining after treatment, corresponding to the moesin band (blue arrow).

C. Intensity plots of total ERM immunoblots show that vehicle control and RKI II treated cultures exhibit bands of comparable intensity, demonstrating that Rho kinase inhibition does not affect the amount of total ERM protein, but does decrease the amount of phosphorylated ERM.
A

- RKI II  + RKI II

pERM

ERM

GAPDH

B

C

Intensity (arbitrary units)

0.00  0.25  0.50  0.75  1.00

- RKI II  + RKI II

pERM  ERM  GAPDH
cell harvests, and confirm that Rho kinase is involved in the phosphorylation of ERM proteins in B35 neuroblastoma cells, as well as neurons.

6.3.3 Rho kinase inhibition significantly inhibits the number of regenerative neurites following transection injury

ERM proteins are re-expressed by more mature neurons exhibiting regenerative neurite sprouting after injury, in particular localised to regenerative neurite growth cones (Chapter 4). In addition, there is increasing evidence that inhibition of Rho kinase is beneficial for neurite outgrowth during attempted regeneration (recent examples include Dergham et al., 2002; Fournier et al., 2003; Monnier et al., 2003; Bertrand et al., 2005; Chan et al., 2005). Therefore, the effect of RKI II on the expression of phosphorylated ERM in regenerative neurites and on the amount of regenerative regrowth of relatively mature cortical neurons was investigated.

Interestingly, phosphorylated ERM expression in regenerative neurites was variable between all experimental conditions, including the ‘low’ (50μM), ‘high’ (250μM) RKI II concentrations and vehicle control preparations. Figure 6.5 A and 6.5 B demonstrate examples of regenerative neurites with variable phosphorylated ERM expression after treatment with the ‘high’ (250μM) concentration of RKI II. Application of RKI II after injury at the ‘low’ (50μM) concentration resulted in no significant difference in the length of regenerative neurite sprouts at 6 hours post-injury (vehicle control 37μm ± 2.7, 50μM RKI II 36μm ± 1.9, p = 0.77) (Figure 6.5 C). The ‘high’ (250μM) concentration also did not significantly influence regenerative neurite length (vehicle control 33μm ± 2.18, 250μM RKI II 29μm ± 1.53, p = 0.13) (Figure 6.5 C). Rho kinase inhibition slightly, but not significantly, increased the number of regenerative neurite sprouts that formed at the ‘low’ 50μM RKI II (vehicle control 13 ± 3.84, 50μM RKI II 16 ± 4.91, p = 0.45), but significantly decreased the number of regenerative neurites at the ‘high’ 250μM concentration of RKI II (vehicle control 14 ± 3.77, 250μM RKI II 5 ± 1.38, p = 0.01) (Figure 6.5 D). Thus, RKI II did not significantly affect the expression of phosphorylated ERM during regenerative responses in these experiments, but the
Figure 6.5 The role of Rho kinase and phosphorylated ERM in post-injury regenerative neurite sprouting. Primary cortical neurons were maintained in culture until 21 DIV, when they were subjected to transection injury. Six hours post-injury, neuron cultures were fixed and immunolabelled with primary antibodies to β-tubulin (red) and phosphorylated ERM (green). A. Following treatment with 'high' (250μM) concentration RKI II, many regenerative neurite sprout growth cones did not express phosphorylated ERM (arrows). B. However, in neuronal cultures subjected to the same treatment ('high' (250μM) concentration RKI II) there were regenerative neurite sprout growth cones that expressed phosphorylated ERM (arrows). Thus, A and B demonstrate the variable expression of phosphorylated ERM in regenerative neurite growth cones after RKI II treatment. C. The average length of regenerative neurites was not significantly affected when treated with 'low' (50μM) or 'high' (250μM) concentration RKI II, compared with their vehicle controls. D. The number of regenerative neurites was significantly affected by treatment with 'high' (250μM) concentration RKI II. Scale bar: A-B = 20μm. * represent p values less than 0.05. Error bars are ±SEM.
Regenerative neurite length

Number of regenerative neurites

A

B

C

D

13-tubulin

pERM

Number of regenerative neurites

Regenerative neurite length

Kill KI II

50, M control

250, M control

50, M control

250, M KI II

Number of neurites/mm

0 5 10 15 20 25

C7)

RC

pERM

0 5 10 15 20 25
number of regenerative neurite sprouts was significantly decreased following ‘high’ 250µM RKI II treatment.

6.3.4 Rho kinase inhibition reduces phosphorylated ERM and neuronal motility in an in vitro assay

Neurons in culture at 21 DIV are capable of undergoing movement across the substratum (Chapter 5). More specifically, in the hours after injury, neurons identified by β-III-tubulin immunolabelling, move into the injury site (Figure 6.6 A). Whether the neurons specifically move into the cell-free injury site in an attempt to heal the wound is thus far unknown. Irrespective of this, the motility assay provided an ideal opportunity to investigate the potential role of ERM proteins in neuron movement and thus potentially migration. Utilising the assay, immunolabelling demonstrated that cells that underwent movement into the injury site express phosphorylated ERM in peripheral growth cone-like structures and filopodia (Chapter 5; Figure 6.6 B), whereas cortical neurons in culture at 21 DIV do not normally express phosphorylated ERM (Chapter 3). This suggests that ERM proteins were re-expressed specifically by motile neurons, and therefore may have a role in the cytoskeletal processes involved in mediating this process.

Next, it was hypothesised that if phosphorylated ERM proteins are re-expressed in 21 DIV neurons undergoing movement, and Rho kinase phosphorylates ERM proteins in neurons, inhibition of Rho kinase may inhibit neuronal movement. Thus, RKI II was applied at 250µM to cortical neurons subjected to transection injury at 21 DIV. The Rho kinase inhibitor caused a marked reduction in neuronal phosphorylated ERM, identified by immunocytochemistry (Figure 6.6 B). Addition of the inhibitor significantly decreased the number of neurons that underwent movement into the injury site (vehicle control 7 neurons/mm ± 1.28, 250µM RKI II 1 neuron/mm ± 0.15; p < 0.05) (Figure 6.6 C), but did not significantly affect the distance moved by individual neurons (vehicle control 25µm ± 3.03, 250µM RKI II 21µm ± 4.32; p = 0.21) (Figure 6.6 C). Combined, these data suggest that Rho kinase and ERM function are important for neuron motility.
Figure 6.6 The effect of Rho kinase inhibition on phosphorylated ERM and post-injury neuron motility. Primary cortical neuron cultures were grown until 21 DIV, when they were subjected to transection injury. A. A post-injury timecourse showed that in the 24 hours after injury, neurons immunolabelled with β-III-tubulin had moved into the injury site (arrows). B. In vehicle treated controls, motile neurons immunolabelled with β-tubulin (red) and phosphorylated ERM (green) showed abundant phosphorylated ERM along neurites and in growth cone-like structures (arrows). After ‘high’ (250μM) concentration RIK II treatment, neurons showed more restricted expression of phosphorylated ERM (arrow). C. After treatment with ‘high’ (250μM) concentration RIK II, significantly fewer neurons had moved into the lesion, 24 hours post-injury. In contrast, the distance moved into the lesion was not significantly affected. Scale bar: A 0h = 100μm; 6h = 90μm; 16h = 65μm; 24h = 80μm. B = 20μm. * represent p values less than 0.05. Error bars are ± SEM.
6.3.5 Rho kinase inhibition differentially effects motility on substrates containing molecular guidance cues

RKI II was applied at the 'high' 250μM concentration to cortical neurons injured at 21 DIV grown on poly-l-lysine, laminin, L1 and Sema 3A substrates to investigate whether Rho kinase is differentially involved in mediating translocation in response to various guidance cues. Addition of the inhibitor significantly decreased the number of neurons that underwent translocation on substrates of poly-l-lysine (vehicle control 7 neurons/mm ± 1.28, 250μM RKI II 1 neuron/mm ± 0.15; p < 0.05, an 86% reduction), laminin (vehicle control 9 neurons/mm ± 0.43, 250μM RKI II 1 neuron/mm ± 0.35; p < 0.05, an 89% reduction) and L1 (vehicle control 4 neurons/mm ± 0.44, 250μM RKI II 2 neurons/mm ± 0.55; p < 0.05; a 50% reduction). The reduction was not significant for the Sema 3A substrate (vehicle control 4 neurons/mm ± 1.10, 250μM RKI II 1 neuron/mm ± 0.36; p = 0.10; a 75% reduction) (Figure 6.7 A). In contrast, RKI II did not significantly decrease the mean distance translocated for neurons cultured on any of the substrates, including poly-l-lysine (vehicle control 25μm ± 3.03, 250μM RKI II 21μm ± 4.32; p = 0.21), laminin (vehicle control 20μm ± 3.69, 250μM RKI II 18μm ± 2.93; p = 0.22), L1 (vehicle control 24μm ± 3.08, 250μM RKI II 27μm ± 4.08; p = 0.19) and Sema 3A (vehicle control 23μm ± 4.65, 250μM RKI II 18μm ± 10.93; p = 0.50) (Figure 6.7 B). Interestingly, RKI II treatment slightly increased the mean distance of translocation on the L1 substrate.
Figure 6.7 The effect of RKI II on neuron motility in response to molecular guidance cues. Cortical neurons grown on substrates of poly-l-lysine, laminin, L1 or Sema 3A were injured at 21 DIV, then treated with 250μM RKI II. 24 hours post-injury they were fixed and immunolabelled for quantitation of the effect of RKI II on post-injury motility on each substrate. A. The number of neurons per mm of lesion when grown on the four different substrates, in the presence of RKI II (black), or DMSO control (white). RKI II treatment resulted in significantly less neurons per mm of lesion on poly-l-lysine, laminin and L1. B. The average distance of neuron translocation in response to substrate bound guidance cues, in the presence of RKI II (black), or DMSO control (white). * represent p values less than 0.05. Error bars are ± SEM.
A

Number of motile neurons

- **ODMSO control**
- **RKI II**

Distance of neuron translocation

- **DMSO control**
- **RKI II**
6.4 DISCUSSION

Several kinases have been implicated in the activation of ERM proteins by phosphorylation in various cell types, and these experiments have examined the specific role of Rho kinase in ERM activation in CNS neurons. The relationship between Rho pathway members and ERM activation was identified when stimulation of RhoA activity in NIH 3T3 cells resulted in the phosphorylation of ERM proteins and the formation of cell surface structures (Shaw et al., 1998), and transfection of NIH 3T3 cells with constitutively active RhoA, but not Rac1 or Cdc42, increased the level of phosphorylated ERM proteins (Matsui et al., 1998). However, in subsequent investigations a constitutively active form of Rho kinase did not increase ERM phosphorylation, and the Rho kinase inhibitor Y27632 did not suppress ERM activation (Matsui et al., 1999). This suggested Rho kinase was not directly involved in ERM phosphorylation, which contrasted with a report indicating Rho kinase phosphorylation of moesin was essential for microvilli formation in COS7 cells (Oshiro et al., 1998). More recently, it has been demonstrated that in some types of kidney-derived cells, ERM proteins can be activated in the absence of Rho, suggesting that a Rho-independent activation mechanism also exists (Yonemura et al., 2002).

These experiments have shown that Rho kinase is involved in the activation of ERM proteins in primary cortical neurons. Application of RKI II caused a significant decrease in phosphorylated ERM positive filopodia of the axonal growth cone and along the axon and dendrites in developing primary cortical neurons, a result that was confirmed by a reduction in the amount of phosphorylated ERM protein in response to RKI II by quantitative immunoblotting, in B35 neuroblastoma cells. However, these data also support the proposal that there are Rho-independent mechanisms of ERM activation (Yonemura et al., 2002), due to the inability of RKI II to abolish phosphorylated ERM immunolabelling in each of these experiments. Furthermore, the inability of RKI II to completely inhibit ERM phosphorylation may be related to the suggestion that moesin is phosphorylated by PKC, while ezrin and radixin are phosphorylated by Rho kinase (reviewed by Louvet-Vallée, 2000). This observation was supported by the current immunoblotting investigations, where bands corresponding to moesin appeared to be
less affected by RKI II. This suggests that the individual ERM proteins are controlled via different activation pathways and may be responsible for specific functions in neurons, rather than acting as a family with completely overlapping functions (for example, Paglini et al., 1998).

These investigations have identified ERM proteins as targets of Rho kinase in primary cortical neurons. However, other targets of Rho kinase include adducin, myosin light chain, myosin light chain phosphatase, collapsin response mediator protein-2, LIM kinase (which in turn phosphorylates cofilin), intermediate filament and the Na+\textsuperscript{+}-H+\textsuperscript{-}-exchanger (reviewed by Bishop and Hall, 2000; Fukata et al., 2001; Giniger, 2002). While RKI II therefore does not exclusively act on ERMs, there are some striking similarities between many of the proposed roles of Rho kinase and ERM proteins in neurons, including neurite initiation, neurite outgrowth and branching, axon guidance, neuronal polarisation and growth cone morphology (Paglini et al., 1998, Dickson et al., 2002; reviewed by Luo, 2000; Govek et al., 2005), which this data supports.

This data is particularly indicative of a link between Rho kinase, ERM activity and neurite outgrowth because RKI II decreased neurite outgrowth, and was concomitant with decreased phosphorylated ERM filopodia of the axonal growth cone, and along the axon and dendrites. By inhibiting Rho kinase and phosphorylated ERM in developing neurons, in vitro, ‘low’ RKI II treatment resulted in increased axon and dendrite length, whereas ‘high’ RKI II decreased axon and dendrite length. It has previously been shown that application of a Rho kinase inhibitor increases neurite outgrowth on inhibitory substrates (Dergham et al., 2002; Borisoff et al., 2003; Fournier et al, 2003), while the effect on more permissive substrates was examined less often and has appeared to be minimal (Borisoff et al., 2003). Data also shows that ERM proteins are involved in neurite outgrowth, although the specific mechanisms are yet to be identified. For example, when radixin and moesin were suppressed in hippocampal neurons using antisense oligonucleotides, the rate of growth cone advance was significantly decreased (Paglini et al., 1998). Furthermore, interestingly these experiments showed opposing effects of ‘low’ and ‘high’ concentrations of RKI II on neurite outgrowth, which may be
a reflection of the importance of growth-associated cellular molecules like the ERMs existing in a tightly controlled balance of activated and inactivated forms in order to exert their appropriate effects on the cell.

The number of primary cortical neuron dendrites was significantly decreased at both 'low' and 'high' concentrations of RKI II. In contrast with our results, inhibition of Rho kinase either with a dominant negative or the inhibitor Y27632 was found to promote neurites in neuroblastoma cell lines (Hirose et al., 1998). ERMs are thought to be involved in neurite initiation, as suppression of radixin/moesin slows hippocampal neurite formation and establishment of polarity (Paglini et al., 1998). In numerous non-neuronal cells ERMs have been implicated in the formation of cell surface structures, such as microvilli and filopodia (Bretscher, 1989; Takeuchi et al., 1994; Amieva and Furthmayr, 1995; Yonemura et al., 1999; Nakamura et al., 2000; Scherer et al., 2001), some of which also showed that ERM activation in this function was under control of Rho signalling (Shaw et al., 1998; Nakamura et al., 2000; Yonemura et al., 2002; Nijhara et al., 2004), evidence which may support a role for Rho kinase activation of ERMs in neurite formation in primary neurons.

Rho kinase inhibition resulted in a significant decrease in phosphorylated ERM positive growth cone filopodia, which was accompanied by a smaller, spiky, less lamellipodial morphology. Others have also reported that Rho kinase inhibition results in growth cones lacking lamellipodia (Borisoff et al., 2003). Loss of ERM function has also been correlated with reduced lamellipodia (Paglini et al., 1998; Castelo and Jay, 1999), as well as decreased growth cone size (Paglini et al., 1998) and growth cone collapse (Gonzalez-Agosti and Solomon, 1996). Identification of an interaction between Rho kinase and ERM proteins in lamellipodial formation would be complementary to the finding that constitutively active Cdc42 expression in NIH 3T3 cells leads to the formation of filopodia and localisation of phosphorylated ERM at the filopodial tips (Nakamura et al., 2000).
Interestingly, in these studies Rho kinase inhibition at ‘low’ and ‘high’ concentrations resulted in similar effects on neurite outgrowth in developing neurons and those undergoing a post-injury regenerative growth response. Rho kinase has been identified as a target for manipulation in the regenerative response to injury, with numerous reports linking Rho kinase inhibition with increased post-injury regrowth (Dergham et al., 2002; Fournier et al., 2003; Monnier et al., 2003; Bertrand et al., 2005; Chan et al., 2005). In using Rho kinase inhibitors to modulate regenerative neurite growth, dose selection appears to be of primary importance. In these experiments, ‘low’ RKI II concentration slightly increased the number of regenerative neurites, while ‘high’ RKI II concentrations resulted in a significant decrease in the number of regenerative neurites. In another recent study, it was shown that application of a low dose of the Rho kinase inhibitor Y27632 was detrimental to spinal cord regeneration, while a higher dose significantly increased the number of axonal sprouts (Chan et al., 2005). The current experimental results demonstrate that RKI II concentrations sufficient for interrupting ERM activation are also detrimental to regenerative neurite formation. ERM proteins are involved in regenerative attempts of cortical neurons after transection injury, as regenerative neurite growth cones expressed ERM proteins, which are not normally expressed in more mature neurons (Chapters 3 and 4). Application of RKI II at ‘low’ and ‘high’ concentrations resulted in variable expression of phosphorylated ERM in regenerative neurite growth cones, which might indicate that some neurites and their growth cones were affected by the inhibitor but not others, which raises the possibility that the different neuronal types in cortical cultures may be differentially dependent on ERM protein function, and therefore be differentially sensitive to the effects of Rho kinase inhibition. Or, it may simply be a reflection of the rapid cyclic nature of ERM phosphorylation and de-phosphorylation in the neuronal growth cone.

Using the model of transection injury, the function of ERMs in neuronal motility was examined. Transection injury resulted in a cell and debris free lesion, but within 24 hours a population of motile neurons had moved into the injury site. These neurons expressed phosphorylated ERM in their peripheral, growth-cone like structures, and RKI II inhibition significantly and dramatically decreased the number of neurons that
underwent movement into the lesion. This is indicative of a potential Rho dependent function in neuronal migration (Kholmanskikh et al., 2003; Yoshizawa et al., 2005). ERM proteins are also involved in migration, in endothelial cells (Simoncini et al., 2006), lymphocytes (Nijhara et al., 2004), neutrophils (Yoshinaga-Ohara et al., 2002) and tumour cells (Sahai and Marshall, 2003). For example, ERM proteins are responsible for re-localising surface cell adhesion molecules to the trailing edge of the cell to reduce its adhesiveness to the substrate (reviewed by Lee et al., 2004). Immunohistochemical analyses of the embryonic brain have indicated ERMs might also be involved in neuron migration during early development of the cerebral cortex, as ERMs are expressed by neurons in the intermediate zone during late embryonic development, a region highly concentrated with migrating neurons (Johnson et al., 2002; Mintz et al., 2003; Chapter 5). It has also been shown that ERM involvement in lymphocyte migration is under the control of Rho kinase (del Pozo et al., 1999), which supports evidence that Rho kinase activation of ERM proteins is required for neuron movement, in vitro.

In addition, the capacity for neuron motility after RKI II treatment was substrate dependent, with an 86% reduction in the number of motile neurons cultured on poly-l-lysine, 89% on laminin, 50% on L1 and 75% on Sema 3A. An interesting possibility is that neuronal translocation is mediated via different intracellular signal transduction pathways and cytoskeletal effectors, in a guidance cue dependent manner. For example, the decrease in translocation was least remarkable on an L1 substrate, and previous investigations into L1 signalling in neuron migration have implicated MAP kinases (Schmid et al., 2004; Thelen et al., 2002), rather than Rho GTPases. In contrast, Rho signalling appears to mediate Sema 3A effects (reviewed by Skaper et al., 2001; Tang, 2003). The current data suggests that intracellular signalling in laminin mediated neuron migration is likely to involve the Rho signalling pathway, due to the marked inhibition of translocation in response to inhibitor application. Previous studies have identified PKC (Bixby et al., 1989), PKA and myosin light-chain kinase (MLCK) (Freire et al., 2002) in laminin induced neuritogenesis, but there is little data relating to intracellular signalling associated with laminin induced neuron migration.
Therefore, these studies suggest that Rho kinase mediated ERM activation is important for specific aspects of neuronal development, nerve cell motility and regenerative growth. The current data also indicates that alternative kinases are involved in ERM activation. Further identifying these kinases, and the specific biological functions they mediate, will be important for our understanding of the complex functions coordinated by the ERM proteins and the cytoskeleton in general.
7 DISCUSSION

This thesis has utilised multiple experimental approaches to investigate the functional significance of the ERM proteins in CNS neurons. These proteins have previously been understudied in the nervous system, especially given that earlier investigations have indicated they are associated with crucial CNS developmental processes, such as neuronal morphogenesis (Gonzalez-Agosti and Solomon, 1996; Castelo and Jay, 1998; Paglini et al., 1998; Dickson et al., 2002). The most compelling evidence of their significance in CNS development was provided when ezrin was identified as an intracellular binding partner of the nervous system specific CAM L1 (Dickson et al., 2002). L1 is vital for normal development of the nervous system (Wong et al., 1995; Fransen et al., 1995), and it is likely that many L1 functions are mediated by its relationship with the ERM proteins. Therefore, this thesis provides experimental data to clarify some previously proposed roles for the ERM proteins, and in addition, has elucidated novel functions for this family of cytoskeletal linker proteins in neurons.

7.1 ROLES OF THE ERM PROTEINS IDENTIFIED IN CNS NEURONS

Initially, the expression of ERM proteins throughout development and maturation of individual CNS neurons was examined. Previous studies have demonstrated that ERMs are specifically localised to axonal and dendritic growth cones in developing CNS neurons (Goslin et al., 1989). The current studies extended on this, by comparing the localisation of the total ERM protein pool, with that of the phosphorylated, or active, ERM protein pool. These aspects were also examined specifically to correlate ERM and L1 expression during development, beyond the early developmental timepoints examined in earlier investigations. Studies confirmed the growth cone localisation of total ERM in developing hippocampal neurons, but also showed they are also localised to fine filopodia emanating from neurite shafts. Phosphorylated ERM showed a similar, but more restricted localisation pattern compared with total ERM protein, demonstrating that at any given developmental timepoint not all cellular ERMs exist in their active state. Perhaps the most notable finding was that, at 21 DIV, when prior investigations have identified that neurons display characteristics of maturity (de Lima et al., 1997; Chuckowree and Vickers, 2003; King et al., 2006), phosphorylated ERM was no longer...
expressed. Combined, this temporal and spatial localisation data strongly indicated roles in developmental neurite outgrowth, as well as neurite outgrowth related functions, such as modulating growth cone morphology and motility, axon pathfinding, as well as a potential role in the intracellular response to guidance cues. Finally, L1 and ERM proteins were co-localised specifically in axonal structures, most notably the growth cone, which is indicative of function in the aforementioned developmental growth processes. Thus, the specific ERM localisation in cultured hippocampal neurons was highly suggestive of their contribution to mechanisms of neuronal morphogenesis.

ERM protein function in developing cortical neurons was assessed by transfection techniques, utilising a dominant negative construct of ezrin. The dominant negative form of the protein is capable of N-terminal binding, but as it is truncated, C-terminal binding to the cytoskeleton is not possible (Crepaldi et al., 1997; Dickson et al., 2002). Compared with GFP transfected controls, perturbation of ERM function resulted in a number of characteristics divergent from normally developing neurons, including slightly increased axon and dendrite outgrowth, increased axon and dendrite branching and decreased axonal growth cone size, particularly due to the loss of lamellipodia.

Further examination of the role of ERM proteins in neurite outgrowth, in particular regenerative neurite outgrowth in relation to the binding partner L1, showed that both proteins are involved in regenerative neurite sprouting in response to trauma. ERM proteins were specifically re-expressed in regenerative neurite sprout growth cones, and supplying regenerating neurons with a substrate containing L1 resulted in significantly longer regenerative neurites. These studies also demonstrated the roles of L1 and neuronal phenotype in growth cone morphology, as growth cones on the L1 substrate were significantly larger and more elaborate than on poly-l-lysine substrates, and hippocampal neurons had significantly larger growth cones than cortical neurons on L1. Furthermore, results demonstrated that hippocampal and cortical neurons differed in their abilities to mount a regenerative response, an important finding since comparisons in regenerative ability between neurons originating in varied cortical areas have rarely been investigated (for example, Becker et al., 2005; Verma et al., 2005).
In an intriguing aspect of this study, a population of highly motile neurons in long-term (21 DIV) cortical neuron cultures was identified. The neurons translocated into the injury site, within 24 hours after transection injury was performed. They were small, multipolar neurons and their morphology, motile ability and cytoskeletal profile did not appear to fit that of a previously described neuronal phenotype. BrdU incorporation and immunocytochemical investigations confirmed that they were differentiated neurons, some of which were polarised. However, they did not express NF-H or MAP2, which might suggest they had not reached the maturity of some other neurons in culture. Interestingly, these post-mitotic neurons' motility was modulated by extracellular guidance cues, in a similar manner to that expected in axon guidance and migration of immature neurons. Perhaps most notable though, was that the motile neurons strongly expressed phosphorylated ERM in their peripheral neurites and growth cone-like structures, while no neurons in other areas of the cultures at that age expressed phosphorylated ERM. This strongly suggested that the neurons specifically expressed phosphorylated ERM as a part of the cytoskeletal mechanisms required for the neurons to undergo translocation.

Due to this novel finding, the expression of phosphorylated ERM, in relation to the migration marker DCX, was examined in the embryonic brain, which revealed co-localisation between ERMs and DCX in neurons of the developing neocortex, in vivo. This further implicated ERM proteins in translocation, and more specifically migration in in vivo neocortical development. This finding is a valuable addition to the limited previous data implicating ERMs in neuron migration (Johnson et al., 2002; Mintz et al., 2003).

Finally, ERM activation has not previously been examined in primary neurons, and confusing data exists on the mechanisms of activation of ERM proteins in non-neuronal cell types. This may be because the mechanisms vary depending on the cell type and the biological outcomes of activation. Specifically, the role of Rho kinase in ERM activation was examined, by application of an inhibitor of Rho kinase. Data showed that
indeed, Rho kinase is involved in the activation of ERM proteins in neurons. Inhibition of this mechanism significantly perturbed a number of ERM related functions, including aspects of development such as axon and dendrite outgrowth, as well as the number of dendrites formed, which was concomitant with a significant decrease in phosphorylated ERM immunolabelling in growth cones and in filopodia along axons and dendrites. Rho kinase inhibition also perturbed aspects of regenerative neurite outgrowth, and significantly disrupted post-injury neuron motility, including the motility observed on different molecular guidance cue containing substrates. However, this data also supports the hypothesis that other kinases are involved in the activation of ERM proteins, because inhibition at high concentrations did not completely abolish phosphorylated ERM immunolabelling, nor were all ERM functions investigated significantly perturbed. Furthermore, glial cell phosphorylated ERM labelling did not appear to be affected by inhibition of Rho kinase.

These findings will significantly contribute to understanding the mechanisms of both nervous system development and the regenerative response to injury.

7.2 IMPLICATIONS OF ERM PROTEIN FUNCTION IN CNS DEVELOPMENT

Many molecules are involved in orchestrating nervous system development, and the data presented in this thesis suggests that the ERM proteins are involved in important and specific developmental CNS functions. Two different methods of ERM perturbation resulted in abnormal neuron morphogenesis. Neuron transfection studies using a dominant negative ezrin construct resulted in morphological changes, particularly by decreasing the number of neurites, increasing neurite outgrowth and branching, and causing reduction in the growth cone size. Interestingly, utilising an inhibitor of Rho kinase to perturb ERM function resulted in similar outcomes, in particular resulting in a significant decrease in the number of dendrites formed and significantly altering neurite outgrowth.
Both experimental approaches to perturbation of ERM proteins caused changes in neurite outgrowth, in that increased axon and dendrite outgrowth was observed in transfection studies, as well as following ‘low’ dose application of RKI II. This suggests that the ERM proteins are involved in neurite outgrowth, but potentially more specifically in mediating retraction. A growing neurite utilises its growth cone structures, the filopodia and lamellipodia, to seek out and then consolidate growth in the appropriate direction. Thus, interrupting normal processes of growth cone or neurite retraction would result in longer neurites. This was also investigated in a study by Paglini and colleagues (1998), where individual filopodia in control neuronal growth cones persisted for no more than ten minutes. In contrast, double suppression of radixin and moesin caused most filopodia to persist longer than thirty minutes. These neurons also showed a significantly increased rate of elongation, and a more than threefold slower rate of retraction (Paglini et al., 1998).

Both perturbation approaches resulted in a decrease in the number of dendrites formed, adding to the previous evidence of a role for ERM proteins in neurite initiation (Paglini et al., 1998). Transfection data also provided further evidence of ERM function in neurite branching, which is an important process for neurite targeting and the establishment of appropriate cellular connections. Transfection with the dominant negative resulted in increased branching of both axons and dendrites. This has previously been reported for axons in hippocampal neurons (Dickson et al., 2002) and cerebellar granule neurons (Cheng et al., 2005), specifically as a result of disrupting the L1-ERM-actin linkage.

It is interesting that the current studies and others have identified striking and significant changes in aspects of growth and morphology following perturbation of one or more ERM proteins at the cellular level, when to date, knockout studies have identified minimal phenotypic effects at the whole animal level. For example, moesin knockout mice showed no morphological abnormalities in any of the tissues examined, nor were ERM related cellular functions affected, including aggregation of platelets, formation of stress fibres in fibroblasts, or microvilli formation in mast cells (Doi et al., 1999). In...
addition, Kikuchi and colleagues (2002) generated a radixin deficient mouse, which was normal at birth, but developed some liver problems within the first eight weeks. More recently, radixin deficient adult mice were shown to be deaf, due to the degeneration of stereocilia, the large microvilli of cochlea hair cells (Kitajiri et al., 2004). Ezrin knockout mice showed retardation in photoreceptor development and microvilli defects of the retina (Bonilha et al., 2006).

The lack of severity of outcomes of knockouts of individual ERM proteins, combined with the demonstration that loss of one protein is not compensated by upregulation of others (Doi et al., 1999) suggests that the individual proteins might be redundant at the tissue level. However, the fact remains that there is specific tissue expression of the individual proteins, which should be interpreted that ERMs are not necessarily interchangeable in all contexts (Mintz et al., 2003). The generation of a knockout of all three ERM proteins, or indeed tissue specific knockouts, may help to answer some of these questions.

Prior to the current investigations, very little was known about ERM protein function in developing CNS neurons, as they have more regularly been the subject of investigation in non-neuronal cells, where their elucidated roles have provided a foundation for investigation of their function in neurons. For example, they are involved in the formation of cell surface microvilli in epithelial and thymoma cells (Takeuchi et al., 1994), and then in subsequent investigations, including this study; ERMs have been implicated in neuron filopodia establishment and dynamics (for example, Paglini et al., 1998). ERMs are also involved in cell adhesion in thymoma cells (Takeuchi et al., 1994), and were subsequently shown to be intracellular components of adhesion systems in neurons (Dickson et al., 2002). Therefore, there are likely to be other neuronal functions that remain to be investigated. For example, ERM proteins are involved in mechanisms of migration in many non-neuronal cell phenotypes, which incited investigation into potential roles in neuronal migration in the present study.
As a result, this thesis has provided evidence that ERMs are involved in developmental neuron migration, particularly shown by their co-localisation with the microtubule-associated, migratory marker DCX, in the embryonic neocortex. There are many identified cytoskeletal associated proteins important for migration (recently reviewed by Bielas and Gleeson, 2004; Bellion et al., 2005; Gressens, 2006). Mutation to the genes encoding these proteins frequently results in disrupted migration, clinically termed lissencephaly disorders. For example, mutation of the DCX gene (encoding DCX) results in an X linked double cortex syndrome, where radial migration is interrupted, and results in a second layer of cortical neurons abnormally gathering under the normal outer cortex (reviewed by Gleeson, 2000). Mutation in the LIS1 gene can have more severe manifestations, such as cobblestone lissencephaly, where the outer layers of the cortex do not form the normal horizontal layered organisation, along with other symptoms including hydrocephalus and cerebellar hypoplasia (Gleeson, 2000). These disorders suggest that perturbing the function of individual proteins involved in neuron migration can have drastic morphological effects. Clinically, they can result in epilepsies, and more subtle alterations may contribute to disorders such as dyslexia and schizophrenia (reviewed by LoTurco and Bai, 2006). Therefore, it would be highly valuable to revisit the ezrin, radixin and moesin knockout mice with the specific focus being to examine the organisation of the cortex. This would be an ideal way to elucidate their more specific functions in developmental neuron migration.

7.3 IMPLICATIONS OF ERM PROTEIN FUNCTION FOR CNS REGENERATION

Previously, very little was known about the role of ERM proteins in neuronal development, and therefore the participation of ERMs in regenerative responses of the adult CNS have not been investigated. The current studies have shown that in an in vitro model of injury of more mature neurons, ERM proteins are re-expressed, both in regenerative neurite sprout growth cones, and in a population of motile neurons that translocate into the site of injury. Furthermore, ERM perturbation by inhibition of Rho kinase was detrimental to aspects of regenerative neurite outgrowth and post-injury neuron motility.
Traditionally there has been pessimism surrounding the capability of the nervous system to undergo regenerative growth. Factors such as an intrinsic loss of the components required for neuronal growth, formation of the glial scar and the presence of inhibitory molecules in the adult CNS contribute to the failure of regeneration (reviewed by Fawcett and Asher, 1999; Morgenstern et al., 2002; Chuckowree et al., 2004; Matsui and Oohira, 2004). However, many of the requirements for developmental growth have been identified in more recent years and then further investigated in injury models and as a result, the goal of functional regeneration now does not seem so unattainable.

One strategy for promoting re-growth after injury might be to use genetic manipulation to upregulate gene expression, and thus protein production, of the cytoskeletal components required for new neurite growth. This thesis has shown that the ERM proteins are involved in aspects of both developmental and regenerative growth, which makes them ideal candidates for manipulation after trauma. Furthermore, their plasma membrane binding partner L1 has been shown in these experiments, and others, to promote regeneration after trauma (Aubert et al., 1998; Brook et al., 2000; Jones et al., 2003; Becker et al., 2004; Chen et al., 2005; Zhang et al., 2005) and, in some cases, has even been associated with functional recovery (Roonprapunt et al., 2003). These are just two developmentally significant proteins that could potentially aid regeneration, and with the discovery of the roles of other proteins, a combined approach to manipulation of the adult injured CNS environment to more closely resemble aspects of development remains a viable and exciting strategy for promoting brain repair.

Another approach to promoting regeneration is the application of pharmacological inhibitors of Rho kinase to promote post-injury neurite growth, because it appears that the signalling mechanisms of many inhibitory molecules converge on the Rho pathway (for example, Wahl et al., 2000; Monnier et al., 2003; Jain et al., 2004). Studies have shown that after injury, levels of Rho kinase are elevated (Dubreuil et al., 2003; 2006), and application of a Rho kinase inhibitor, in numerous different experimental models, has been shown to promote regenerative growth (Borisoff et al., 2003; Dergham et al.,
2002; Fournier et al., 2003; Monnier et al., 2003; Bertrand et al., 2005). However, the data presented in this thesis serves as a caution that molecules such as Rho kinase exist in a controlled balance and their inhibition does not necessarily always translate to better outcomes, and dose selection appears to be of primary importance (Chan et al., 2005). This was demonstrated in the current investigation because addition of RKI II at both the 'low' and 'high' concentrations slightly reduced the average length of regenerative neurites, compared with controls. Furthermore, application of RKI II at the concentration that effectively blocked ERM activation resulted in a significant decrease in the number of regenerative neurites. These studies did, however, demonstrate that Rho kinase inhibition during developmental and regenerative periods causes similar outcomes, particularly with respect to formation of neurites and neurite length, which is evidence that the signalling pathways involved in the mechanisms of growth in more mature neurons are similar to those of growth during development.

Identification of the phosphorylated ERM expressing motile neuron population in long-term cortical cultures after transection injury was both novel and potentially relevant to post-injury regeneration. These neurons were approximately 21 days old, and their characteristics suggested they had progressed through several post-mitotic developmental stages. Therefore, their motile ability does not fit with the traditional view of neuron migration, which has previously only been reported in precursor and early post-mitotic neurons. In fact, the potential for more mature neurons to undergo translocation has not previously been widely considered. Indeed it seems highly unlikely that neuron phenotypes which posses long axons and elaborate dendritic networks could become motile, however this data suggests that there are small, more mature neurons with short processes that could potentially migrate through brain regions. Identification of such populations in vivo may potentially provide a new avenue for exploration with respect to regenerative strategies.

These experiments also observed the role of molecular guidance cues in the motility observed after injury. Molecular guidance cues including laminin, L1 and Sema 3A significantly effected neuron motility, which showed that post-mitotic neurons remain
Chapter 7 - Discussion

responsive to guidance cues, which in turn modulated their behaviour in a similar manner to that which has been elucidated in developmental migration (for example, Persohn and Schachner, 1987; Liesi, 1990; Marin et al., 2001). Furthermore, Rho kinase inhibition reduced motility differentially depending on the molecular guidance cue. This was indicative of the signalling pathways that mediate the actions of guidance cues, therefore it would be interesting to now determine whether ERM proteins are a downstream constituent of specific molecular guidance signalling pathways, in relation to nerve cell migration, as well as neurite outgrowth.

Whether the motile neuron population identified in these experiments has the same characteristics in vivo is still uncertain, but the possibility of using molecular guidance cues to attract the neurons to the injury site for regenerative purposes is an exciting possibility that warrants future investigations. Manipulation of the pattern of expression of guidance cues in the adult CNS after injury has been suggested for promoting regrowth and establishing correct targets (reviewed by Koerberle and Bahr, 2004), but less widely considered as a method for guiding implanted stem cells or progenitor neurons produced in the ventricular zone to sites of injury in the adult brain. Implanting stem cells may provide more promising results, as whether neural progenitors are even produced throughout life in human's remains controversial (reviewed by Au and Fishell, 2006). Finally, the ability of the motile neuron population at 21 DIV to respond to guidance cues provides additional evidence that neurons in the developing and more mature nervous system share the same intrinsic properties.

The idea that aspects of CNS development must be recapitulated in order for regenerative growth to occur in the more mature nervous system has received widespread support (for example, Doucet and Petit, 2002; Emery et al., 2003; Chuckowree et al., 2004; Koerberle and Bahr, 2004; Filbin, 2006; Harel and Strittmatter, 2006). However, there appears to be few reports providing direct evidence that aspects of regeneration recapitulate development. For example, the re-expression of growth-associated cytoskeletal components, such as GAP-43 (Bisby et al., 1988; Li et al., 1996; Christman et al., 1997; Avwenagha et al., 2003; Zhang et al., 2005; Ikegami et al., 2005)
has been demonstrated, but for many other aspects this still appears to be speculative, such as the expression of molecular guidance cues in the mature CNS. Therefore, this study contributes greatly to this theory, as it has provided many examples of common mechanisms in development and regeneration of the more mature injured nervous system. Examples include; that ERM proteins were expressed in the growth cones of developing neurons and then re-expressed in regenerative neurite growth cones, and that L1 promoted developmental and regenerative neurite outgrowth. In addition, regenerative neurite growth cones and developmental neurite growth cones displayed the same morphology when grown on poly-l-lysine and L1 substrates. Phosphorylated ERM was expressed by migrating neurons in the developing neocortex, as well as by a motile neuron population at 21 DIV. Furthermore, post-mitotic neurons at 21 DIV responded to guidance cues in the same way that has been reported for more immature neurons, and finally, inhibition of Rho kinase in developing neurons and more mature neurons post-injury resulted in decreased neurite formation, as well as similar, dose dependent effects on neurite outgrowth. In summary, data relating to the commonality of mechanisms of development and regeneration encompasses multiple steps of neuron growth, including molecular guidance, intracellular signal transduction and cytoskeletal-associated protein expression.

7.4 CONCLUSIONS

- **ERM proteins mediate a number of aspects of neuronal morphogenesis, including neurite initiation, outgrowth and branching.**
- **ERM proteins and their binding partner L1 are involved in developmental and regenerative neurite outgrowth.**
- **ERMs are localised to translocating neurons, in development and post-injury, suggesting a role in neuron migration.**
- **The regenerative response of more mature neurons to injury induces recapitulation of development, including the mechanisms of cytoskeletal proteins, intracellular signalling pathways and molecular guidance cues.**
- **Neurons derived from the neocortex and hippocampus may vary in their ability to undergo regenerative growth.**
• *ERM proteins are ideal candidates for manipulation in therapeutic strategies to promote recovery following CNS trauma.*
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9 APPENDIX – SOLUTIONS

GENERAL SOLUTIONS

0.01M PBS, pH 7.4

- 100mL 9% NaCl (90g of NaCl (Sigma) per 1L Milli-Q® water)
- 40mL Na₂HPO₄ (BDH) (28.4g per 1L Milli-Q® water)
- 10mL NaH₂PO₄·2H₂O (Sigma) (31.2g per 1L Milli-Q® water)
- 850mL Milli-Q® water

CELL CULTURE SOLUTIONS

0.001% poly-l-lysine

- 450mL sterile 0.01M PBS
- 50mL 0.01% poly-l-lysine (Sigma)

10mM HEPES buffer

- 1.19g HEPES (BDH)
- 500mL 0.01M PBS

Combine and heat sterilise

Imaging buffer

- 15.5mL 4M NaCl (23.4g per 100mL Milli-Q® water)
- 2.5mL 2M KCl (Sigma; 7.46g per 50mL Milli-Q® water)
- 50mL 2M CaCl₂ (BDH; 14.7g per 50mL Milli-Q® water)
- 250mL 2M MgCl₂ (Sigma; 20.33g per 50mL Milli-Q® water)
- 7.5mL 2M Dextrose (D-glucose) (Sigma; 18.02g per 50mL Milli-Q® water)
- 6.25mL 2M HEPES (26.03g per 50mL Milli-Q® water)

Adjust to pH 7.3 with 2M NaOH, make up to 500mL with Milli-Q® water, filter sterilise and store at 4°C.
IMMUNOCYTOCHEMISTRY SOLUTIONS

4% Paraformaldehyde (PFA)
40g PFA (Sigma)
40g Sucrose (Sigma)
100mL 9% NaCl
400mL Na₂HPO₄
500mL NaH₂PO₄·2H₂O
Heat while stirring in a fume hood.

10% Trichloroacetic acid (TCA)
1g Trichloroacetic acid (Sigma)
10mL Milli-Q® water

0.3% Triton/PBS
600µL Triton X (Fluka)
200mL 0.01M PBS

WESTERN BLOTTING SOLUTIONS

Bis/Acrylamide
29.2g acrylamide (Sigma)
0.8g N‘N‘-bis-methylene-acrylamide (Sigma)
Make up to 100mL with Milli-Q® water, filter through Whatman filter paper and store at 4°C, in the dark

1.5M Tris-HCl, pH 8.8
27.23g Tris base (Bio-Rad)
~80mL Milli-Q® water
Adjust to pH 8.8 with 10M HCl and make up to 150mL with Milli-Q® water. Store at 4°C.
0.5M Tris-HCl, pH 6.8
6g Tris base
~60mL Milli-Q® water
Adjust to pH 6.8 with 10M HCl and make up to 100mL with Milli-Q® water. Store at 4°C.

10% Sodium Dodecyl Sulfate (SDS)
10g SDS (BDH)
Dissolve in 90mL of Milli-Q® water with gentle stirring and adjust to 100mL with Milli-Q®. Store at room temperature.

5X Running Buffer, pH 8.3
9g Tris base
43.2g Glycine (Bio-Rad)
3g SDS
Combine and add 600mL of Milli-Q® water. Store at 4°C. Prior to use combine 100mL Running Buffer with 400mL Milli-Q® water.

Transfer Buffer
3.03g Tris base
14.4g Glycine
200mL Methanol (Sigma)
Combine reagents in a fume cabinet and make up to 1L with 800mL of Milli-Q® water. Store at room temperature.

Tris buffered saline (TBS)
4.84g Tris base
58.4g NaCl
1.5L Milli-Q®
Adjust to pH 7.5 with 10M HCl and make up to 2L with Milli-Q® water.
Tris buffered saline with Tween (TTBS)

1.1L TBS
550uL Tween-20 (Bio-Rad)

TTBS/5%FCS/5% skim milk powder

100mL TTBS
5mL FCS (Gibco)
5g skim milk powder
Store at 4°C for a limited time.

12% Separating gel

3.35mL Milli-Q® water
2.5mL 1.5M Tris-HCl, pH 8.8
100µL 10% SDS
4mL Bis/Acrylamide
Add quickly immediately prior to pouring
50µL 10% APS (Bio-Rad)
5µL TEMED (Bio-Rad)
Combine ingredients in the listed order and pour gel immediately. Allow to polymerise for 45 minutes.

7.5% Separating gel

4.85mL Milli-Q® water
2.5mL 1.5M Tris-HCl, pH 8.8
100µL 10% SDS
2.5mL Bis/Acrylamide
Add quickly immediately prior to pouring
50µL 10% APS
5µL TEMED
Combine ingredients in the listed order and pour gel immediately. Allow to polymerise for 45 minutes.

**4% Stacking gel**

- 6.1mL Milli-Q® water
- 2.5mL 0.5M Tris-HCl, pH 6.8
- 100μL 10% SDS
- 1.33mL Bis/Acrylamide
- 50μL 10% APS
- 10μL TEMED

**Coomassie stain**

- 1g Coomassie blue R-250 (Bio-Rad)
- 40mL methanol, 10%
- 10mL acetic acid (Spectrum)

**Coomassie destain**

- 40mL methanol, 10%
- 10mL acetic acid
- 50mL Milli-Q® water

**2X Sample buffer**

- 2.5mL 0.5M Tris HCl pH 6.8
- 2mL glycerol (Sigma)
- 200μL 2-β mercaptoethanol (Serva)
- 2mL 10% SDS (in water)

Make up to 10mL with Milli-Q® water, add a small amount of bromophenol blue, aliquot and freeze
PLASMID DNA PREPARATION SOLUTIONS

NZY + Broth
10g casein hydrosolate (NZ amine) (Oxoid)
5g yeast extract (Oxoid)
5g NaCl
Adjust to pH 7.5 with NaOH and autoclave

Add the following supplements before use:
12.5mL 1M MgCl₂ (Sigma)
12.5mL M MgSO₄ (Sigma)
10mL 2M filter sterilised glucose solution or 20mL of 20% (w/v) glucose
Filter sterilise

LB medium and plates
475mL Milli Q water
5g Bacto-tryptone (Oxoid)
2.5g Bacto-yeast extract (Oxoid)
5g NaCl
7.5g Bacto-agar (for plates only) (Oxoid)

Shake until dissolved and adjust pH to 7.0 with 5N NaOH, make volume of solution to 500mL. Autoclave and allow media to cool to before adding antibiotics at 30µg per mL.

Antibiotics
Kanamycin (10mg/mL) and Ampicillin/Carbenicillin (50mg/mL) are used at a final concentration of 30µg/mL.

50X TAE
242g Tris Base
57.1mL glacial acetic acid
100mL 0.5M EDTA pH8.0 (BDH)
Make up to 1L with Milli-Q® water

0.8% Agarose Gel
0.4g Agarose (Invitrogen)
1mL 50x TAE
50mL Milli-Q® water

Microwave for two minutes. Add 2μl of Ethidium Bromide swirl, pour. Run gel in TAE.