THE RECEPTOR PROTEIN TYROSINE PHOSPHATASE-MU SIGNALING PATHWAY DIFFERENTIALLY REGULATES E-CADHERIN, N-CADHERIN AND R-CADHERIN-MEDIATED AXON OUTGROWTH

By

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LIST OF ABBREVIATIONS

A - Anterior
AS PTPµ - Antisense PTPµ
C-S PTPµ - Catalytically Inactive Mutant form of PTPµ
CA - Constitutively Active
CAM - Cell Adhesion Molecule
Cdc42 - Cell Division Cycle 42
CMF - Calcium-Magnesium-Free Hank’s Buffered Saline
COOH - Carboxyl-Terminus
D - Dorsal
D1 - Membrane Proximal Tyrosine Phosphatase Domain
D2 - Membrane Distal Tyrosine Phosphatase Domain
DAG - Diacylglycerol
DN - Dominant Negative
DRG - Dorsal Root Ganglia
E - Embryonic Day
EC - Extracellular Cadherin Domain
ECM - Extracellular Matrix
EDTA - Ethylenediaminetetraacetic Acid
EGF - Epidermal Growth Factor
ERM - Ezrin-Radixin-Moesin
FN III - Fibronectin Type III Repeat
GAP - GTPase Activating Protein
GCL - Ganglion Cell Layer
GDI - Guanine Nucleotide Dissociation Inhibitor
GEF - Guanine Nucleotide Exchange Factor
GFP - Green Fluorescent Protein
GPI - Glycosylphosphatidylinositol
GTPase - Rho Subfamily of Small G-Proteins
HAV - Histadine-Alanine-Valine
HLH - Helix-Loop-Helix
HSV - Herpes Simplex Virus
Ig - Immunoglobulin
IgSF - Immunoglobulin Superfamily
ILM - Inner Limiting Membrane
INL - Inner Nuclear Layer
IPL - Inner Plexiform Layer
IQGAP - IQ Motif Containing GTPase Activating Protein
IRES - Internal Ribosome Entry Site
JM - Juxtamembrane Sequence
kDa - KiloDalton
LAR - Leukocyte Common Antigen-Related Receptor
LNCaP - Human Prostate Carcinoma Cells
MAM - Meprin-A5 Neuropilin-Mu
MDCK - Madin-Darby Canine Kidney Cells
MO - Morpholino Oligonucleotides
N - Nasal

NCAM - Neural Cell Adhesion Molecule

NE - Neuroepithelium

NFL - Nerve Fiber Layer

NgCAM - Neuron-Glia Cell Adhesion Molecule

OFL - Optic Fiber Layer

ONL - Outer Nuclear Layer

OPL - Outer Plexiform Layer

OLM - Outer Limiting Membrane

P - Posterior

PC12 - Cell Line Derived from a Pheochromocytoma of the Rat Adrenal Medulla

PI - Phosphoinositol

PDK - Phosphoinositide Dependent Kinase

PKC - Protein Kinase C

PLC - Phospholipase C

PRL - Photoreceptor Layer

PS - Phosphatidylserine

PTP - Protein Tyrosine Phosphatase

PTK - Protein Tyrosine Kinase

Rac1 - Ras-Related C3 Botulinum Toxin Substrate 1

RACK1 - Receptor for Activated C Kinase 1

RGC - Retinal Ganglion Cell

RhoA - Ras Homologous Member A
RPE - Retinal Pigmented Epithelium

RPTK - Receptor Protein Tyrosine Kinase

RPTP - Receptor Protein Tyrosine Phosphatase

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SGFS - Stratum Griseum et Fibrosum Superficial

SO - Stratum Opticum

SPTPµ-Tat - Scrambled PTPµ Wedge Peptide Tat Tagged

T - Temporal

Tiam1 - T-Lymphoma and Metastasis 1 Protein

V - Ventral

WLAR-Tat - LAR Wedge Peptide Tat Tagged

WPTPµ-Tat - PTPµ Wedge Peptide Tat Tagged

WT PTPµ - Wild-Type PTPµ
Understanding the complexity of how retinal ganglion cell (RGC) axons migrate out of the retina, along the retinotectal pathway to reach their target in the brain remains a central question facing neurobiologists today. RGC axon outgrowth initiated by cell adhesion molecules (CAMs) requires both adhesion to the CAM and activation of cell surface receptors initiating intracellular signaling cascades. The focus of this dissertation is on the role of E-cadherin, N-cadherin and R-cadherin in axon outgrowth in the chick visual system. In addition, we investigated the role of protein tyrosine phosphatase-mu (PTPµ) and its associated proteins in cadherin-mediated axon outgrowth. E-cadherin, N-cadherin and R-cadherin are three members of the classical cadherin family of CAMs and are expressed in the chick visual system during peak RGC axon outgrowth. N-cadherin and R-cadherin have been shown to promote neurite outgrowth. Homophilic binding of N-cadherin mediates neurite outgrowth that is dependent upon PTPµ function. PTPµ associates with E-cadherin, N-cadherin and R-cadherin. We hypothesized that E-cadherin and R-cadherin-mediated neurite outgrowth requires PTPµ. We detected E-cadherin expression in RGCs and in the tectum. In
retinal organ culture studies, we demonstrated that E-cadherin promotes neurite outgrowth via homophilic binding. Conversely, R-cadherin promotes neurite outgrowth through heterophilic binding with N-cadherin. We also demonstrated that E-cadherin and R-cadherin-mediated neurite outgrowth requires PTP\(\mu\) function. Observation of distinct growth cone morphologies on all three substrates, led us to hypothesize that unique signaling pathways are involved in E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth. Inhibition studies in retinal organ cultures demonstrated that Rac1 activity is required for E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth while Cdc42 activity is required for N-cadherin and R-cadherin-mediated neurite outgrowth via IQGAP1. The scaffolding protein IQGAP1, binds to PTP\(\mu\), Cdc42 and Rac1. PTP\(\mu\) also binds to RACK1, an intracellular receptor for activated protein kinase C (PKC). We demonstrated a unique requirement for the PKC\(\delta\) serine-threonine kinase in E-cadherin and R-cadherin-mediated neurite outgrowth. Investigating cadherin-mediated axon outgrowth will shed light on axon growth during development and regeneration as well as contribute to the understanding of cadherin signaling in cell-cell adhesion and cell migration.
CHAPTER 1

Introduction
1.1 Historical Perspective

At the turn of the 19th century, an on-going debate existed as to the organization of the nervous system. On one end, the reticularists believed that the nervous system is made up of a single, large network of continuous nerve cells. Opposing this theory were the neuronists who proposed that the nervous system is made up of independent elements, or cells. Despite growing support for the cell theory proposed by Theodore Schwann and Matthias Schleiden stating that the cell is the basic functional unit of life (Schleiden, 1838; Schwann, 1839), this theory was not accepted to be applicable to the nervous system. It was only through advances in microscopy and histological techniques that a greater understanding of the nervous system was attained.

In northern Spain, Santiago Ramon y Cajal was developing such techniques for staining the nervous system. Based on earlier work by Camillo Golgi, Cajal modified the “Golgi stain” which involves fixing tissue in potassium bichromate, ammonia and finally silver nitrate solution, to observe embryonic nervous tissue in greater detail (Golgi, 1873). From these detailed observations Cajal characterized the growth cone, proposed that information travels in one direction through the neuron, starting at the dendrite through the cell body to the axon and confirmed the neuronist perspective of the nervous system now known as the Neuron Doctrine (Waldeyer-Hartz, 1891). Cajal also suggested that due to the lack of physical barriers in the nervous system, chemical gradients must exist to direct migrating axons to their target. Cajal’s contribution to the understanding of the organization of the nervous system eventually lead to the award of the Nobel Prize for Physiology along with Camillo Golgi for their work on comparative neuroanatomy in 1906.
Moving into the 20th century, Roger Sperry made a significant contribution to the understanding of the nervous system by demonstrating the fundamental importance of neural connectivity in neuronal function (Sperry, 1945). From his observations, Sperry further proposed that in order for this connectivity to occur, each target neuron must have a distinct cell surface identification tag. Additionally, growing axons must have a complementary tag allowing them to seek out and identify their specific target in the brain. This theory came to be known as the chemoaffinity hypothesis (Sperry, 1963). The general chemoaffinity hypothesis stating that all axons have differential cell surface tags that are the result of cellular differentiation and that their target cells have corresponding tags is now widely accepted.

Pioneering work by Holtfreter, Moscano and Steinberg further supported Sperry’s hypothesis. These scientists found that culturing mechanically or chemically disassociated cells from different tissues together resulted in re-aggregation of the cells into their respective tissue type (Moscona, 1961; Steinberg, 1978; Townes and Holtfreter, 1955). In the nervous system, co-culturing single cell suspensions of disassociated neural and mesodermal germinal layers resulted in the proper segregation of tissue specific cell complexes (Townes and Holtfreter, 1955). Based on these observations, scientist deduced that the re-aggregation of tissue specific cells is driven by changes in the expression level and adhesive properties of complementary cell-surface tags distinct to each tissue cell. These findings also paved the way for the differential adhesion hypothesis stating that the sorting out of different cell types into separate tissues during development requires differences in cell adhesion (Steinberg, 1963).
In recent years, neurobiologists have shifted their focus to the identification of these molecular tags and characterization of how these tags are regulated and transduced into directed growth. One class of cell-surface tags capable of mediating cell adhesion and consequently morphogenesis and tissue architecture are aptly termed cell adhesion molecules (CAMs). This family of CAMs includes the integrins, immunoglobulin (Ig) superfamily of CAMs, cadherins and selectins (Kamiguchi, 2007). The following dissertation contributed to the knowledge of three classical cadherins, E-cadherin, N-cadherin and R-cadherin in axon outgrowth.

1.2 The Chick (Gallus gallus) Visual System

The chick visual system is an excellent tool for studying axon growth and migration. Because chickens in general are highly visually dependent organisms, they have developed large eyes accompanied by a large visual processing center in the brain called the optic tectum (analogous to the superior colliculus in mammals). The embryonic chick itself is easily accessible, develops rapidly (21 days to maturation) and as vertebrates, their developmental process is similar to that of higher organisms. The chick visual system is made up of the retina, retinotectal pathway (optic nerve, optic chiasm and optic path) and the visual processing centers of the brain (optic tectum, opticus principalis of the thalamus, nucleus of the basal optic root, external pretectal nucleus and area pretectalis) (Mey and Thanos, 2000). The retina and retinal ganglion cell axon projections are the primary focus of this dissertation. Therefore, the emphasis on chick visual system development will be placed on the retina. For a detailed review

1.2.1 Chick Eye Development

Approximately 26-33 hours after fertilization, the primordial eye can be identified as a lateral outgrowth from the embryonic forebrain called the optic vesicle (Hamburger and Hamilton, 1951). Each optic vesicle develops a stalk-like connection to the central nervous system that will eventually give rise to the rudimentary optic nerve. Invagination of the primary optic vesicle forms the optic cup. This process also forms a groove at the ventral side of the eye that connects with the optic stalk, called the optic fissure. The neural retina is derived from the inner layer of the optic cup while the outer layer becomes the sclera, choroids coat, and retinal pigmented epithelium (RPE). Eye development is not complete until two days before hatching when light stimulation can be detected (Oppenheim, 1968; Rager, 1979).

1.2.2 The Neural Retina

Retinal development progresses from a pseudostratified neuroepithelium to a multi-laminated sensory organ. Six different types of neurons and one type of glial cell (Müller glia) constitute the mature retina (Fig. 1.1). Retinal ganglion cells (RGCs) are the first cell type of the retinal neuroepithelium to leave the cell cycle and differentiate. This cell differentiation begins at embryonic E3 and is followed by rod and cone photoreceptors, amacrine cells, horizontal cells and finally bipolar cells. By E5, two layers of the retina are clearly defined, the outer ventricular zone adjacent to the RPE
and the mantle or inner neuroblastic layer adjacent to the vitreous. In general, cell proliferation occurs in the ventricular zone followed by cell migration to the mantle. Retinal development proceeds in a centrifugal gradient, meaning the cells in the central retina, dorsal-temporal to the optic fissure are more mature than those at the periphery of the retina (Rager et al., 1993). By E8, the majority of cells have stopped dividing. At this stage in development ten distinct layers of the retina are clearly defined; RPE, photoreceptor layer (PRL), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), nerve fiber layer (NFL), and inner limiting membrane (ILM) (Fig. 1.1).

There are approximately 2.5 million RGCs in the chick retina, compared to 1.5 million in the human eye (Bao, 2008). Seven morphological types of RGCs varying in dendritic field, cell body size and dendrite branching density have been identified (Chen and Naito, 1999; Chen et al., 2004; Thanos et al., 1992). Despite these differences, all RGCs share the ability to project an axon. In doing so, RGCs are the only neurons of the retina with the ability to transmit information gathered from the light sensing photoreceptors directly to the optic tectum in the brain. The migratory path of the axon from the RGC to its target in the optic tectum can be divided into several defined phases; intraretinal axon targeting, axon exit out of the eye to form the optic nerve, contralateral crossing at the optic chiasm and innervation at the optic tectum/visual processing center of the brain (Mey and Thanos, 2000). Each of these events is discussed below.
Figure 1.1 Layers of the Retina. The vertebrate retina receives visual information as light and transforms this information into signals, which are transmitted to the brain. As development progresses neuroepithelial cells proliferate, differentiate and migrate to precise cell layers within the retina. The mature retina is composed of 6 types of neurons and one glial cell type, the Müller glia. Each neuronal cell type projects an axon and dendritic processes. The layers of the retina that form synapses are termed plexiform layers. As light enters the retina it passes through the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), outer limiting membrane (OLM), and the photoreceptor layer (PRL) where light is translated into signals. These signals then travel back to the RGCs, the only neuronal cell type to projects axons out of the retina to the brain. The inner limiting membrane (ILM) and the retinal pigmented epithelium (RPE) border the retina at the vitreal or ventricular surface, respectively. Adapted from: (Dowling and Boycott, 1966)
Pigmented Epithelium

Rod Photoreceptors

Cone Photoreceptors

Amacrine Cells

Bipolar Cells

Horizontal Cells

Müller Glia

Ganglion Cells

ONL

NFL

ILM

IPL

INL

OPL

ONL

OLM

PRL

RPE

Light \downarrow

vitreous
1.2.3 Retinotectal Pathway

1.2.3a Intraretinal Axon Targeting

RGC axon extension towards the vitreal surface of the retina, migration along the optic fiber layer and eventual exit out of the retina through the optic disk is referred to as intraretinal axon targeting (Goldberg and Coulombre, 1972). Initially proliferating retinal neuroepithelial cells extend a ventricular and vitreal process or endfoot. These endfeet span the full thickness of the neuroepithelium thus anchoring the cell within the retina. As the RGC becomes polarized, the cell body migrates towards the mantle and the ventricular endfoot begins to retract. Concurrently, the RGC axon emerges from the vitreal endfoot (Brittis et al., 1995b; Snow and Robson, 1995; Watanabe and Raff, 1992) or a de novo vitreal process as seen in zebrafish (Zolessi et al., 2006). Initiation of axon extension is regulated by molecular cues in the surrounding neuroepithelium (Zolessi et al., 2006) along with CAMs such as integrins and cadherins (Lilienbaum et al., 1995; Riehl et al., 1996).

Once the axon reaches the vitreal surface it must turn towards the optic disc and migrate along GCL forming the optic fiber layer. The layers adjacent to the GCL and OFL are inhibitory to migrating axons (Stier and Schlosshauer, 1995), therefore maintaining RGC axons in the OFL. RGC axons extend onto the ventricular surface when inhibitory molecules such as chondroitin sulfate proteoglycan (CSPG), which normally resides in a gradient at the vitreal surface, are miss-expressed at the ventricular surface (Brittis and Silver, 1994). Perturbation of Ig CAMs (Brittis et al., 1995a), integrins (Agius et al., 1996; Stone and Sakaguchi, 1996), cadherins (Doherty and Walsh, 1991; Wanner and Wood, 2002), RPTPs (Ensslen et al., 2003; Ledig et al.,
1999a), and laminin (Bonner and O'Connor, 2001) result in axon initiation and elongation defects demonstrating the importance of cell adhesion and cell signaling in axon outgrowth.

1.2.3b Axon Exit via the Optic Disc

Pioneer axons leave the retina and enter the ventral side of the optic stalk to form the optic nerve. As later RGC axons migrate towards the optic disc, they fasciculate with pioneer axons to form small bundles or fascicles (Van Vactor, 1998). These axons are added peripherally and ventrally to the optic stalk maintaining their positional information obtained in the retina. Fasciculation is mediated by adhesive interactions among growing axons (Bao, 2008; Erskine and Herrera, 2007; Oster et al., 2004) and typically occurs when axons migrate on less permissive substrata resulting in increased axon-axon association.

1.2.3c Contralateral Crossing at the Optic Chiasm

Around E3.5 to E4, RGC axons from both eyes reach the ventral midline to form the optic chiasm (Mey and Thanos, 2000). RGC axons maintain their positional information in a topographic manner as they reach the optic chiasm. It is here that each RGC axon must decide whether to cross the chiasmal midline and innervate the contralateral optic tract or remain uncrossed and innervate the ipsilateral optic tract. For the chick and other species with laterally located eyes, RGC axons from the nasal and temporal retina completely cross the midline to innervate the contralateral optic tract (Fig. 1.2). In species with binary vision or forward-facing eyes, RGC axons from the
Figure 1.2 Retinotectal Projections. The visual field is inverted by the lens as light enters the eye. Consequently, the inverted field of view is projected onto the retina. In order to ensure that the image is inverted once again in the tectum, RGC axons maintain their positional information along the retinotectal pathway. At the optic tectum, RGC axons establish an orderly arrangement of connections reconstructing a topographical representation of the visual field. At the optic chiasm, RGC axons cross the midline to innervate the contralateral optic tectum. Dorsal RGC axons (green) project to the ventral tectum, while the ventral RGC axons (purple) project to the dorsal tectum. Nasal RGC axons (red) project to the posterior tectum, while the temporal RGC axons (blue) project to the anterior tectum. Anterior (A), dorsal (D), nasal (N), posterior (P), temporal (T), ventral (V).
nasal retina cross over the midline and project onto the contralateral optic tract while RGC axons from the temporal retina do not cross the midline and project ipsilaterally.

1.2.3d Innervation of the Optic Tectum

Following along the optic tract, the first RGC axons reach the anterior ventral optic tectum at E6. From E7 to E12 RGC axons continue to advance in an anterior to posterior direction along the outermost most layer of the optic tectum, the stratum opticum (SO). Around E9 RGC axons innervate the outer layers of the tectum, called the stratum griseum et fibrosum superficiale (SGFS) subsequently creating a mirror image of the visual field onto the contralateral tectal quadrants (Fig. 1.2). The visual field is inverted in the eye, therefore, RGC axons form the temporal region of the retina innervate the anterior region of the tectum while the RGC axons from the nasal region of the retina innervate the posterior region of the tectum. In forming the topographic map, RGC axons typically overshoot their target in the optic tectum (Nakamura and O'Leary, 1989; Yates et al., 2001). While it was predicted that topographic map formation is mediated by axon projections, studies demonstrate that axon branching and arborization mediate proper topographic map formation (Yates et al., 2001). RGC axon arborization and synapse formation with neurons of the optic tectum begins at E9 and is completed by E19 (Thanos and Bonhoeffer, 1987).

1.2.4 The Growth Cone

Located at the leading edge of a migrating axon is a dynamic, specialized structure called the growth cone. As the growth cone projects out into the extracellular environment, receptor molecules on its surface recognize and interact with molecular
guidance cues found on the surface of other axons, non-neuronal cells (glial cells) or in the extracellular matrix (Fig. 1.4). These cues are then transmitted to intracellular signaling cascades, which eventually lead to the assembly, disassembly or rearrangement of the cytoskeleton in order to regulate axon growth and navigation. Therefore the morphology of the growth cone reflects the signaling pathways activated in response to cues in the extracellular environment.

Growth cone anatomy consists of three regions, the peripheral, transitional and central domain (recently reviewed in Ishikawa and Kohama, 2007; Pak et al., 2008) (Fig. 1.3). The peripheral domain at the leading edge of the axon consists of filopodia and lamellipodia. Filopodia or microspikes are long thin fingerlike projections extending in front of and to the sides of the growth cone. Each filopodia contains bundles of actin filaments and are responsible for adhesion to a given substrate (Hall, 2005; Le Clainche and Carlier, 2008). Often referred to as veils due to their thin, flat, fan like appearance, lamellipodia contain a meshwork of actin filaments. Transverse bundles of actin filaments are present in the transitional domain while the central domain near the axon is void of actin superstructures. The central region of the growth cone contains organelles, vesicles and microtubules.

1.3 Cell Adhesion Molecules that Mediate Neurite Outgrowth

In order for an RGC axon to migrate long distances along a given trajectory, the growth cone must encounter a number of molecular cues with adhesive and growth promoting properties (Fig. 1.4). Three superfamilies of cell adhesion molecules have been identified to play an important role in chick RGC axon growth: the integrins, the
**Figure 1.3 The Growth Cone.** At the leading edge of a migrating axon is a dynamic structure called the growth cone. The morphology of the growth cone is divided into three regions. Broad fan shaped lamellipodia and long thin filopodia are found in the peripheral domain of the growth cone. Changes in the arrangement of the actin cytoskeleton (red) determine the morphology of the peripheral domain of the growth cone. The central domain of the growth cone (yellow), adjacent to the axon is void of actin superstructures. The transitional domain (brown) resides between the central and peripheral domain. Adapted from: (Luo, 2000; Pak et al., 2008)
**Figure 1.4 Molecular Tags for Axon Outgrowth.** Guidance cues found on the surface of other axons, non-neuronal cells (glia cells) or in the extracellular matrix are required for RGC axons to maintain their topographical information along the retinotectal pathway and innervate their appropriate targets in the brain. These molecular guidance cues are recognized by the growth cone and can be positive (permissive/attractive), negative (inhibitory/repulsive) or affect the directionality (guidance) of the growth cone. For RGC axon migration, growth cones must come into contact with molecular cues that are adhesive and permissive. Cell adhesion molecules such as laminin, L1/NgCAM, NCAM, PTPμ, and the cadherins play an important role in chick RGC axon growth. Adapted from: (Mey and Thanos, 1992)
Soluble guidance molecules

Non-neuronal cells

Laminin

Integrins  CAMs  L1/NgCAM  Cadherins  PTPμ
immunoglobulin superfamily of cell adhesion molecules (IgSF CAMs), and the cadherins (Bao, 2008; Haupt and Huber, 2008; Matthews et al., 2008). Key regulators of chick neurite outgrowth in each of these superfamilies are discussed below.

1.3.1 Integrins

The extracellular environment is a rich landscape for growth promoting molecules. Laminin, an adhesive glycoprotein, is one of the earliest guidance molecules to be identified in the extracellular matrix (ECM) (McKerracher et al., 1996; Timpl et al., 1979). Laminin is secreted from cells into the ECM and is the main non-collagenous glycoprotein found in the basement membrane (Aumailley and Smyth, 1998). During development, the basement membrane that lines the surface of the optic cup gives rise to the inner limiting membrane of the retina (Mey and Thanos, 2000). Structurally, laminin is composed of three subunits joined together in parallel in a coiled-coiled manner: two distinct light chains of approximately 200 kDa designated b and g, and one heavy a chain of 400 kDa (Beck et al., 1990; Burgeson et al., 1994; Kroger and Niehorster, 1990; Tunggal et al., 2000). Laminin-1, -5, -ß1, -ß2, and -1 chains have been identified in the vertebrate ILM (Bystrom et al., 2006). Laminin is broadly expressed in the chick visual system (Cohen et al., 1987; Dong et al., 2002). After 24 hours in culture chick RGC axons extend long neurites out onto a laminin substrate during peak axon extension in the retina (Cohen et al., 1986; Hall et al., 1987). Other molecules that comprise the ECM such as collagen IV and fibronectin also promote RGC axon outgrowth but to a far lesser extent (Hall et al., 1987; Rogers et al., 1983).
Laminin is recognized by integrin receptors expressed on the surface of migrating axons (Tzu and Marinkovich, 2008). Integrins are a family of cell surface receptors that interact with and attach to the extracellular matrix to transduce intracellular signals. Integrins exist as noncovalent heterodimers containing two distinct chains, the alpha (α) subunit and the beta (β) subunit (Denda and Reichardt, 2007). The integrin α6 and β1 subunits are expressed by chick RGCs (de Curtis et al., 1991) and are co-distributed with laminin in the retina (Cann et al., 1996; de Curtis and Gatti, 1994). Antibody blocking experiments directed against the integrin α6 subunit (de Curtis, 1993; de Curtis and Reichardt, 1993) or integrin β1 subunit (Cohen et al., 1986; Hall et al., 1987) block RGC neurite outgrowth on laminin in vitro suggesting that α6β1 is the laminin receptor for chick RGC axons in vivo. However, perturbation of α6β1 in the visual system in vivo has not been demonstrated.

1.3.2 Immunoglobulin Superfamily of Cell Adhesion Molecules

Proteins containing an immunoglobulin (Ig) structural domain belong to the Ig superfamily of CAMs (IgSF CAMs). Typically these family members are cell surface receptors made up of an extracellular segment, transmembrane segment and cytoplasmic segment that associates with the cytoskeleton (Skaper, 2005). All IgSF CAMs have at least one Ig domain that is approximately 70-110 amino acids in length with two characteristic cysteine residues approximately 55 to 75 residues apart. IgSF CAMs mediate Ca^{2+} independent homophilic and heterophilic binding through this Ig domain (Brummendorf and Rathjen, 1998). Most IgSF CAMs, but not all, have fibronectin type III (FN III) repeats that are approximately 90 amino acids in length and
are structurally related to the FN III repeats found in fibronectin (Main et al., 1992). The cytoplasmic segments of IgSF CAMs vary in their structure and function. Many IgSF CAMs have enzyme activity such as with IgSF kinases or IgSF phosphatases (Ensslen-Craig and Brady-Kalnay, 2004).

1.3.2a Neural Cell Adhesion Molecule (NCAM)

The first IgSF CAM to be identified is the neural cell adhesion molecule, NCAM (Brackenbury et al., 1977; Thiery et al., 1977). The extracellular segment of NCAM is made up of five Ig domains and two FN III repeats (Cunningham et al., 1987). The fifth Ig domain is post-translationally modified by the addition of polysialic acid (PSA) moieties that regulate NCAM cell-cell adhesion (Close et al., 2003; Walsh et al., 1997) and neurite fasciculation (Rutishauser et al., 1988). NCAM binds homophilically to mediate cell-cell adhesion (Brackenbury et al., 1977; Hoffman et al., 1982). Heterophilic interactions of NCAM include L1/NgCAM, neurocan and axonin-1 (Brummendorf and Rathjen, 1998). During development, NCAM mediates neurite fasciculation and neurite outgrowth through intracellular signaling pathways (Bonfanti, 2006; Povlsen, 2008). NCAM deficient mice demonstrate fasciculation and pathfinding errors of hippocampal axons (Cremer et al., 1997). Antibody perturbation of NCAM in retinal organ cultures result in RGC growth cone stalling (Brittis et al., 1995a), while intraocular injections disrupt optic stalk projections (Silver and Rutishauser, 1984). Finally, disruption of PSA induces RGC guidance defects in the retina (Monnier et al., 2001).
1.3.2b Neuron-Glia Cell Adhesion Molecule (L1/NgCAM)

Neuron-glia CAM (NgCAM), the chick homologue to L1, is another IgSF CAM that plays an important role in axon growth and guidance. The extracellular segment of L1/NgCAM is made up of six Ig domains and five FN III repeats (Burgoon et al., 1991). Within the visual system L1/NgCAM is solely expressed by RGC axons and in the SO of the optic tectum (Daniloff et al., 1986; Rager et al., 1996). Based on this expression pattern, scientists reasoned that the primary function of L1/NgCAM is to regulate axon growth and guidance. In fact, genetic mutation or deletion of L1/NgCAM in mice and humans results in severe neurological defects (Kamiguchi et al., 1998; Rolf et al., 2001; Yamasaki et al., 1997). Homophilic binding of L1/NgCAM is required for L1/NgCAM-mediated neurite outgrowth (Lemmon et al., 1989) and plays a role in axon fasciculation (Stallcup and Beasley, 1985). Heterophilic binding occurs between L1/NgCAM and NCAM, TAG-1/axonin-1, contactin/F3/F11 or integrins (β1 and β3) (Brummendorf and Rathjen, 1998). L1 associates with the cytoskeleton via ezrin-radixin-moesin (ERM) proteins (Dahlin-Huppe et al., 1997; Dickson et al., 2002). Antibody perturbation of L1/NgCAM in vitro results in RGC fasciculation defects (Rathjen et al., 1987) and RGC growth and guidance defects in vivo (Brittis et al., 1995a; Schlosshauer and Dutting, 1991), suggesting a role for L1/NgCAM in retinotectal map formation. In fact, L1 deficient mice show defects in RGC axon arborization in the superior colliculus (Demyanenko and Maness, 2003).

Although NCAM and L1/NgCAM contribute to RGC axon outgrowth, guidance and targeting, it is clear that other molecules must be required. Some studies have identified NgCAM-related cell adhesion molecule (NrCAM), close homologue of L1
(CHL1), and axonin-1 (the chick homologue of contactin-2) as important in chick axon migration and guidance (Fitzli et al., 2000; Stoeckli and Landmesser, 1995; Zelina et al., 2005). Clearly more work is needed to identify and characterize the cell adhesion molecules responsible for the proper formation of the retinotectal pathway.

1.4 The Classical Cadherins

Sperry’s chemoaffinity hypothesis that complementary cell surface tags exist between each neuron and its target suggests a requirement for the expression of proteins with a remarkable range of diversity on neurons. One family of calcium dependent cell adhesion molecules, the cadherins, meets this requirement. Over 100 vertebrate cadherins have been reported (Venter et al., 2001). In the visual system, cadherin expression patterns are restricted to distinct fiber tracts during development (Hirayama and Yagi, 2006; Redies et al., 2003; Suzuki et al., 1997). Furthermore, RGC axons preferentially select fiber tracts expressing the same cadherin (Treubert-Zimmermann et al., 2002), suggesting a role for the cadherins in axon targeting. Finally, differential distribution of cadherins along the plasma membrane regulates nascent synaptic positioning and synaptic connections (Shapiro et al., 2007; Takeichi, 2005). Indeed, the molecular diversity of the cadherin family makes them ideal candidates to regulate chick visual system development.

The cadherins are classified by their structural and sequence similarities, however all cadherins possess at least one extracellular cadherin (EC) domain approximately 110 amino acids long. Beyond this shared similarity the cadherin family members have considerable structural diversity. There are five distinct cadherin
subfamilies: classical (type I and type II/atypical), fat-like, seven-pass transmembrane, desmosomal and protocadherins (Halbleib and Nelson, 2006; Takeichi, 2007). Though some groups have proposed two additional subfamilies; DCad102F-like and protein kinase cadherins (Tepass et al., 2000). In addition to EC domains, cadherins can possess epidermal growth factor (EGF), flamingo, mucin, and laminin A-G repeats (Angst et al., 2001; Tepass et al., 2000). Cadherins also differ in their association with the cell membrane. While most cadherins have a transmembrane segment, Flamingo cadherin has a seven-pass transmembrane domain with similarity to G-protein linked receptors (Pettitt, 2005). Another cadherin, T-cadherin (CDH13, H-cadherin) lacks a transmembrane and cytoplasmic region and is instead linked to the membrane by glycosylphosphatidylinositol (GPI) anchor (Ranscht and Dours-Zimmermann, 1991). The structural diversity of the cadherins translates to their diversity in function. While the classical cadherins mediate strong cell-cell adhesion, other cadherin family members are not involved in cell-cell adhesion or only mediate weak cell-cell adhesion (Pettitt, 2005).

The classical cadherins were the first subfamily of cadherins to be identified. This family of cadherins is further subdivided into the type I and type II or atypical cadherins. E-cadherin (epithelial), N-cadherin (neural), R-cadherin (retinal) and P-cadherin (placental) make up the four members of the classical type I cadherin subfamily. Structurally, these cadherins have a pro-domain, five EC domain repeats, a transmembrane segment and a highly conserved cytoplasmic segment. The pro-domain must be cleaved by furin family proteases in order to mediate cell adhesion (Henrich et al., 2003; Posthaus et al., 2003). Each EC domain is approximately 110 amino acids
long. Unique to classical (type I) cadherins is a His-Ala-Val (HAV) sequence located in the EC domain farthest from the membrane (EC1) that is required for cell adhesion and neurite outgrowth (Doherty et al., 2000; Patel et al., 2006).

Classical cadherins are calcium dependent adhesion molecules (Fig. 1.5). Accordingly, calcium ions associate with the linker region that connects two tandem EC domains together (reviewed in Patel et al., 2006; Sotomayor and Schulten, 2008). Calcium binding makes the EC domains rigid and resistant to proteolytic degradation (Hyafil et al., 1981). In addition, the presence of calcium causes a conformational change to expose one conserved tryptophan (Trp2) side chain. The Trp2 side chain of one cadherin monomer is proposed to insert itself into an EC1 domain hydrophobic pocket intramolecularly or insert itself into the EC1 domain hydrophobic pocket of another cadherin monomer to mediate adhesion (Patel et al., 2003).

Cadherin-mediated adhesion is accomplished through trans interactions, meaning the extracellular segment of one cadherin will recognize and bind to the extracellular segment of another cadherin presented on an adjacent cell (Leckband and Prakasam, 2006; Patel et al., 2003). This trans interaction likely requires one or more EC cadherin domains. Lateral cis interactions, where cadherin monomers on the surface of the same cell dimerize, have also been suggested to play a role in the formation of trans cadherin interactions (Klingelhofer et al., 2002; Nagar et al., 1996; Takeda et al., 1999; Tomschy et al., 1996; Yap et al., 1997). However, a functional role for cis dimerization in cadherin-mediated adhesion is controversial (Troyanovsky et al., 2007).
**Figure 1.5 The Classical (Type I) Cadherins.** E-cadherin (yellow) and N-cadherin (red) participate in calcium dependent homophilic *trans* binding to mediate cell adhesion and migration. R-cadherin (orange) participates in homophilic interactions with itself and heterophilic interactions with N-cadherin (orange/red). In order to function as adhesion molecules the classical cadherins must associate with the actin cytoskeleton. The COOH terminal cytoplasmic tail of the cadherin binds directly to β-catenin. In turn, β-catenin associates with actin-associated proteins such as α-catenin, α-actinin, vinculin or formin. P120 catenin binds to the membrane proximal region of the cadherin cytoplasmic segment. This cadherin/catenin complex tethers the cadherin to the actin cytoskeleton, allowing for cell adhesion and migration.
β-catenin α-catenin p120 α-actinin Vinculin Formin

E-cadherin N-cadherin R-cadherin R-cadherin/N-cadherin

Ca++

Actin
The highly conserved cytoplasmic segment, 70-90% sequence similarity between the classical cadherins (Nollet et al., 2000), must be tethered to the actin cytoskeleton for proper cell-cell adhesion (Nagafuchi and Takeichi, 1988). At approximately 170 amino acids in length, the cytoplasmic segment associates with a number of actin-associated proteins including the catenins (Suzuki and Takeichi, 2008). The catenins are a group of intracellular proteins of the armadillo repeat containing gene family that link transmembrane proteins to the cytoskeleton. β-catenin binds directly to a short 30 COOH amino acid sequence (amino acids 677 to 706) of the cadherin cytoplasmic segment (Jou et al., 1995; Nagafuchi and Takeichi, 1988; Nagafuchi and Takeichi, 1989; Ozawa et al., 1990; Stappert and Kemler, 1994). A core region containing 8 conserved serine residues is required for β-catenin binding (Stappert and Kemler, 1994). Partial substitution of up to five serine residues does not disrupt cadherin/β-catenin binding, while mutation of all eight serine residues results in the loss of β-catenin binding to E-cadherin (Stappert and Kemler, 1994). Tyrosine phosphorylation of β-catenin also results in loss of cadherin-actin association and therefore regulates cadherin function (Lilien and Balsamo, 2005; McLachlan and Yap, 2007). Plakoglobin (also known as γ-catenin) also binds to the cytoplasmic segment of the cadherins at the same site as β-catenin (Jou et al., 1995). Therefore plakoglobin binding to the cadherins is mutually exclusive with β-catenin. P120-catenin binds to the cytoplasmic segment of cadherins in the juxtamembrane (JM) sequence near the membrane (amino acids 587 to 615) (Reynolds et al., 1994; Shibamoto et al., 1995; Yap et al., 1998) and regulates the stability of the cadherin complex (McLaughlin and Yap, 2007). Mutation of a short core sequence, DEEGGGEED, within the p120 binding
domain of E-cadherin results in a loss of p120 binding (Thoreson et al., 2000). Another
catenin, α-catenin binds directly to the actin cytoskeleton (Rim et al., 1995) to β-catenin
and several other actin binding proteins such as α-actinin, ZO1, vinculin, afadin and
spectrin (Aberle et al., 1996; Huber et al., 1997; Nieset et al., 1997; Obama and Ozawa,
1997).

Based on the above findings, the conventional dogma of cadherin association
with the cytoskeleton has been that the cytoplasmic segment of the cadherin binds to β-
catenin, which binds to α-catenin, which in turn binds to the actin cytoskeleton.
Recently, rigorous direct binding studies with purified proteins and measurement of
protein dynamics in live cells (Drees et al., 2005; Yamada et al., 2005) have revealed
that cadherin association with the actin cytoskeleton is not so clear-cut. While α-
catenin binds to the actin cytoskeleton and to β-catenin, this binding is mutually
exclusive (Drees et al., 2005). Therefore other actin binding proteins such as afadin,
ajuba, formin, vinculin, α-actinin, and ZO1 might be necessary for cadherin association
with the actin cytoskeleton (Weis and Nelson, 2006).

1.4.1 Classical Cadherins in Cell Adhesion

Classical Cadherins mediate cell-cell adhesion and cell sorting through
predominantly homophilic trans interactions (Halbleib and Nelson, 2006). For
instance, cells expressing a particular cadherin (such as E-cadherin) on the surface of
one cell will only recognize and bind to E-cadherin on the surface of another cell.
Rigorous analysis of cadherin binding has been preformed in aggregation assays using
L-cells, one of a few cell lines that do not express endogenous cadherins and naturally
do not aggregate. When cells are transfected with cDNA encoding E-cadherin, N-cadherin or R-cadherin and mixed together in an aggregation assay, E-cadherin expressing cells aggregate separately demonstrating that E-cadherin is a homophilic binding protein (Inuzuka et al., 1991a; Matsunami et al., 1993; Shan et al., 2000). The only example of heterophilic binding among these three cadherins is between N-cadherin and R-cadherin (Inuzuka et al., 1991a; Inuzuka et al., 1991b; Matsunami et al., 1993; Shan et al., 2000). Interestingly, N-cadherin and R-cadherin expressing cells segregate within the chimeric aggregates demonstrating a preference for homophilic binding over heterophilic binding (Inuzuka et al., 1991a; Matsunami et al., 1993; Shan et al., 2000). This N/R-cadherin binding is attributed to their sequence similarity (74%) compared to other cadherins (43-58%). While it is clear that the cadherins mediate direct cell-cell binding, the role of the classical cadherins in dynamic cell-cell interactions such as cell migration is slow to emerge.

1.4.2 Classical Cadherins in Cell Migration

Cadherins are typically associated with stable cell-cell adhesion. For example, E-cadherin is the principle component of ultrastructurally defined complexes found at cell-cell junctions called adherens junctions. Often found in polarized epithelial cells, adherens junctions and therefore E-cadherin itself functions to provide tissue integrity and cell polarity (Wheelock et al., 2008). Evidence for the requirement of E-cadherin in cell migration during development has been observed in Drosophila. During Drosophila oogenesis, DE-cadherin (the E-cadherin homologue) contributes to the proper migration of border cells (Niewiadomska, 1999). Follicle cells that surround the
oocyte and germline cells express DE-cadherin during development (Godt and Tepass, 1998; Niewiadomska, 1999). Perturbation of DE-cadherin results in disruption of border cell and centripetal cell migration (Niewiadomska, 1999; Oda et al., 1997). When Drosophila mutants express DE-cadherin lacking the cytoplasmic domain, fused to α-catenin, defects in border cells were also observed (Pacquelet and Rorth, 2005). The DE-cadherin mutant described is able to mediate cell-cell adhesion (Pacquelet and Rorth, 2005). Therefore the cytoplasmic domain, which is known to associate with regulatory proteins in the cytosol, is required for DE-cadherin-mediated border cell migration. Of note, decreased expression of DE-cadherin in Drosophila (Niewiadomska, 1999) or E-cadherin in zebrafish (Montero et al., 2005) correlates with decreased cell migration. Further studies are needed to investigate the role of cadherin cytoplasmic segment association with other regulatory proteins during the dynamic process of cell migration where cadherin-mediated contacts are continually broken and remade.

1.4.3 Classical Cadherins in the Chick Retina

Originally, the classical cadherins were named based on the tissue in which they were first identified. Despite their nomenclature inference, many cell types in different tissues express the classical cadherins. Of the four classical cadherins, N-cadherin and R-cadherin have been described in detail in the chick visual system. Expression patterns of N-cadherin and R-cadherin differ throughout the retina and retinofugal pathway (Inuzuka et al., 1991a; Inuzuka et al., 1991b; Takeichi et al., 1993; Wohrn et al., 1998), suggesting distinct roles during development.
N-cadherin is widely expressed in the chick visual system. Therefore, considerable attention has been placed on the role of N-cadherin in visual system development (Suzuki and Takeichi, 2008). Expression of N-cadherin is detected early in development, at the time of neural retina formation (Inuzuka et al., 1991a; Matsunaga et al., 1988). By E4.5 high levels of N-cadherin expression are seen uniformly throughout the undifferentiated retinal neuroepithelium with intense staining at the outer limiting membrane (Inuzuka et al., 1991a; Lagunowich and Grunwald, 1989). By E8, N-cadherin expression is observed in the outer limiting membrane, the outer plexiform layer, the inner plexiform layer, and is highest in the retinal ganglion cell layer (Wohrn et al., 1998). Overall N-cadherin expression decreases at E10, but is still uniform throughout the inner plexiform layer (Wohrn et al., 1998). By E14 N-cadherin expression is restricted to the outer plexiform layer and outer limiting membrane (Matsunaga et al., 1988). N-cadherin, is also expressed by photoreceptors of the outer nuclear layer at this stage (Wohrn et al., 1998).

R-cadherin is evident in the visual system at stage 20 (E3, 3.5) in outer layer of optic cup which gives rise to optic stalk (Inuzuka et al., 1991a; Wohrn et al., 1998). As retinal eye development progresses, R-cadherin expression is visible in the outer limiting membrane at E6 and extends to the pigmented epithelium. Expression increases in these layers at E8 (Wohrn et al., 1998). By E10 staining for R-cadherin is observed in the outer limiting membrane, the outer plexiform layer, the inner nuclear layer, the inner plexiform layer, and the retinal ganglion cell layer (Wohrn et al., 1998). Unlike N-cadherin, R-cadherin expression in the inner plexiform layer is selectively expressed (Wohrn et al., 1998). In addition, expression of N-cadherin more so then R-
cadherin is observed in the outer limiting layer (Wohrn et al., 1998). As development continues, the inner plexiform layer continues to differentiate. R-cadherin expression in this layer displays different intensities (Wohrn et al., 1998), suggesting that differentiated cell types within the inner plexiform layer selectively express R-cadherin. At late stages of development, the nerve fiber layer is positive for R-cadherin, but the optic stalk is negative (Redies and Takeichi, 1993b).

E-cadherin (also referred to as L-CAM in the chick) is broadly expressed in all epithelia (Wheelock et al., 2008), and is observed in the retinal pigmented epithelium (RPE) of the chick visual system. Data pertaining to E-cadherin expression in the chick retina and optic tectum is lacking in the literature (Yamagata et al., 1995). E-cadherin is observed in the RGC layer of the mouse retina (Faulkner-Jones et al., 1999; Xu, 2002) and is broadly expressed in all cells of the Drosophila retina (Hayashi and Carthew, 2004), suggesting that E-cadherin may be present in the chick retina. Chapter 2 addresses the expression of E-cadherin in the retina and optic tectum during chick visual system development.

Like E-cadherin, few papers address the expression pattern of P-cadherin in the chick visual system. This may be due to the lack of chick specific P-cadherin antibodies. P-cadherin is expressed during embryonic development of the eye (Nose and Takeichi, 1986). As development progresses, expression is limited to the lens and the RPE (Nose and Takeichi, 1986). P-cadherin expression in the visual system often overlaps with E-cadherin and N-cadherin expression (Takeichi, 1988). This expression pattern is also observed in the mouse (Xu, 2002).
1.4.4 Classical Cadherins in Neurite Outgrowth

1.4.4a N-cadherin

Based on the extensive expression pattern described above for N-cadherin, scientists have placed an emphasis on the role of N-cadherin in visual system development (Shapiro et al., 2007). Since the identification of N-cadherin (Volk and Geiger, 1984), multiple studies have confirmed the significance of N-cadherin in neurulation, regionalization of neuroectoderm, neuronal migration, axon growth and fasciculation (Bixby and Zhang, 1990; Detrick et al., 1990; Drazba, 1990; Matsunaga et al., 1988; Riehl et al., 1996). For instance, *Xenopus* RGCs treated with dominant negative N-cadherin fail to extend an axon (Riehl et al., 1996). In N-cadherin deficient zebrafish, neuroepithelial cell polarity is lost leading to defects in retinal lamination (Pujic and Malicki, 2004). In addition, defects in retinotectal projections at the optic chiasm and RGC axon arborization at the ipsilateral optic tectum were observed (Masai et al., 2003). Antibody perturbation studies in the chick (Inoue and Sanes, 1997) and *Drosophila* mutants null for the N-cadherin homologue DN-cadherin (Iwai et al., 1997; Lee et al., 2001; Yonekura et al., 2007) display similar defects in the visual system. Taken together, these studies demonstrate an important functional role for N-cadherin in RGC axon growth. However, the signaling mechanisms regulating N-cadherin-mediated neurite outgrowth are not clear. A comparative analysis of the regulatory molecules required for N-cadherin, R-cadherin and E-cadherin-mediated neurite outgrowth is presented in Chapter 3.
1.4.4b R-cadherin

It was not until almost a decade after R-cadherin was first identified in chick (Inuzuka et al., 1991a) that the mechanisms behind R-cadherin in the visual system began to emerge. Cadherin 4, the zebrafish R-cadherin homologue, is required for zebrafish retinal development, differentiation and retinotectal projection formation (Liu et al., 1999a). In R-cadherin deficient zebrafish, using antisense morpholino oligonucleotides (MO), neuroepithelial cell differentiation defects are observed in the retina, demonstrating a role for R-cadherin in retinal differentiation and lamination. Retinal cells that are able to differentiate, they target to the appropriate lamina and project an axon (Babb et al., 2005). Along the retinotectal pathway, RGC axons project to the optic tectum in R-cadherin MO-injected embryos but fail to properly arborize within the neuropil (Babb et al., 2005), reiterating the importance of the cadherin cytoplasmic segment in cadherin-mediated events. Cytoplasmic domain deletion of R-cadherin inhibits RGC axon formation and extension (Babb et al., 2005). Cells expressing this R-cadherin mutant display a rounded soma shape and have significantly fewer process (Babb et al., 2005). The broad expression pattern of R-cadherin in the retina combined with the defects observed in zebrafish axon projection, suggest an essential role for R-cadherin in visual system development.

1.4.4c E-cadherin

The role of E-cadherin in axon development and growth is limited. Mouse zygotes deficient for E-cadherin are embryonic lethal, with defects in morula differentiation and preimplantation (Larue et al., 1994; Riethmacher et al., 1995),
emphasizing the importance of E-cadherin in early embryonic development. Due to the fundamental requirement for E-cadherin during development and lack of E-cadherin deficient RGC conditional knockout mice, it is difficult to identify the functional role of endogenous E-cadherin in the visual system. Expression of dominant negative DE-cadherin in Drosophila neuroblasts, secondary neurons and glial cells results in irregularities in neuroblast differentiation, axon projection through the cortex and a general loss of proximal axon tracts (Dumstrei et al., 2003a) displaying a role for E-cadherin in Drosophila neurogenesis. Chapter 2 addresses the functional role of E-cadherin in the chick visual system.

1.5 Rho Family of Small GTPases

1.5.1 Regulation of the Actin Cytoskeleton

Dynamic rearrangement of the cytoskeleton regulates cell shape and cell migration (Ridley, 2008; Rivero and Cvrckova, 2007). The cell cytoskeleton is made up of actin filaments, microtubules and intermediate filaments. Changes in these cellular components can be stimulated by extracellular guidance cue or changes in cell adhesion (Doherty and McMahon, 2008). The Rho subfamily of small G-proteins (Rho GTPases) regulates growth cone morphology and subsequent axon migration through dynamic rearrangement of the actin cytoskeleton (Govek et al., 2005; Koh, 2006; Linseman and Loucks, 2008). The most extensively characterized Rho GTPases are Cdc42 (cell division cycle 42), Rac1 (Ras-related C3 botulinum toxin substrate 1) and RhoA (Ras homologous member A). Activation of these GTPases in fibroblasts
generates the formation of filopodia, lamellipodia and stress fibers, respectively (Nobes and Hall, 1995).

1.5.2 Rho GTPase Regulation, GAPs, GEFs and GDIs

Rho GTPases act as molecular switches, cycling between GTP-bound “on” and GDP-bound “off” states to control cell signaling events (Fig. 1.6) (recently reviewed in Bos et al., 2007; Buchsbaum, 2007; Linseman and Loucks, 2008). The GTPases are activated by guanine nucleotide exchange factors (GEFs). GEFs promote the exchange of GDP for GTP on the GTPase. Inactivation of GTPases occurs when bound GTP is hydrolyzed to GDP. GTPases have intrinsic GTPase activity. However, the rate of hydrolysis is very slow. The GTPase activating proteins (GAPs) increase intrinsic GTPase activity, acting as a catalyst for the conversion of GTP bound GTPases to GDP bound forms. Guanine nucleotide dissociation inhibitors (GDIs) bind to GDP-bound GTPases and prevent the exchange of GDP for GTP (DerMardirossian and Bokoch, 2005). GDIs also bind to GTP-bound GTPases to block both intrinsic and GAP-catalyzed GTPase activity. Another regulatory function of GDIs is to modulate the cycling of GTPases. Rho GTPases are sequestered by GDIs in the cytosol until the cell is stimulated. When GTPases are released from the GDIs, they are free to be activated and associate with their effector targets at the cell membrane.
Figure 1.6 Regulation of the Rho GTPases. The Rho GTPases exist within the cell in an active GTP bound state or an inactive GDP bound or unbound state. GTP bound, active GTPases typically reside at the plasma membrane where they interact with effector target molecules. Conversion of an inactive GDP bound GTPase requires the action of guanine nucleotide exchange factors (GEFs) to catalyze the exchange of GDP for GTP. GTP hydrolysis, which is intrinsically slow, is catalyzed by GTPase activating proteins (GAPs). Guanine nucleotide dissociation inhibitors (GDIs) sequester inactive GTPases in the cytosol by binding to GDP bound GTPases and blocking the exchange of GDP for GTP or binding to GTP bound GTPases to block intrinsic and GAP-catalyzed GTPase activity.
Several GEFs are expressed in the nervous system and play a role in neurite outgrowth (Koh, 2006). GEFs typically contain pleckstrin homology (PH) domains that contribute to membrane targeting (Stam et al., 1997) and membrane ruffling (Michiels et al., 1997). GEFs also contain tandem Dbl homology (DH) domains that are responsible for exchange activity (Rossman et al., 2005). The invasion induced T-lymphoma and metastasis 1 protein (Tiam1) GEF activates Rac1 (Habets et al., 1994). Tiam1/Rac1 activity has been shown to be required for the maintenance of E-cadherin-mediated cell-cell adhesion (Kraemer et al., 2007; Malliri et al., 2004). Tiam1 is expressed in the nervous system and overexpression of Tiam1 induces neurite outgrowth in N1E-115 neuroblastoma cells (Leeuwen et al., 1997). Another GEF, Trio, also activates Rac1 in addition to RhoA (Bellanger et al., 1998a; Bellanger et al., 1998b; Briancon-Marjollet et al., 2008; Chhatrniwala et al., 2007). Originally identified as a leukocyte common antigen-related receptor (LAR) binding protein (Debant et al., 1996), Trio binds to the catalytically inactive D2 PTPase domain of LAR. Trio is unique in that it has two GEF domains (Debant et al., 1996). The first GEF domain has Rac1 specific activity and is essential for axon extension in PC12 cells (Estrach et al., 2002). The second GEF domain has RhoA specific activity (Bellanger et al., 1998a; Bellanger et al., 1998b). Trio is also expressed by axons and regulates neurite outgrowth and axon pathfinding in Drosophila (Awasaki et al., 2000; Bateman et al., 2000; Newsome et al., 2000).
1.5.3 Association with the Classical Cadherins

Evidence for the Rho GTPases in cadherin-mediated cell-cell adhesion has been studied in MDCK epithelial cells. Rac1 co-localizes with E-cadherin at sites of cell-cell contact (Nakagawa et al., 2001). Overexpression of constitutively active Rac1 in MDCK cells increases E-cadherin, β-catenin and actin filaments at sites of cell-cell contact (Takaishi et al., 1997). E-cadherin-mediated cell adhesion requires Rac1 and Cdc42 (Kodama et al., 1999; Kuroda et al., 1997). In addition to E-cadherin, Rac1 and Cdc42 activity is required for R-cadherin-mediated cell migration. Exogenous expression of R-cadherin increases cell motility and correlates with increased Cdc42 and Rac1 activity (Johnson et al., 2004). Taken together, Rac1 and Cdc42 play a regulatory role in cadherin-mediated cell adhesion and cell migration.

One potential mechanism for the regulation of E-cadherin by Rac1 and Cdc42 is through the scaffolding protein IQGAP1 (IQ motif containing GTPase activating protein 1). IQGAP1 negatively regulates E-cadherin-mediated cell adhesion by binding to β-catenin (Kuroda et al., 1998). Subsequently, α-catenin disassociates from the cadherin/catenin complex and cell adhesion is lost (Kuroda et al., 1998). Activated Cdc42 and to a lesser extent Rac1 binds to IQGAP1 (Hart et al., 1996; Swart-Mataraza et al., 2002; Zhang, 1997). This binding blocks the association of IQGAP1 with β-catenin (Fukata et al., 1999). As a result, β-catenin is free to associate with the E-cadherin/catenin complex making Rac1 and Cdc42 activation key factors in cadherin-mediated cell adhesion.
1.5.4 Role in Neurite Outgrowth

In addition to cell adhesion, Rho GTPases have been implicated in axon guidance and neurite outgrowth (Koh, 2006; Linseman and Loucks, 2008). Looking over the past ten years a general trend for GTPase activity in neurite outgrowth has emerged. Rac1 and Cdc42 act as positive regulators of neurite outgrowth while RhoA negatively regulates neurite outgrowth (Govek et al., 2005; Koh, 2006). More recently, studies in *Xenopus* suggest a delicate balance of GTPase activity is required for the regulation of neurite outgrowth (Woo and Gomez, 2006). Either inhibition of Rac1 or overexpression of active Rac1 results in a decrease in the rate of neurite outgrowth and defects in growth cone contact dynamics (Woo and Gomez, 2006). In the chick visual system, Cdc42 and Rac1 are expressed during peak axon extension and are involved in PTPμ-mediated growth cone rearrangement and neurite outgrowth (Major and Brady-Kalnay, 2007; Rosdahl et al., 2003). To date, specific Rho GTPases required for E-, N- and R-cadherin-mediated neurite outgrowth are unknown.

1.6 Tyrosine Phosphorylation in the Visual System

The first molecules to be identified as topographic guidance cues are the Eph family of receptor protein tyrosine kinases (RPTKs) and their ligands, the ephrins (reviewed in McLachlan and Yap, 2007). The Eph/ephrin RPTKs are found in a gradient within the visual system and are proposed to control the initial projection of RGC axons along the anterior-posterior axis of the optic tectum. While Eph/ephrin RPTKs have been shown to regulate patterning of the retinotectal map, additional guidance cues are necessary for proper patterning of the visual system.
The requirement for protein tyrosine phosphorylation as a critical component of protein regulation invariably implicates protein tyrosine dephosphorylation as equally important. Nevertheless, it took nearly a decade after the characterization of kinases to identify the first protein with tyrosine phosphatase activity (Tonks et al., 1988a; Tonks et al., 1988b). Since that time, the protein tyrosine phosphatases (PTPs) have grown to include a diverse family of proteins that are divided into two large subfamilies, the cytoplasmic PTPs and the receptor PTPs (RPTPs). All PTPs contain at least one catalytically active tyrosine phosphatase domain with a unique HC(X)5R motif. The RPTPs are transmembrane proteins that are further subdivided into five different types based on the similarity of their extracellular domain structure (Fig. 1.7). The RPTPs have emerged as major regulatory molecules in axon growth, guidance, and synapse formation (Ensslen-Craig and Brady-Kalnay, 2004; Johnson and Van Vactor, 2003).

One member of the RPTP family, PTPµ, is of particular interest due to its expression in the visual system and its ability to couple extracellular mediated events directly to intracellular signaling pathways.

1.6.1 The PTPµ Subfamily of RPTPs

PTPµ, along with PTPκ, PTPρ, and PTPλ (PCP-2), belong to the RPTP type IIb subfamily of phosphatases. The adhesion molecule like EC segment of the type IIb RPTPs contain an N-terminal MAM (Meprin-A5 neuropilin-Mu) domain, an Ig domain and four FN III repeats (Brady-Kalnay and Tonks, 1995; Ensslen-Craig and Brady-Kalnay, 2004). The fourth fibronectin domain, proximal to the membrane, is cleaved.
Figure 1.7 RPTP Superfamily. All receptor protein tyrosine phosphatases (RPTPs) contain one or more tyrosine phosphatase domains in their cytoplasmic segment. Subsequently, this family of molecules is classified by the various structural characteristics in their extracellular segment. PTPµ belongs to the type IIb subfamily of RPTPs. At the N-terminus is the MAM domain, which is necessary for cell-cell adhesion and cell sorting. The Ig domain, similar to IgSF CAMs is required for homophilic binding. PTPµ also has four FN III repeats. The cytoplasmic segment of PTPµ has a unique juxtamembrane segment sharing sequence homology to the cytoplasmic segment of the cadherins. Despite having two tyrosine phosphatase domains (D1 and D2), only the membrane proximal D1 domain is catalytically active. Adapted from: (Ensslen-Craig and Brady-Kalnay, 2004)
by furin at a conserved consensus cleavage site RXXR but remains tightly associated (Anders et al., 2006; Brady-Kalnay and Tonks, 1994; Jiang et al., 1993; Serra-Pages et al., 1994; Streuli et al., 1992).

The striking similarity of the EC segment to IgSF CAMs, led to the hypothesis that type IIb RPTPs function as cell-cell adhesion molecules. Sf9 insect cells do not naturally aggregate. Expression of PTPµ, PTPκ, PTPρ or PTPλ alone or in combination in Sf9 cells results in the sorting out of PTP specific aggregates (Brady-Kalnay et al., 1993; Cheng et al., 1997; Gebbink et al., 1993; Sap et al., 1994; Yu et al., 2008; Zondag et al., 1995), demonstrating that PTPµ, PTPκ, PTPρ and PTPλ participate in homophilic cell-cell binding. This sorting out of cell populations is reminiscent of the experiments conducted by Holtfreter, Moscano and Steinberg. Despite their high structural and sequence similarity, heterophilic interactions were not observed. Expression of PTPµ EC domains, coated onto fluorescent beads (Covaspheres) demonstrates that the Ig domain is necessary and sufficient for homophilic binding (Brady-Kalnay and Tonks, 1994). Expression of EC deletion constructs in Sf9 cells, reveals that the MAM domain is also necessary but not sufficient for cell-cell aggregation (Zondag et al., 1995), suggesting that the MAM domain plays more of discriminatory role in cell-cell binding. Like the classical cadherins, trans as well as cis dimmers have been observed for RPTPs (Ensslen-Craig and Brady-Kalnay, 2004). In fact, recent studies demonstrate the importance of the MAM domain in PTPµ cis dimerization (Aricescu et al., 2006; Cismasiu et al., 2004). PTPµ-mediated cell-cell aggregation does not require the cytoplasmic segment or PTPµ catalytic activity
(Brady-Kalnay et al., 1993; Gebbink et al., 1993). The same holds true for PTP\(\mu\)-mediated cell adhesion (Hellberg et al., 2002).

1.6.2 Regulation of RPTP Catalytic Activity

RPTPs are unique in their ability to couple extracellular-mediated events directly to intracellular signaling pathways via their tyrosine phosphatase domains. The intracellular segment of each type IIb RPTP contains a juxtamembrane (JM) segment sharing sequence similarity with the cytoplasmic segment of the cadherins (Brady-Kalnay and Tonks, 1994; Tonks et al., 1992) and two tandem tyrosine phosphatase domains (D1 and D2). Only the membrane proximal tyrosine phosphatase D1 domain is catalytically active (Brady-Kalnay et al., 1993; Gebbink et al., 1993). Making a single point mutation in the essential cysteine to a serine (C-S) in the D1 domain of the RPTP LAR results in a 99% loss of phosphatase activity (Pot et al., 1991). The second tyrosine phosphatase D2 domain is catalytically inactive due to naturally occurring mutations. Beyond this functional requirement, little is known in terms of mechanisms that regulate RPTP tyrosine phosphatase activity.

Analysis of the crystal structure of the PTP\(\mu\) D1 domain, identified a distinct helix-loop-helix (HLH) wedge-shaped sequence in the JM segment N-terminal the D1 PTP\(\mu\) catalytic domain (Hoffmann et al., 1997). The functional role of this HLH-sequence in PTP\(\mu\) is unknown. However RGC retinal explants cultured in the presence of a peptide resembling the HLH wedge-shaped sequence (WPTP\(\mu\)-Tat) results in perturbation of PTP\(\mu\)-mediated neurite outgrowth (Xie et al., 2006) which has been shown to require PTP\(\mu\) catalytic activity (Ensslen-Craig and Brady-Kalnay, 2005),
implicating a role for the wedge sequence in regulating PTPµ catalytic activity. WPTPµ-Tat binds to itself in bead binding assays but not to the wedge peptide LAR (WLAR-Tat), another member of the type II RPTP subfamily (Xie et al., 2006), reiterating that the WPTPµ-Tat is specific and does not interact with other RPTP family members. In addition, WLAR-Tat does not perturb PTPµ-mediated neurite outgrowth (Xie et al., 2006) demonstrating the specificity of the PTPµ wedge sequence. RPTP inter/intramolecular interactions regulate catalytic activity of the RPTPs (Bixby, 2001; Brady-Kalnay, 2001; Ensslen-Craig and Brady-Kalnay, 2004; Johnson and Van Vactor, 2003). Inter/intramolecular regulation of phosphatase activity is demonstrated by the association of the JM segment of PTPµ with the D2 domain of PTPµ (Feiken et al., 2000; Gebbink et al., 1993). This interaction is thought to be due to folding back of the D2 domain to bind the JM segment on the same molecule, subsequently blocking substrate access to the catalytic site (Feiken et al., 2000). The WPTPµ-Tat peptide may regulate PTPµ catalytic activity by blocking these inter/intramolecular interactions.

Heterodimerization of PTPµ with other RPTPs on the same cell, has also been proposed to regulate PTPµ function. For example, the D1 and D2 domains of PTPµ interact with each other in addition to PTPα, PTPε, PTPσ and LAR (Blanchetot et al., 2002). Therefore, regulation of PTPµ function by cis heterodimerization is dependent upon the expression of multiple RPTPS within a given cell. In the visual system, RGCs express PTPµ, PTPσ, PTPδ and LAR (Johnson and Holt, 2000; Ledgi et al., 1999). For that reason, interaction between these RPTPs could be relevant to PTPµ-dependent regulation of the visual system development.
1.6.3 PTPµ-Mediated Neurite Outgrowth

PTPµ is expressed in a gradient within the developing chick retina and optic tectum (Burden-Gulley et al., 2002), and is a potential topographic guidance molecule. PTPµ is required for proper lamination within the retina (Ensslen et al., 2003). Perturbation of PTPµ in E5 retinal organ cultures in vitro results in defective RGC axon growth characterized by inappropriate axon elongation along the ventricular surface of the retina and duplication of the optic fiber layer (Ensslen et al., 2003). At E8, a time of peak axon extension (Mey and Thanos, 2000; Thanos and Mey, 2001), PTPµ is expressed in a high temporal, low nasal gradient in the retina and a high anterior, low posterior gradient in the tectum (Burden-Gulley et al., 2002). Temporal RGCs project axons to the anterior portion of the optic tectum while nasal RGCs extend axons to the posterior optic tectum (Mey and Thanos, 2000; Thanos and Mey, 2001), therefore PTPµ may function in a complementary gradient during retinotectal projection. PTPµ differentially regulates nasal versus temporal RGC neurite outgrowth in vitro. Throughout development of the chick visual system high levels of PTPµ are repulsive for temporal RGC neurite outgrowth (Burden-Gulley et al., 2002), suggesting that PTPµ can act as a “stop” cue to migrating axons (Ensslen-Craig and Brady-Kalnay, 2004). Low levels of PTPµ are only permissive to ventral nasal RGC axons at E8, during peak axon extension (Burden-Gulley et al., 2002). Furthermore, PTPµ expression and catalytic activity is required for PTPµ-mediated neurite outgrowth and repulsion (Ensslen-Craig and Brady-Kalnay, 2005). PTPµ mediates axon guidance during development in vitro, however the functional role of PTPµ phosphorylation and association with other proteins in axon outgrowth in vivo is yet to be established.
1.6.4 PTPμ Signaling via Rho GTPases

PTPμ-mediated neurite outgrowth is also regulated by the Rho GTPases, Rac1 and Cdc42. Retinal explants cultured in the presence of dominant negative (DN) or constitutively active (CA) TAT-tagged Cdc42, Rac1 or RhoA results in distinct requirements for the Rho GTPases in PTPμ-mediated neurite outgrowth. At E8, PTPμ is repulsive to temporal RGCs but permissive to ventral nasal RGCs (Burden-Gulley et al., 2002). Rac1 inhibition is required for PTPμ-dependent repulsion of temporal RGC neurons (Major and Brady-Kalnay, 2007). Moreover, PTPμ-dependent nasal RGC neurite outgrowth and temporal RGC neuron repulsion is significantly reduced by the addition of Cdc42-DN (Major and Brady-Kalnay, 2007).

1.6.5 PTPμ Signaling via IQGAP1

PTPμ binds directly to IQGAP1 (Phillips-Mason et al., 2006). IQGAP1 was originally identified as RasGAP (Weissbach et al., 1994), despite the fact that IQGAP1 does not exhibit RasGAP activity nor bind to Ras. Instead, IQGAP functions to bind and stabilize activated Cdc42 and to a lesser extent Rac1 in their GTP-bound state (Hart et al., 1996; Swart-Mataraza et al., 2002; Zhang, 1997). IQGAP1 contains a number of protein-interacting domains such as a calponin homology domain, a WW domain, and four IQ motifs (Hart et al., 1996; Joyal et al., 1997; Weissbach et al., 1994), implying that IQGAP1 functions as a scaffolding protein. In fact, IQGAP1 recruits activated GTPase family members to cytoskeletal proteins (Brown, 2006) to regulate cadherin-mediated adhesion (Fukata et al., 1999; Izumi et al., 2004; Kuroda et al., 1998; Li et al., 1999). Several proteins of the cadherin/catenin complex associate with IQGAP1 such
as E-cadherin (Kuroda et al., 1998; Li et al., 1999), N-cadherin (Lui et al., 2005) and β-catenin (Briggs et al., 2002; Kuroda et al., 1998). Activated Cdc42 and to a lesser extent, Rac1 increase the association between PTPµ and IQGAP1 (Phillips-Mason et al., 2006). Furthermore IQGAP1 is required for PTPµ-mediated neurite outgrowth (Phillips-Mason et al., 2006). Therefore, PTPµ may regulate Cdc42 activity via IQGAP1 in PTPµ-mediated neurite outgrowth. A role for IQGAP1 in cadherin-mediated neurite outgrowth has not been demonstrated.

1.6.6 PTPµ Signaling via RACK1 and PKCδ

Another scaffolding protein, the Receptor for Activated C Kinase 1 (RACK1) binds to the D1 domain of PTPµ (Mourton et al., 2001). RACK1 is composed of seven WD repeats, each generating one blade of a propeller composed of four anti-parallel β-sheets proposed to mediate protein-protein interactions (Garcia-Higuera et al., 1996; Neer et al., 1994; Ron et al., 1994). RACK1 was originally identified as an intracellular receptor for activated protein kinase C (PKC) (Ron et al., 1994). This interaction mediates PKC translocation to the plasma membrane to facilitate substrate phosphorylation (Mochly-Rosen and Gordon, 1998; Schechtman and Mochly-Rosen, 2001). In theory each of the seven WD repeats can bind to seven different proteins. RACK1 binds to more than a dozen different proteins including, but not limited to phospholipase Cγ (PLCγ), the Src cytoplasmic protein tyrosine kinase (PTK), RasGAP, p120GAP and the p85 subunit of PI3 kinase (reviewed in Sklan, 2006). RACK1 also binds to PKC substrates containing pleckstrin homology (PH) domains such as β-spectrin, dynamin-1 (Rodriguez et al., 1999). The GEFs, Tiam1 and Trio also contain
PH domains (Chhatriwala et al., 2007; Habets et al., 1994), suggesting that they might associate with RACK1. However, direct binding of Trio or Tiam1 to RACK1 has not been demonstrated.

The protein kinase C family of lipid-dependent serine/threonine kinases are subdivided into three groups based on their cofactor requirement for activation and are known as classical, novel and atypical (Gould and Newton, 2008). The phosphatidylserine (PS) cofactor activates all PKCs. Novel and classical PKCs require PS and diacylglycerol (DAG), with a unique requirement for calcium binding by classical PKCs. Phosphorylation by phosphoinositide dependent kinase (PDK-1) and autophosphorylation of serine/threonine residues is also required for PKC activation (Gould and Newton, 2008).

PKCδ has been implicated in the regulation of E-cadherin-mediated adhesion and formation of adherens junctions (Hellberg et al., 2002; Lewis et al., 1994; Skoudy, 1995). Our lab demonstrated that inhibition of PKCδ activity is required for the restoration of E-cadherin-mediated adhesion in human prostate carcinoma cells (LNCaP) (Hellberg et al., 2002). LNCaP cells lack endogenous PTPµ and are unable to adhere to E-cadherin (Hellberg et al., 2002), despite normal expression levels of the proteins in the cadherin/catenin complex (Hellberg et al., 2002). Re-expression of wild-type PTPµ also restores E-cadherin-mediated cell adhesion (Hellberg et al., 2002), suggesting that the PTPµ/PKC pathway regulates cadherin-mediated cell-cell adhesion. PKCδ is also required for PTPµ-mediated neurite outgrowth (Rosdahl et al., 2002). PTPµ is found in a complex with RACK1 (Mourton et al., 2001) and PKCδ in cultured RNEs and in E8 retinal tissue (Rosdahl et al., 2002). Lastly, PTPµ-dependent nasal
neurite outgrowth and temporal RGC axon repulsion requires PKCδ activity (Ensslen and Brady-Kalnay, 2004; Rosdahl et al., 2002). The adhesive and cell-signaling properties of PTPμ and its associated proteins (Fig. 1.8) are influential in axon guidance and cadherin-mediated events during nervous system development. Experimental analysis demonstrating a role for the PTPμ associated proteins IQGAP1 and PKCδ are described in Chapter 3.

1.7 Summary

E-cadherin, N-cadherin and R-cadherin-dependent cell-cell contact and cell adhesion are required for development of the visual system. Distinct expression patterns of E-cadherin, N-cadherin and R-cadherin throughout the retina and optic tectum point to specific roles for these cadherins in axon growth and guidance. PTPμ has emerged as a regulatory guidance molecule in axon outgrowth in the visual system. This dissertation addresses the role of PTPμ and its associated proteins in E-cadherin, N-cadherin and R-cadherin-mediated RGC neurite outgrowth in vitro. Additionally, the role of the PTPμ signaling partners Rac1, Cdc42, IQGAP1 and PKCδ in E-cadherin, N-cadherin and R-cadherin-mediated RGC neurite outgrowth in vitro is examined. The following two chapters describe in detail the growth promoting capabilities of homophilic E-cadherin, N-cadherin and heterophilic R/N-cadherin-mediated neurite outgrowth of chick RGCs. These studies demonstrate a role for PTPμ in E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth. Furthermore, distinct regulatory proteins are identified for each classical cadherin in neurite outgrowth. Identification and characterization of PTPμ substrates are discussed in future directions.
Figure 1.8 Potential PTPµ Signaling Pathways. The cytoplasmic segment of PTPµ binds to E-cadherin, IQGAP1 and RACK1. PTPµ is required for N-cadherin-mediated neurite outgrowth and associates with the cadherin/catenin complex, potentially regulating N-cadherin directly or indirectly through cadherin associated proteins. PTPµ-mediated neurite outgrowth requires IQGAP1, Rac1 and Cdc42. IQGAP1 binds Rac1 and Cdc42 and may recruit Rac1 and/or Cdc42 to the membrane to interact with PTPµ. Regulation of PKCδ is also required for PTPµ-dependent nasal RGC neurite outgrowth and temporal RGC repulsion. Since RACK1 binds PTPµ and PKCδ, it is proposed that RACK1 links PKCδ to PTPµ-mediated neurite outgrowth.
CHAPTER 2

E-cadherin Promotes Retinal Ganglion Cell Neurite Outgrowth in a Protein Tyrosine Phosphatase-mu Dependent Manner*

* This chapter represents results reported in (Oblander et al., 2007).
2.1 Abstract

During development of the visual system, retinal ganglion cells (RGCs) require cell-cell adhesion molecules and extracellular matrix proteins for axon growth. In this study, we demonstrate that the classical cadherin, E-cadherin, is expressed in RGCs from E6 to E12 and promotes neurite outgrowth from all regions of the chick retina at E6, E8 and E10. E-cadherin is also expressed in the optic tectum. E-cadherin adhesion blocking antibodies specifically inhibit neurite outgrowth on an E-cadherin substrate. The receptor-type protein tyrosine phosphatase, PTPμ, associates with E-cadherin. In this manuscript, we demonstrate that antisense-mediated down regulation of PTPμ, overexpression of catalytically inactive PTPμ, and perturbation of endogenous PTPμ using a specific PTPμ inhibitor peptide results in a substantial reduction in neurite outgrowth on E-cadherin. Taken together these findings demonstrate that E-cadherin is an important adhesion molecule for chick RGC neurite outgrowth and suggest that PTPμ expression and catalytic activity are required for outgrowth on an E-cadherin substrate.
2.2 Introduction

The chick visual system serves as a well-established model to investigate the molecular mechanisms involved in axon growth and guidance. Retinal ganglion cells (RGCs) are the first cells to differentiate within the retina at embryonic (E) day 4 (Mey and Thanos, 2000; Thanos and Mey, 2001). Development within the retina proceeds in a central-to-peripheral gradient, with cells in the temporal region of the retina being the most differentiated. RGCs first extend an axon toward the optic fissure, and then travel out of the eye along the optic nerve to the chiasm where they cross and continue on the retinofugal pathway to their target, the optic tectum. Retinal axons reach the anterior portion of the tectum by E6 and extend along the tectal surface to form the stratum opticum (SO). Temporal axons innervate the anterior surface while nasal axons extend to the posterior tectum at E10. RGCs extend axons toward the optic tectum in response to various molecular cues on the surface of other cells or in the extracellular environment (Mey and Thanos, 2000).

Cell adhesion molecules are important for the formation of the visual system (Hirano et al., 2003; Kiryushko, 2004; Thiery, 2003a). Classical cadherins are cell surface integral membrane glycoproteins that mediate cell-cell adhesion, cell migration and cell sorting via calcium-dependent, homophilic interactions (Gumbiner, 2005). Cadherins are tethered to the actin cytoskeleton by their association with the catenins, \(\alpha\)-catenin, \(\beta\)-catenin, plakoglobin and p120 (Lilien and Balsamo, 2005). N-cadherin is predominantly expressed in the developing nervous system and mediates axon guidance and synapse formation (Kiryushko, 2004; Redies, 2000; Takeichi, 2005). Previous studies have demonstrated that N-cadherin promotes neurite outgrowth in vitro and in
vivo (Bixby and Zhang, 1990; Riehl et al., 1996). Within the chick retina, N-cadherin has been shown to be regulated by tyrosine phosphorylation (Lilien and Balsamo, 2005; Lilien et al., 2002).

Receptor protein tyrosine phosphatases (RPTPs) are expressed in the developing chick visual system and a subset of RPTPs has been suggested to play a role in retinotectal pathfinding (Brady-Kalnay, 2001; Ensslen-Craig and Brady-Kalnay, 2004; Johnson and Van Vactor, 2003). RPTPμ (PTPµ) is comprised of CAM-like extracellular domains that mediate cell-cell adhesion and associates with E-, N-, R- and VE-cadherin and the catenins, α-catenin, β-catenin and p120 (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995; Hiscox and Jiang, 1998; Hiscox and Jiang, 1999; Sui et al., 2005; Zondag et al., 2000).

Another classical cadherin, E-cadherin is expressed by mouse RGCs (Faulkner-Jones et al., 1999; Xu, 2002). However, a role for E-cadherin in neurite outgrowth has not been examined. In this study, we used a retinal explant model system to demonstrate that E-cadherin promotes neurite outgrowth of RGCs when used as a culture substrate in vitro. E-cadherin is expressed in the chick retina from E6 to E12 and promotes neurite outgrowth from all regions of the retina. Neurite outgrowth is specific to E-cadherin since outgrowth on an E-cadherin substrate is inhibited by addition of E-cadherin adhesion blocking antibodies. We have shown previously that PTPµ is present in a complex with E-cadherin in other systems (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995). In order to determine the physiological significance of an association between PTPµ and E-cadherin in neurite outgrowth, the expression level of PTPµ was perturbed in retinal explants. The phosphatase activity of PTPµ was
also perturbed in retinal explants. Down-regulation of PTPµ expression through antisense techniques and overexpression of catalytically inactive PTPµ resulted in a substantial reduction in neurite outgrowth on an E-cadherin substrate. In addition, perturbation of endogenous PTPµ in retinal explants using a specific PTPµ inhibitor peptide also resulted in a decrease in both N-cadherin and E-cadherin-mediated neurite outgrowth. These findings indicate that PTPµ expression and catalytic activity are required for neurite outgrowth by RGCs on an E-cadherin substrate.
2.3 Materials and Methods

2.3.1 Immunoblot Analysis

Tissue lysates were prepared by dissecting nasal retina from temporal retina at various developmental stages in ice-cold calcium-magnesium-free Hank’s buffered saline (CMF) and transferred to cold lysis buffer (20 mM Tris pH 7.6, 1% Triton X-100, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.1 mM ammonium molybdate, 0.2 mM phenyl arsine oxide, 0.3% protease inhibitor cocktail (P8340; Sigma). The tissue was lysed by vigorous trituration and incubated on ice for 20 minutes. The triton insoluble material was removed by centrifugation (5,000 rpm for 5 minutes in an Eppendorf Microcentrifuge), and the protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976). Equal amounts of protein were loaded per lane and separated by SDS-PAGE (6% gels). Proteins were transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH) and immunoblotted as described previously using an antibody generated against PTPµ (SK18 or SK15) (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994), E-cadherin (610182; BD Biosciences, San Diego, CA) or N-cadherin (610920; BD Biosciences). To verify equal protein load per lane, the immunoblots were stripped and reprobed (Reblot Plus; Chemicon International, Temecula, CA) with a monoclonal antibody generated against vinculin (V9131; Sigma, St. Louis, MO). All immunoblot data were acquired on a Bio-Rad Fluor-S Max MultiImager system (Bio-Rad, Hercules, CA), using the Quantity One (Bio-Rad) image processing software. For quantitation, bands were normalized to vinculin.
2.3.2 Immunohistochemistry

Retina and brain were dissected out in ice-cold CMF. Tissue was fixed in 3.7% formaldehyde for 30-45 minutes at room temperature followed by a PBS rinse. Tissue was taken through alcohol dehydration and then embedded in paraffin wax. Coronal sections were taken of the retina in that the blade cut across the eye, parallel to the optic fissure. Sections were cut on a microtome at 12 µm intervals. Next, sections were dried for 1 hour, cleared with xylene and taken through alcohol rehydration. After rinsing in PBS, sections were heated at 37°C in 10 mM sodium citrate, pH 6, 3 times for 6 minutes each to unmask antigenic sites. Sections were allowed to cool for 20 minutes before incubating in 3% H₂O₂ for 20 minutes to block endogenous peroxidase activity. Sections were blocked with 1.5% horse serum/PBS. In order to block endogenous avidin/biotin activity, sections were incubated with avidin D followed by biotin (Avidin/Biotin Blocking Kit; Vector Laboratories, Burlingame, CA). Sections were then incubated in monoclonal anti-E-cadherin antibody (BD Biosciences) in blocking buffer overnight at 4°C. After rinsing in PBS, sections were incubated in biotinylated secondary antibody (Vectastain Elite avidin-biotin complex (ABC) kit; Vector Laboratories) in blocking buffer for 25 minutes at room temperature. Sections were rinsed and then incubated in ABC reagent in PBS for 45 minutes at room temperature. After PBS rinses, sections were incubated with diaminobenzidine (DAB) solution (Vector Laboratories) for 5-10 minutes and then rinsed with PBS. DAB produces a brown precipitate, making protein expression in the retinal pigmented epithelium (RPE) indistinguishable from the brown melanin found in the RPE. Sections were dehydrated through a graded ethanol series and then coverslipped using Permount mounting.
medium (Fisher Scientific, Hampton, NH). All images were collected using an RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI) mounted on an Olympus BX 60 Upright Microscope (Tokyo, Japan).

2.3.3 Neurite Outgrowth Assays

Human E-cadherin-Fc and N-cadherin-Fc were obtained from R&D Systems (Minneapolis, MN). Laminin was obtained from Sigma. Briefly, 35 mm tissue culture dishes were coated with nitrocellulose in methanol (Lagenaur and Lemmon, 1987) and allowed to dry. Several different lots of substrate were used over the course of the experiments resulting in variability in the concentration of substrate used. 0.25-0.50 µg of E-cadherin-Fc, 0.06-0.15 µg of N-cadherin-Fc or 2.50-4.00 µg of laminin was spread across the center of each dish and incubated for 20 minutes at room temperature. Remaining binding sites on the nitrocellulose were blocked with 2% BSA in CMF, and the dishes were rinsed with RPMI 1640 medium (Hyclone, Logan, UT).

Embryonic day 8 (stage 32-33 according to Hamburger and Hamilton, 1951) chick eyes were dissected in cold CMF and the retinal explants were prepared as described (Burden-Gulley and Brady-Kalnay, 1999; Drazba, 1990; Halfter et al., 1983). Briefly, neural retinas were flattened on concavalin-coated nitrocellulose filters and cut into 350µm-wide explants. Explants were placed retinal ganglion side down onto substrate coated dishes and cultured in RPMI-1640, 10% fetal bovine serum (Hyclone), 2% chick serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.025 µg/ml amphotericin (Sigma).
For growth cone visualization, Lab-TekII Chamber Slides (Fisher Scientific) were coated with 0.01% poly-L-lysine overnight, rinsed 5 x with distilled H₂O and allowed to dry overnight. The slides were then coated with E-cadherin, N-cadherin or laminin substrate as described above. Retinas were prepared as described above. Before placing the explant retinal ganglion side down onto the substrate coated slide, Dil crystals (Invitrogen) were placed on the tissue. Culture medium containing serum was then added and explants were incubated for 20 hours.

For antibody inhibition studies, N-cadherin blocking antibody, NCD2 (Hatta and Takeichi, 1986) at a final concentration of 11 µg/ml, or E-Cadherin blocking antibody, goat anti-L-CAM (chick E-cadherin) (Renaud-Young, 2002) at a final concentration of 1 mg/ml. The goat anti-L-CAM (chick E-cadherin) antibody was a kind gift from Drs. Bruce Cunningham and Warren Gallin. The antibodies were added to the culture media in each substrate-coated culture dish and incubated at room temperature for 30 minutes prior to addition of the explant. Explants were incubated for 20 hours in the presence of the blocking antibody.

For viral perturbation studies, 7.5 µl of replication-defective herpes simplex virus (HSV) encoding green fluorescent protein (IRES-GFP), wildtype PTPµ (WT), antisense PTPµ (AS) or catalytically inactive PTPµ (C-S), as previously described (Ensslen et al., 2003), in RPMI-1640 alone was added at the time of explanting. The virus was allowed to incubate at 37°C for 2 hours. Culture media containing serum was then added. All explants were incubated at 37°C for 20 hours, fixed in 4% paraformaldehyde, 0.1% glutaraldehyde and imaged.
For PTPµ inhibitor peptide studies, a PTPµ wedge peptide (WPTPµ-Tat) or scrambled control (SPTPµ-Tat), was added as previously described (Xie et al., 2006). A final concentration of 5.5 µM peptide was added at the time of explanting. Both peptides include a membrane-penetrant Tat-derived sequence at the C terminus, which promotes cellular uptake of the peptide (Wadia and Dowdy, 2002). All explants were incubated at 37°C for 20 hours, fixed in 4% paraformaldehyde, 0.1% glutaraldehyde and imaged.

2.3.4 Retinal Neuroepithelial Cell Infection

Embryonic day 6 RNE cultures were prepared as previously described (Burden-Gulley and Brady-Kalnay, 1999). Briefly, E6 chick retinas were dissected in cold CMF and dissociated in 0.25% Trypsin, 4 Na EDTA (Invitrogen) for 20 minutes at 37°C shaking, followed by vigorous trituration. Cells were resuspended, plated at a concentration of 5 x 10^5 and allowed to attach overnight at 37°C in RPMI-1640, 10% fetal bovine serum, 2% chick serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.025 µg/ml amphotericin. RNE cells were then infected with 2µl PTPµ AS HSV or IRES-GFP HSV for 2 hours in RPMI-1640 alone, followed by 18 hours of incubation in RPMI-1640, 10% fetal bovine serum, 2% chick serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.025 µg/ml amphotericin in the presence of HSV.

2.3.5 Quantitation of Neurite Outgrowth

Neurite outgrowth from specific regions of the retina was analyzed using a SPOT RT digital camera and image acquisition software (Diagnostic Instruments, Inc.,
Sterling Heights, MI). In short, the length of the five longest neurites per given area of the explant were measured perpendicular to the explant tissue. To calculate neurite density, images were analyzed using Metamorph software version 6.3r4 (Universal Imaging, Downington, PA). The data from all similar experiments were combined, analyzed by Student’s $t$ test and graphed (Microsoft Excel, 10.0.0 2001).
2.4 Results

2.4.1 Expression of E-cadherin in the Visual System

Molecules that regulate axon outgrowth can be expressed in a gradient within the chick visual system. Since RGCs from nasal versus temporal regions of the retina extend axons to distinct locations in the tectum, we examined nasal versus temporal E-cadherin expression at several developmental time points corresponding to peak RGC axon growth in the retina and tectum (Mey and Thanos, 2000). Lysates were made, separated by SDS-PAGE and immunoblotted for E-cadherin (Fig. 2.1). E-cadherin is expressed during development from E6 to E12, the earliest and latest time-points examined (Fig. 2.1), and is expressed in the nasal and temporal regions of the retina. N-cadherin is expressed in the retina from E8 to E10 as tested by immunoblot analysis (Burden-Gulley and Brady-Kalnay, 1999; Lagunowich and Grunwald, 1989; Matsunaga et al., 1988). PTPμ is also expressed in the retina (Burden-Gulley and Brady-Kalnay, 1999; Burden-Gulley et al., 2002). Full length PTPμ migrates at ~200 kDa whereas the proteolytically processed form of PTPμ that contains the cytoplasmic domain migrates at 100 kDa (Brady-Kalnay and Tonks, 1994). In retinal lysates, an additional 95 kDa immunoreactive band is also present (Burden-Gulley and Brady-Kalnay, 1999; Burden-Gulley et al., 2002). Full length PTPμ increases in size, possibly due to glycosylation or alternative splicing. To ensure equal protein loading immunoblots were stripped and reprobed with antibodies to vinculin (Fig. 2.1).

To further characterize the expression of E-cadherin in the developing retina, E8 retinas (stage 32) were sectioned and immunohistochemically labeled with an anti-E-cadherin antibody (Fig. 2.2 A). E8 retinas were used since this time point in
Figure 2.1. Immunoblot of E-cadherin, N-cadherin and PTPµ in the Developing Chick Retina. Lysates from nasal or temporal retina were prepared from E6, E8, E10 and E12 chicks, separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with an antibody to E-cadherin, N-cadherin or PTPµ (SK18). E-cadherin protein migrates at ~120 kDa, while N-cadherin migrates at ~130 kDa. Full length PTPµ is ~200 kDa whereas the proteolyticaly processed form of PTPµ containing the cytoplasmic domain migrates at ~100 kDa (Brady-Kalnay and Tonks, 1994). A 95 kDa immunoreactive band is also present. Each immunoblot was stripped and reprobed with antibodies against vinculin to verify equal protein load.
Figure 2.2. Expression of E-cadherin in Chick Retina and Optic Tectum at E8.

Coronal sections of E8 chick retina (A-F) and sagittal sections of tectum (G, H) were immunohistochemically labeled with antibodies against E-cadherin (A, B, G). The nuclei of each serial section were stained with Hematoxylin (C, D, H). A no primary antibody control is shown (E, F) as an indicator of background staining. E-cadherin expression is present in the retinal ganglion cells (A, arrowheads). In the optic tectum, E-cadherin is also expressed (G). RGC, retinal ganglion cell layer; RPE, retinal pigmented epithelium; P, posterior; D, dorsal; A, anterior; V, ventral; SGFS, stratum grisium et fibrosum; SO, stratum opticum; NE, neuroepithelium. Scale bar (A) 200 μm, (F) 50 μm, (H) 500 μm.
development coincides with peak RGC axon extension (Thanos and Mey, 2001). Coronal sections of the retina were taken in order to view both the dorsal and ventral region of the retina. E-cadherin is expressed in the retinal ganglion cells and optic fiber layer (Fig. 2.2 A, B). Serial sections of retina were stained with Hematoxylin to indicate the nuclear location of the RGC cell bodies (Fig. 2.2 C, D), or incubated in the absence of primary antibody (Fig. 2.2 E, F) as a control.

We then examined the expression of E-cadherin in the optic tectum. By E8, RGC axons have migrated out of the retina, across the optic chiasm and are innervating the anterior region of the tectum (Thanos and Mey, 2001). Retinal axons extend along the tectal surface to form the stratum opticum (SO). Temporal axons innervate the anterior surface while nasal axons extend to the posterior tectum at E10. E-cadherin is expressed in E8 optic tectum in the stratum opticum (SO), the outermost layer of the tectum, and the stratum griseum et fibrosum superficiale (SGFS), where RGC axons innervate (Fig. 2.2 G). E-cadherin was also expressed in the neuroepithelium of the tectum (Fig. 2.2 G). At E8, undifferentiated neuroepithelium is most prominent in the anterior portion of the tectum and gives rise to differentiating cells which migrate to the pial surface (LaVail and Cowan, 1971).

2.4.2 E-cadherin Promotes Neurite Outgrowth

Early in embryogenesis, one or two leading RGC axons migrate along the optic stalk toward the optic tectum (Thanos and Mey, 2001). As development continues, successive waves of axons project along the neuronal and glial cells within the optic nerve (Thanos and Mey, 2001). Thus, cadherins expressed on the surface of these cells
can serve as a “substrate” for axonal migration. To determine whether E-cadherin promotes neurite outgrowth, we used a well-established in vitro model to investigate neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999; Lagenaur and Lemmon, 1987). Purified recombinant E-cadherin-Fc chimera was coated on tissue culture dishes and used as a substrate to culture chick retinal explants. Neurite outgrowth on an E-cadherin substrate was observed from retinal explants taken at E6, E8 and E10, after 20 hours in culture (Fig. 2.3 A, B, C). Neurite length and density was similar between all time points examined, suggesting that E-cadherin is equally effective at promoting neurite outgrowth at these ages. Neurite outgrowth on E-cadherin was similar in length and density to that observed on N-cadherin (Fig. 2.6 D, G).

Growth cones located at the distal tip of the axon allow neurons to interact with the extracellular environment. Each growth cone recognizes cues in the extracellular environment and on the surface of adjacent cells via membrane-associated proteins such as the cadherins (Hirano et al., 2003; Kiryushko, 2004). These interactions lead to intracellular signaling events, which induce cytoskeletal rearrangements that ultimately regulate axon guidance. Dil labeling of RGCs illustrates that the morphology of the growth cones present on an E-cadherin substrate consists of large, broad lamellipodia with a few short filopodia (Fig. 2.4 C). In contrast, growth cones on N-cadherin had smaller lamellipodia with several short filopodial processes (Fig. 2.4 B), which is consistent with previous published work (Bixby and Zhang, 1990; Payne et al., 1992). Growth cones with small lamellipodia were observed on laminin (Fig. 2.4 A). The differences in growth cone morphology observed on each cadherin substrate suggest
Figure 2.3. E-cadherin Promotes RGC Neurite Outgrowth at Various Stages of Development. E6 (A), E8 (B) and E10 (C) chick retinal explants were isolated and cultured on an E-cadherin substrate for 20 hours. Scale bar, 200 μm.
**Figure 2.4. Growth Cone Morphology.** Dil labeling demonstrates that growth cones on a laminin substrate (A) appear to have small lamellipodia with few, short filopodia. Growth cones on an N-cadherin substrate (B) have larger lamellipodia in addition to short filopodial processes. On an E-cadherin substrate (C), growth cones have very large, broad lamellipodia with short filopodial processes. Scale bar, 10 µm.
that distinct signaling mechanisms may be involved in E-cadherin versus N-cadherin-dependent neurite outgrowth.

The 3-dimensional position of the RGC cell body within the retina determines which positional cues the RGC cell body and therefore its axon will respond to. Previous studies have shown that at E8, N-cadherin-mediated neurite outgrowth predominantly occurs from RGCs originating from the ventral-nasal, ventral-temporal and dorsal-temporal retina while little to no growth occurs from RGCs from the dorsal-nasal region (Burden-Gulley et al., 2002). In order to identify which regions of the retina promote neurite outgrowth on an E-cadherin substrate, explants from distinct regions of the retina were isolated and cultured in vitro. In contrast to N-cadherin, robust neurite outgrowth on E-cadherin was observed from all regions of the retina (Fig. 2.5 B). Laminin, which has been shown to promote robust neurite outgrowth from all regions of the retina (Burden-Gulley et al., 2002), was used as a control (Fig. 2.5 A).

Classical cadherins are predominantly homophilic binding proteins (Gooding, 2004; Ivanov et al., 2001). To confirm that neurite outgrowth on E- or N-cadherin substrates is specific, E8 retinal explants were cultured on an E-cadherin, N-cadherin or laminin substrate in the presence or absence of adhesion-blocking antibodies. Neurite outgrowth on an E-cadherin substrate was blocked when cultured in the presence of antibodies against the extracellular domain of chick E-cadherin (Fig. 2.6 I). These E-cadherin blocking antibodies had no effect on N-cadherin-mediated outgrowth (Fig. 2.6 F). Antibodies against the extracellular domain of chick N-cadherin (Hatta and Takeichi, 1986) had no effect on E-cadherin-mediated neurite outgrowth (Fig. 2.6 H). However, N-cadherin adhesion blocking antibodies did block neurite outgrowth on an
Figure 2.5. Neurite Outgrowth on E-cadherin and Laminin is Independent of RGC Cell Body Origin. Explants from E8 chick retina were cut parallel to the optic fissure and explants from retina were cultured on E-cadherin (B) or laminin (A) substrates. Images were acquired after 20 hours in culture from a location corresponding to the outer third of each explant. Each number indicates the explant number (e.g. 1 and 6 are most peripheral). Dorsal (D), ventral (V), nasal (N), temporal (T). Scale bar, 200 µm.
Figure 2.6. E-cadherin-Mediated Neurite Outgrowth is Specifically Blocked by E-cadherin Adhesion Blocking Antibodies. Retinal explants from E8 chick embryos were cultured on a laminin (A, B, C), N-cadherin (D, E, F) or E-cadherin (G, H, I) substrate in the presence of adhesion blocking antibodies to N-cadherin (B, E, H) or E-cadherin (C, F, I). Antibodies against N-cadherin inhibited neurite outgrowth on an N-cadherin (E) substrate, whereas they had no effect on neurite outgrowth on laminin (B) or E-cadherin (H) substrates. Similarly, antibodies against E-cadherin inhibited neurite outgrowth on an E-cadherin (I) substrate, while they had no effect on neurite outgrowth on laminin (C) or N-cadherin (F) substrates. Scale bar, 200 μm.
N-cadherin substrate (Fig. 2.6 E). Neurite outgrowth on laminin was unaffected by E- and N-cadherin adhesion blocking antibodies (Fig. 2.6 B, C). Taken together, these data suggest that neurite outgrowth on an E-cadherin substrate is due to specific E-cadherin binding.

2.4.3 PTPµ Expression and Catalytic Activity are Required for E-cadherin-mediated Neurite Outgrowth

Previously our laboratory has demonstrated that PTPµ is expressed in the retina (Fig. 2.1), interacts with N-cadherin, and is required for N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999). We have also shown that PTPµ interacts directly with E-cadherin in other cell types (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995). We therefore hypothesized that PTPµ expression might be required for E-cadherin-mediated neurite outgrowth. In order to test this, we infected E8 retinal explants with herpes simplex virus (HSV) encoding antisense PTPµ (AS) (Ensslen et al., 2003). Previous studies have demonstrated that infection of cultured retinal neuroepithelial cells (RNE) with PTPµ AS HSV reduces expression of full length PTPµ by 60% (Ensslen et al., 2003). We confirm that PTPµ AS HSV decreased full length PTPµ by 64%, cleaved PTPµ (100 kDa) decreased by 59% and the 95 kDa band decreased by 51% when normalized to vinculin. In addition, infection of RNE with PTPµ AS HSV had no significant effect on E- or N-cadherin expression (Fig. 2.7). Retinal explants infected with PTPµ AS HSV were cultured on either an E-cadherin, N-cadherin or laminin substrate. Neurite outgrowth was observed after 20 hours of incubation in the presence of the virus (Fig. 2.8). Neurite length decreased by 63% and
Figure 2.7. PTPµ Antisense HSV Infection Does Not Alter Cadherin Expression.

E6 retinal neuroepithelial cells were infected with IRES-GFP HSV or PTPµ AS HSV for 20 hours. Lysates from infected RNE cells were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an antibody to E-cadherin, N-cadherin or PTPµ (SK15). Full-length (200 kDa) PTPµ expression decreased by 64%, cleaved PTPµ (100 kDa) decreased by 59% and the 95 kDa band decreased by 51% in the presence of PTPµ AS HSV, while there was no significant effect on E- or N-cadherin expression as measured by densitometry. Each immunoblot was stripped, reprobed with antibodies against vinculin to verify equal protein load, normalized to vinculin and quantitated by densitometry.
density decreased by 77% when retinal explants were cultured on an E-cadherin substrate in the presence of PTP\(\mu\) AS HSV (Fig. 2.8 K, Fig. 2.9). Similar to previously reported data using PTP\(\mu\) antisense retrovirus (Burden-Gulley and Brady-Kalnay, 1999), neurite length and density of retinal explants grown on an N-cadherin substrate in the presence of PTP\(\mu\) AS HSV decreased by 51% and 76% respectively (Fig. 2.8 G, Fig. 2.9). PTP\(\mu\) AS HSV had no effect on neurite outgrowth of retinal explants grown on a laminin substrate (Fig. 2.8 C, Fig. 2.9), indicating that the amount of virus used is not toxic and does not exhibit nonspecific effects on neurite outgrowth. These results suggest that PTP\(\mu\) expression is required for E-cadherin to mediate neurite outgrowth.

In addition to PTP\(\mu\) expression, PTP\(\mu\) tyrosine phosphatase activity is required for N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999). Since infection with HSV encoding PTP\(\mu\) AS does not tell us whether PTP\(\mu\) adhesion or phosphatase activity is regulating E-cadherin-mediated neurite outgrowth we investigated the requirement for PTP\(\mu\) catalytic activity in E-cadherin-mediated neurite outgrowth. In order to test this, E8 retinal explants were infected with HSV encoding either wild-type PTP\(\mu\) (WT) or full length catalytically inactive PTP\(\mu\) (C-S) (Ensslen et al., 2003) and cultured on either an E-cadherin, N-cadherin or laminin substrate (Fig. 2.8). Overexpression of PTP\(\mu\) WT also had no effect on laminin (Fig. 2.8 B) and N-cadherin-mediated neurite outgrowth (Fig. 2.8 F) as previously published (Burden-Gulley and Brady-Kalnay, 1999). We also demonstrate that PTP\(\mu\) WT had no effect on E-cadherin-mediated neurite outgrowth (Fig. 2.8 J, Fig. 2.9). After 20 hours of incubation in the presence of PTP\(\mu\) C-S HSV, neurite length decreased by 49% and density decreased by 79% when cultured on an E-cadherin substrate (Fig. 2.8 L,
**Figure 2.8. PTPμ Expression and Catalytic Activity are Required for Neurite Outgrowth on N-cadherin and E-cadherin.** E8 chick retinal explants were infected with HSV encoding GFP control (A, E, I), PTPμ WT (B, F, J), PTPμ AS (C, G, K) or PTPμ C-S (D, H, L) and cultured on laminin (A, B, C, D), N-cadherin (E, F, G, H) or E-cadherin (I, J, K, L) substrates for 20 hours. No difference in neurite length or density was observed in cultures infected with GFP, PTPμ WT, PTPμ AS, or PTPμ C-S when cultured on laminin (A, B, C, D). Overexpression of PTPμ WT also had no effect on laminin (B), N-cadherin (F) or E-cadherin (J) substrates. Infection with PTPμ AS or PTPμ C-S resulted in a dramatic decrease in neurite outgrowth when cultured on N-cadherin (G, H) or E-cadherin (K, L) substrates. Scale bar, 200 μm.
Figure 2.9. Quantitation of PTPμ Perturbation on Neurite Outgrowth. Infection of E8 chick retinal explants with HSV encoding GFP control (white bars) or PTPμ WT (gray bars), had no effect on laminin, N-cadherin or E-cadherin-dependent neurite outgrowth. PTPμ AS HSV (black bars) infection decreased neurite length (A) by 51% on N-cadherin and by 63% on E-cadherin substrates while neurite density (B) decreased by 76% on N-cadherin and by 77% on E-cadherin. PTPμ C-S HSV (hatched bars) infection also decreased neurite length (A) by 52% on N-cadherin and by 49% on E-cadherin substrates, while neurite density (B) decreased by 70% on N-cadherin and by 79% on E-cadherin. PTPμ AS and PTPμ C-S HSV infection had no effect on laminin-dependent neurite outgrowth. Asterisk denotes statistically significant changes in neurite length or density compared to control. n = 5 for laminin, n = 5 for N-cadherin, n = 6 for E-cadherin.
Fig. 2.9). Neurite length and density of retinal explants grown on an N-cadherin substrate in the presence of PTPµ C-S HSV decreased by 52% and 70% respectively (Fig. 2.8 H, Fig. 2.9). PTPµ C-S HSV had no effect on neurite outgrowth of retinal explants grown on a laminin substrate (Fig. 2.8 D, Fig. 2.9). Taken together, we demonstrate that PTPµ catalytic activity is also required for E-cadherin-mediated neurite outgrowth.

To test whether endogenous PTPµ function is required for E-cadherin and N-cadherin-mediated neurite outgrowth, a PTPµ specific peptide inhibitor was used. The peptide resembles the HLH wedge-shaped sequence (Hoffmann et al., 1997), located in the juxtamembrane domain near the D1 PTPµ catalytic domain (Xie et al., 2006). The peptide utilized mimics inter/intramolecular interactions and is proposed to regulate catalytic activity of the phosphatase (for reviews see Bixby, 2001; Brady-Kalnay, 2001; Ensslen-Craig and Brady-Kalnay, 2004). The PTPµ wedge peptide (WPTPµ-Tat) binds to itself in a bead binding assay but not to the wedge peptide LAR (WLAR-Tat), another member of the type II RPTP subfamily (Xie et al., 2006), demonstrating that the WPTPµ-Tat is specific and does not interact with other RPTP family members. In addition, WPTPµ-Tat but not WLAR-Tat was shown to perturb PTPµ-mediated neurite outgrowth (Xie et al., 2006), which requires PTPµ catalytic activity (Ensslen-Craig and Brady-Kalnay, 2005).

E8 retinal explants were cultured in the presence of the PTPµ wedge peptide (WPTPµ-Tat), which includes a Tat-derived domain linked to the C terminus for uptake of the peptide into the cell, or scrambled control (SPTPµ-Tat) and cultured on either an E-cadherin, N-cadherin or laminin substrate for 20 hours (Fig. 2.10). Incubation with
WPTPμ-Tat had no effect on laminin-dependent neurite outgrowth (Fig. 2.10 B) when compared to SPTPμ-Tat control (Fig. 2.10 A). Neurite length (Fig. 2.10 G) decreased by 46% on N-cadherin and by 80% on E-cadherin substrates, while neurite density (Fig. 2.10 H) decreased by 84% on N-cadherin and by 90% on E-cadherin in the presence of WPTPμ-Tat when compared to SPTPμ-Tat control. Perturbation of E-cadherin and N-cadherin-mediated neurite outgrowth using the PTPμ specific wedge peptide inhibitor, confirms the importance of PTPμ catalytic activity in cadherin-dependent neurite outgrowth.
Figure 2.10. PTPμ-specific Inhibitor Peptide Blocks Neurite Outgrowth on E-cadherin and N-cadherin. E8 chick retinal explants were cultured on laminin (A, B), N-cadherin (C, D), or E-cadherin (E, F) substrates for 20 hours in the presence of either scrambled control (SPTPμ-Tat) (A, C, E) or the PTPμ wedge peptide (WPTPμ-Tat) (B, D, F) at a final concentration of 5.5 μM. Cultures incubated with WPTPμ-Tat had no effect on laminin-dependent neurite outgrowth (B) when compared to SPTPμ-Tat control (A). Neurite length (G) decreased by 46% on N-cadherin and by 80% on E-cadherin substrates, while neurite density (H) decreased by 84% on N-cadherin and by 90% on E-cadherin in the presence of WPTPμ-Tat (hatched bars) when compared to SPTPμ-Tat control (white bars). Asterisk denotes statistically significant changes in neurite length or density compared to control. n = 4 for laminin, n = 5 for N-cadherin n = 6 for E-cadherin. Scale bar, 200 μm.
2.5 Discussion

Although many cell adhesion molecules are expressed within the nervous system, only a subset of these molecules have been shown to be permissive to axon outgrowth \textit{in vivo}. In order to address the functional role of CAMs in axon extension, an \textit{in vitro} RGC neurite outgrowth assay using various CAMs as substrates is utilized. Integrins and their ligands the extracellular matrix (ECM) molecules, the immunoglobulin superfamily of cell adhesion molecules (CAMs) and cadherins comprise the three primary classes of proteins known to mediate neurite outgrowth (Kiryushko, 2004). Integrin receptors are present on the surface of RGCs and signal to the cell to extend neurites onto certain ECM molecules including fibronectin and laminin (Kiryushko, 2004). L1, an Ig superfamily CAM, is known to promote neurite outgrowth from RGCs (Burden-Gulley, 1995; Kamiguchi, 2003; Skaper, 2005). Within the cadherin superfamily only two classical cadherins, N-cadherin and R-cadherin have been shown to promote neurite outgrowth (Bixby and Zhang, 1990; Redies and Takeichi, 1993b). Previous studies have shown that E-cadherin is expressed in mouse RGCs (Faulkner-Jones et al., 1999; Xu, 2002). However, the role of E-cadherin in neurite outgrowth is unknown. In this study we show that E-cadherin is expressed within the chick visual system and identified a functional role for E-cadherin in promoting neurite outgrowth from RGCs.

In this manuscript, we demonstrate that E-cadherin is expressed in the retina from E6 to E12. At E8, E-cadherin is expressed by the RGCs of the retina and is also present in the chick tectum. In order to stimulate the elongation of retinal axons, RGCs require molecules with growth permissive properties (Hirano et al., 2003; Kiryushko,
2004; Thiery, 2003a). We demonstrate that RGC neurons extended neurites onto an E-cadherin substrate early in retinal development at E6, throughout peak axon extension at E8 to E10. E-cadherin is a homophilic binding protein, meaning E-cadherin on the surface of one cell has the ability to interact in trans with an E-cadherin molecule on the surface of another cell (Gooding, 2004; Ivanov et al., 2001). We show that neurite outgrowth on an E-cadherin substrate was blocked by the addition of an E-cadherin function blocking antibody. These data suggest that E-cadherin-mediated neurite outgrowth is specific to E-cadherin.

Distinct differences in neurite outgrowth were observed on an E-cadherin substrate versus N-cadherin. Neurite outgrowth on an E-cadherin substrate was robust from all regions of the retina at E8, whereas little to no neurite outgrowth is observed from the dorsal-nasal region of the retina on N-cadherin (Burden-Gulley et al., 2002). Growth cones on an E-cadherin substrate had large, broad lamellipodia with very few short filopodia in contrast to growth cones on N-cadherin with smaller lamellipodia and several short filopodia, indicating that different downstream signaling molecules may be regulating E-cadherin versus N-cadherin-mediated neurite outgrowth.

Expression of E-cadherin during embryonic development is classically associated with epithelial cell organization and maintenance of stable cell-cell adhesion (Thiery, 2003b). Epithelial cells express E-cadherin, however down-regulation of E-cadherin or loss of E-cadherin function occurs during epithelial-mesenchymal transition (EMT) (Larue and Bellacosa, 2005; Thiery, 2003b). In contrast to the role of E-cadherin in maintaining cell-cell adhesion in epithelial cells, recent findings in D. melanogaster have indicated a role for E-cadherin in axon growth and cell migration.
Drosophila epithelial (DE) cadherin is expressed in postembryonic neuroblasts which form the Drosophila brain and is required for proper axon tract formation (Dumstrei et al., 2003a; Dumstrei et al., 2003b). Border cells also express DE-cadherin and require DE-cadherin for migration during oogenesis (Niewiadomska, 1999). Lack of DE-cadherin in border cells blocks cell migration, and expression of extracellular DE-cadherin alone is unable to rescue border cell migration (Pacquelet and Rorth, 2005). These data highlight the importance of the DE-cadherin cytoplasmic domain in DE-cadherin-mediated cell migration.

Intracellular tyrosine phosphorylation of cadherins is associated with a loss of cadherin-mediated adhesion and destabilization of adherens junctions (Andl and Rustgi, 2005; Brunton, 2004; Erez et al., 2005). In addition, dephosphorylation of E-cadherin or E-cadherin associated proteins may be required for proper cell adhesion (Beltran and Bixby, 2003; Brady-Kalnay, 2001; Lilien and Balsamo, 2005). In the retina, PTPμ is primarily expressed on RGCs and is developmentally regulated (Burden-Gulley and Brady-Kalnay, 1999; Ensslen et al., 2003). PTPμ interacts with the E-cadherin/catenin complex in many cell types (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995). Our laboratory has previously reported that expression and catalytic activity of PTPμ are required for neurite outgrowth on an N-cadherin substrate (Burden-Gulley and Brady-Kalnay, 1999). In this study, we report that PTPμ expression and catalytic activity is also required for neurite outgrowth on an E-cadherin substrate. Although distinct downstream signaling pathways between E-cadherin and N-cadherin-mediated neurite outgrowth may be involved, it is clear that PTPμ expression and catalytic activity are required for both E-cadherin and N-cadherin-mediated neurite outgrowth.
One possible mechanism for the regulation of E-cadherin-mediated neurite outgrowth by PTPμ is through the recruitment of other regulatory proteins to the cadherin/catenin complex. The protein kinase C (PKC) family of serine/threonine kinases has been implicated in the regulation of E-cadherin-mediated adhesion and formation of adherens junctions (Hellberg et al., 2002; Lewis et al., 1994; Skoudy, 1995). PKC is able to bind the receptor for activated protein kinase C 1 (RACK1) (Ron et al., 1999), a scaffolding protein known to regulate signaling pathways in the central nervous system (Sklan, 2006). Within the chick retina, RACK1, PKCδ and PTPμ are found in complex together (Rosdahl et al., 2002). RACK1 and PTPμ have also been found in complex in epithelial cells and regulate E-cadherin dependent adhesion (Chattopadhyay et al., 2003). Regulation of PKCδ activity is required for restoration of E-cadherin-mediated adhesion in LNCaP cells (Hellberg et al., 2002). It is possible that PTPμ recruits RACK1/PKCδ to the cadherin/catenin complex at the cell surface where PKCδ may regulate E-cadherin-mediated cell adhesion. Future studies will investigate the PTPμ signaling pathways required for E-cadherin dependent neurite outgrowth.
2.6 Acknowledgements

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CHAPTER 3

Distinct Signaling of the Classical Cadherins, E-cadherin, N-cadherin and R-cadherin Differentially Regulates Growth Cone Morphology and Neurite Outgrowth
3.1 Abstract

E-cadherin, N-cadherin and R-cadherin, three type I classical cadherins, are expressed in the visual system and have distinct roles during development in axon growth and guidance. While functional roles of the classical cadherins have been identified, the signaling pathways activated in response to cadherin-mediated adhesion for some classical cadherins remain unclear. In this study, we investigated the signaling pathways required for E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth using a chick retinal explant model system. Our lab has previously demonstrated the importance of the receptor protein tyrosine phosphatase PTPmu in E-cadherin and N-cadherin-mediated neurite outgrowth. Here we demonstrate a role for PTPmu in R-cadherin-mediated neurite outgrowth. We further demonstrate that the Rho GTPase Rac1 is required for E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth while Cdc42 is required for N-cadherin and R-cadherin-mediated neurite outgrowth. IQGAP1, a scaffolding protein known to bind to activated Cdc42, is also required for N-cadherin and R-cadherin-mediated neurite outgrowth but not E-cadherin-mediated neurite outgrowth. Finally, we identified a unique requirement for the PKCδ serine-threonine kinase in E-cadherin and R-cadherin-mediated neurite outgrowth. Taken together, our data indicate that distinct signaling pathways are required for a specific cadherin to mediate neurite outgrowth.
3.2 Introduction

Proper formation of the visual system requires a myriad of interactions between migrating axons and the extracellular environment. Retinal ganglion cell (RGC) axons recognize growth promoting, attractive and repulsive cues that are secreted or presented on the surface of other axons and non-neuronal cells such as cell adhesion molecules (CAMs). The sum of these interactions determine the path taken by the axon (Skaper, 2005). One subfamily of CAMs, the classical cadherins, is expressed during development and has been shown to play a key role in axon growth, guidance and synapse formation (Takeichi, 2007).

The classical cadherins, E-cadherin, N-cadherin and R-cadherin, are expressed in restricted and complimentary patterns throughout the retina and retinofugal pathway (Inuzuka et al., 1991a; Inuzuka et al., 1991b; Takeichi et al., 1993; Wohrn et al., 1998). While all three cadherins promote neurite outgrowth in vitro (Bixby and Zhang, 1990; Oblander et al., 2007; Redies and Takeichi, 1993b), their unique pattern of expression suggests that they play distinct roles during visual system development. Early in development, N-cadherin is expressed uniformly throughout the retina (Matsunaga et al., 1988) and down regulation of N-cadherin during development in vivo results in RGC axon projection defects in Xenopus (Riehl et al., 1996), axon pathfinding errors in chick (Treubert-Zimmermann et al., 2002) and defects in Drosophila axon fasciculation (Iwai et al., 1997). R-cadherin is selectively expressed in the retina (Honjo et al., 2000; Inuzuka et al., 1991a; Inuzuka et al., 1991b; Liu et al., 1999b; Wohrn et al., 1998) and perturbation of R-cadherin in zebrafish results in improper arborization of RGC axons within the neuropil (Babb et al., 2005). E-cadherin is expressed by RGCs (Faulkner-
Down-regulation of E-cadherin in *Drosophila* results in proximal axon tract trajectory defects (Dumstrei et al., 2003a). While it is evident that the classical cadherins are required for axon growth and guidance, the precise signaling cascades activated in response to cadherin-mediated adhesion and subsequent axon outgrowth remains unclear.

E-cadherin, N-cadherin and R-cadherin are cell surface integral membrane glycoproteins that mediate cell-cell adhesion, cell migration and cell sorting (Halbleib and Nelson, 2006). Each cadherin mediates cell-cell adhesion through calcium-dependent homophilic (and/or heterophilic) binding, utilizing extracellular cadherin repeats. Intracellularly, the highly conserved cytoplasmic tail tethers cadherins to the actin cytoskeleton via an association with the catenin family of proteins, α-catenin, β-catenin, plakoglobin and p120 (Nelson, 2008; Yap et al., 2007). Association of cadherin with the cytoskeleton is required for cadherin-mediated cell-cell adhesion. The molecules that interact with the cadherin cytoplasmic tail determine the outcome of cadherin-mediated events (McLachlan and Yap, 2007).

We utilized a well-established chick *in vitro* model system (Burden-Gulley and Brady-Kalnay, 1999; Lagenaur and Lemmon, 1987) to investigate the signaling pathways required for E-cadherin, N-cadherin and R-cadherin-mediated RGC axon outgrowth. Our lab has previously demonstrated that the receptor protein tyrosine phosphatase PTPμ associates with E-cadherin, N-cadherin and R-cadherin and is required for E-cadherin and N-cadherin-mediated neurite outgrowth (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995; Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007). In the current study we demonstrate a role for PTPμ in R-cadherin-
mediated neurite outgrowth. Interestingly, we observe unique growth cone morphologies on each cadherin substrate, suggesting that distinct signaling mechanisms underlie neurite outgrowth on a particular cadherin substrate. E-cadherin growth cones have large lamellipodia, N-cadherin growth cones are smaller with several filopodia and R-cadherin growth cones display a hybrid morphology of moderate lamellipodia with short filopodia. We present evidence that E-cadherin mediated neurite outgrowth requires Rac1 and PKCδ serine-threonine kinase activity. N-cadherin-mediated neurite outgrowth requires Rac1 and IQGAP1/Cdc42 activity. An R-cadherin substrate promotes neurite outgrowth through R-cadherin/N-cadherin heterophilic binding. Interestingly, R-cadherin-mediate neurite outgrowth requires Rac1 and IQGAP1/Cdc42 activity but has a unique requirement for PKCδ serine-threonine kinase. While PTPµ function is required for E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth, each cadherin signals through distinct downstream pathways.
3.3 Materials and Methods

3.3.1 Antibodies

Monoclonal antibodies to human E-cadherin, N-cadherin and R-cadherin were purchased from BD Biosciences (San Diego, CA). A monoclonal antibody to the extracellular 92–593 amino acids of human N-cadherin was purchased from Affinity Bioreagents (Golden, CO). A rabbit polyclonal antibody to the extracellular 92–356 amino acids of E-cadherin (795) was a kind gift from Dr. Robert Brackenbury. The rat monoclonal blocking antibody to chick N-cadherin (NCD-2) was generously provided by Dr. Gerald Grunwald (Thomas Jefferson University, Philadelphia, PA) from hybridoma cells generated by Dr. Masatoshi Takeichi (Hatta and Takeichi, 1986). The mouse monoclonal antibody to chick R-cadherin (RCD-2), was a kind gift from Masatoshi Takeichi, and has been previously described (Redies et al., 1992). HRP conjugated antibody to Human IgG (Fc specific) was purchased from Jackson ImmunoResearch laboratories (West Grove, PA).

3.3.2 Neurite Outgrowth Assays

Human E-cadherin-Fc, N-cadherin-Fc and R-cadherin-Fc were obtained from R&D Systems (Minneapolis, MN). Laminin was obtained from Sigma (St. Louis, MO) or Biomedical Technologies, Inc. (BTI) (Stoughton, MA). Briefly, 35 mm tissue culture dishes were coated with nitrocellulose in methanol (Lagenaur and Lemmon, 1987) and allowed to dry. Several different lots of substrate were used over the course of the experiments resulting in variability in the concentration of substrate used. 0.35 µg of E-cadherin-Fc, 0.065 µg of N-cadherin-Fc, 0.35 µg R-cadherin-Fc, 1 µg of BTI
laminin or 4 µg of Sigma laminin was spread across the center of each dish and
incubated for 20 minutes at room temperature. Remaining binding sites on the
nitrocellulose were blocked with 2% BSA in CMF pH 7.2, and the dishes were rinsed
with RPMI 1640 medium (Hyclone, Logan, UT).

Embryonic day 8 (stage 32-33 according to Hamburger and Hamilton, 1951)
White Leghorn chick eyes were dissected in cold calcium-magnesium-free Hank's-
buffered saline (CMF) pH 7.2 and the retinal explants were prepared as described
(Burden-Gulley and Brady-Kalnay, 1999; Drazba, 1990; Halfter et al., 1983). Briefly,
neural retinas were flattened on concanavalin-coated nitrocellulose filters and cut into
350 µm-wide explants. Explants were placed retinal ganglion side down onto substrate
coated dishes and cultured in RPMI-1640, 10% fetal bovine serum (Hyclone), 2% chick
serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.025
µg/ml amphotericin (Sigma). All explants were incubated at 37°C for 24 hours, fixed in
4% paraformaldehyde, 0.1% glutaraldehyde and imaged.

For antibody inhibition studies, the function blocking rat monoclonal anti-
chicken N-cadherin blocking antibody, NCD-2 ascites (Hatta and Takeichi, 1986) was
used at 1:100. An isotype matched mouse antibody was generated by our lab and used
at an equivalent concentration. Explants were incubated for 24 hours in the presence of
the blocking antibody.

For growth cone visualization, 22 mm square cover glass from Corning
(Corning, NY) was coated with 0.01% poly-L-lysine overnight, rinsed 5x with distilled
H₂O and allowed to dry overnight. The cover glass was then UV sterilized and coated
with nitrocellulose followed by E-cadherin, N-cadherin, R-cadherin or laminin substrate
as described above. Retinas were prepared as described above. Before placing the explant retinal ganglion side down onto the substrate-coated slide, DiI crystals (Invitrogen) were placed directly on the tissue. Culture medium containing serum was then added.

For PTPµ inhibitor peptide studies, 6.5 µM of the PTPµ wedge peptide (WPTPµ-Tat) or scrambled control (SPTPµ-Tat) was added at the time of plating as previously described (Oblander et al., 2007; Xie et al., 2006). Each peptide includes a membrane-penetrant Tat-derived sequence at the C terminus, which promotes cellular uptake of the peptide (Wadia and Dowdy, 2002).

For viral perturbation studies, 7 µl of replication-defective herpes simplex virus (HSV) encoding green fluorescent protein (GFP IRES) or dominant negative (DN) Rho GTPases (Cdc42, Rac1 and RhoA), in RPMI-1640 alone was added at the time of plating. The virus was allowed to incubate at 37°C for 2 hours. Culture media containing serum was then added.

We utilized the HSV-1 vector pHSV-IRES-GFP-MCS previously generated by our lab (Ensslen et al., 2003), to generate all three dominant negative Rho GTPase vectors. In brief, retroviral constructs encoding DN Cdc42 (N17Cdc42/pBSTRI), DN Rac1 (N17Rac1/pBSTRI) or DN RhoA (N19RhoA/pBSTRI) were cut with BamHI and NotI. Each fragment was then ligated to pHSV-IRES-GFP-MCS cut with BglII and NotI. All of the pBPSTR1 constructs have been described previously (Wong et al., 2001). HSV was produced as previously described (Ensslen et al., 2003).

For Rac I inhibition studies, a cell permeable small molecule that specifically inhibits Rac1 GDP/GTP exchange activity by blocking Rac1 interaction with the Rac-
specific GEFs Trio and Tiam1 (IC$_{50} \sim 50$ µM) was used (Calbiochem, San Diego, CA). A final concentration of 60 µM Rac1 inhibitor was added at the time of plating.

For IQGAP1 inhibition studies, an IQGAP1 competitive peptide corresponding to the Cdc42 and Rac1 binding site on IQGAP1 or scrambled control, was added as previously described (Phillips-Mason et al., 2006). A final concentration of 12 µM peptide was added at the time of plating. Both peptides include a membrane-penetrant Tat-derived sequence at the N terminus, which promotes cellular uptake of the peptide (Wadia and Dowdy, 2002).

For PKCδ inhibition studies, the cell-permeable protein kinase C inhibitor Rottlerin (Calbiochem), that exhibits selectivity for PKCδ was added at the time of plating at a final concentration of 0.6 µM. DMSO at an equivalent concentration was added to control dishes at the time of plating.

3.3.3 Quantitation of Neurite Outgrowth

Neurite outgrowth was analyzed using a SPOT RT digital camera and image acquisition software (Diagnostic Instruments, Inc., Sterling Heights, MI). In short, the length of the five longest neurites per given area of the explant were measured perpendicular to the explant tissue. Neurite density was calculated by quantitating the area of the image occupied by neurites. Images were analyzed using Metamorph software version 6.3r4 (Universal Imaging, Downingtown, PA). The data from all similar experiments were combined, analyzed by Student’s $t$ test and graphed (Microsoft Excel, 10.0.0, 2001).
3.3.4 Immunoblot Analysis

E8 chick retinas were dissected in ice-cold CMF, transferred to lysis buffer (20 mM Tris pH 7.6, 1% Triton X-100, 2 mM CaCl$_2$, 150 mM NaCl, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.1 mM ammonium molybdate, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin), dounced and incubated on ice for 30 minutes. The triton insoluble material was removed by centrifugation (10,000 rpm for 3 minutes in an Eppendorf Microcentrifuge), and the protein concentration of the supernatant was determined by the BCA Protein Assay Kit (Pierce). 100 ng E-cadherin Fc, N-cadherin Fc, R-cadherin Fc or 125 µg E8 chick retina lysate was loaded per lane and separated by SDS-PAGE (6% gels). Proteins were transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH) and immunoblotted with the antibodies described above. Immunoblot data was acquired on a Bio-Rad Fluor-S Max MultiImager system (Bio-Rad, Hercules, CA), using the Quantity One (Bio-Rad) image processing software.
3.4 Results

In order to examine the signaling pathways required for E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth, our lab utilized a well-established chick in vitro model system (Burden-Gulley and Brady-Kalnay, 1999; Lagenaur and Lemmon, 1987). In brief, we culture embryonic day 8 (stage 32) retinal explants on a purified cadherin substrate and observe axon extension from retinal ganglion cells (RGCs). Neurite outgrowth requires adhesion and signaling to the actin cytoskeleton (Bouquet and Nothias, 2007; Takeichi, 2007), therefore the neurite outgrowth assay is ideal for investigating adhesion molecule function. By using a purified cadherin substrate, we were able to examine specific cadherin-mediated, adhesion-dependent signaling events between RGC axons and the cadherin substrate. E8 retinal explants are used because this is the time point in development when peak RGC axon extension to the optic tectum in the brain is observed (Thanos and Mey, 2001).

Bixby and Zhang first demonstrated that purified N-cadherin was a potent substrate for chick ciliary ganglion neurons in 1990. Subsequently, our lab has used purified N-cadherin to demonstrate the ability of N-cadherin to promote neurite outgrowth from chick RGCs (Burden-Gulley and Brady-Kalnay, 1999; Burden-Gulley et al., 2002; Oblander et al., 2007). Purified E-cadherin has also been demonstrated to promote neurite outgrowth from chick RGCs at various stages of development (Oblander et al., 2007). Previous studies have demonstrated the ability of E6 chick retinal explants to extend axons onto R-cadherin-transfected neuroblastoma cells (Redies and Takeichi, 1993b), which express little if any endogenous cadherins (Matsunaga et al., 1988). While this data establishes the ability of R-cadherin to
promote neurite outgrowth, detailed analysis of neurite outgrowth from chick RGCs on a purified R-cadherin substrate has not been demonstrated.

3.4.1 Purified R-cadherin Promotes Neurite Outgrowth

In order to confirm the ability of purified R-cadherin to promote chick RGC neurite outgrowth, we used recombinant human R-cadherin-Fc chimera as a substrate in the neurite outgrowth assay. At E6, E7, E8, E9 and E10, neurite outgrowth was observed on a R-cadherin substrate after 24 hours in culture (Fig 3.1 A-E). At E6, when R-cadherin expression is first observed in the retina (Inuzuka et al., 1991a; Wohrn et al., 1998), very few, short neurites extended out onto a R-cadherin substrate (Fig. 3.1A). Longer, more fasciculated neurites were observed at E7 (Fig. 3.1B). Similar to E-cadherin and N-cadherin-mediated neurite outgrowth, peak neurite outgrowth on R-cadherin was observed at E8 (Fig. 3.1C). This time point correlates with R-cadherin protein expression in the retina (Inuzuka et al., 1991a). At E9 and E10 (Fig. 3.1D, E), neurite outgrowth is still observed on an R-cadherin substrate although neurites are not as long as those observed at E8.

Observations of chick RGC neurite-outgrowth on an E-cadherin or N-cadherin substrate indicate that the 3-dimensional position of the RGC cell body within the retina determines its response to a given substrate. For example, robust neurite outgrowth is observed from all regions of the retina on an E-cadherin substrate (Oblander et al., 2007), while N-cadherin-mediated neurite outgrowth is limited to the temporal and ventral-nasal region of the retina (Burden-Gulley et al., 2002). Since robust neurite outgrowth was observed at E8, we next examined the ability of R-cadherin to promote
Figure 3.1. R-cadherin Promotes RGC Neurite Outgrowth at Various Stages of Development. E6 (A), E7 (B), E8 (C), E9 (D) and E10 (E) chick retinal explants were isolated and cultured on an R-cadherin substrate for 24 hours. Scale bar, 200 µm.
Figure 3.2. Neurite Outgrowth on R-cadherin is Independent of RGC Cell Body Origin. E8 chick retina explants were sectioned parallel to the optic fissure. Explants from each region of the retina were cultured on an R-cadherin substrate. Images were acquired after 24 hours in culture from a location corresponding to the outer third of each explant. Each number indicates the explant number (e.g. 1 and 6 are most peripheral). Dorsal (D), ventral (V), nasal (N), temporal (T). Scale bar, 200 µm.
neurite outgrowth from distinct regions of the retina. Unlike N-cadherin, R-cadherin-mediated neurite outgrowth was observed from all regions of the retina. The most robust RGC neurite outgrowth was primarily in the ventral region of the retina (Fig. 3.2). Neurites from the dorsal region of the retina were shorter and less dense than those in the ventral region. Similar to N-cadherin (Burden-Gulley et al., 2002), temporal neurites appeared to be more fasciculated than those observed in the nasal region of the retina, suggesting that N-cadherin and R-cadherin are not optimal substrates for temporal RGC axon outgrowth.

E-cadherin and N-cadherin-mediated neurite outgrowth occurs via homophilic binding (Oblander et al., 2007; Redies and Takeichi, 1993b); E-cadherin expressed on the surface of the RGC axon recognizes and bind to E-cadherin on the surface of a surrounding axon, cell or substrate. Previous studies demonstrated that partially purified rabbit IgG raised against chick N-cadherin was able to block neurite outgrowth on R-cadherin expressing cells, suggesting that heterophilic binding events take place (Redies and Takeichi, 1993b). To confirm that R-cadherin-mediated neurite outgrowth is due to heterophilic binding, E8 retinal explants were cultured on laminin, E-cadherin, N-cadherin or R-cadherin substrate in the presence or absence of a function-blocking monoclonal antibody to chick N-cadherin (NCD-2). The NCD-2 antibody is chick specific and will therefore only bind to N-cadherin expressed in the retinal explant, not the human N-cadherin substrate. NCD-2 had no effect on laminin or E-cadherin-mediated neurite outgrowth (Fig. 3.3E, F). As demonstrated previously (Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007), NCD-2 blocked neurite outgrowth on an N-cadherin substrate (Fig. 3.3G), implicating that N-cadherin homophilic binding is
required for N-cadherin-mediated neurite outgrowth. NCD-2 also blocked neurite outgrowth on an R-cadherin substrate (Fig. 3.3H). These results indicate that R-cadherin-mediated neurite outgrowth occurs due to a heterophilic interaction between N-cadherin on the surface of the RGC axon and R-cadherin on the surface of the dish.

To confirm the specificity of the NCD-2 antibody to chick N-cadherin the following immunoblots were performed. 100 ng of human E-cadherin-Fc, N-cadherin-Fc, R-cadherin-Fc or E8 chick retina lysate were examined by SDS gel electrophoresis. The NCD-2 antibody recognized one band in the E8 chick retina lysate corresponding to N-cadherin (Fig. 3.4A), indicating that the NCD-2 blocking antibody was specific to chick N-cadherin. Furthermore, NCD-2 does not recognize human N-cadherin-Fc and does not cross react with human R-cadherin-Fc or chick R-cadherin. It is important to note that each cadherin-Fc comprises the extracellular portion of the cadherin fused to the Fc region of human IgG. Therefore, antibodies to the intracellular domain of E-cadherin (Fig. 3.4B), intracellular domain of N-cadherin (Fig. 3.4C) and a chick specific R-cadherin antibody (RCD-2) (Fig. 3.4D) were used to confirm the presence of each cadherin in the E8 chick retina lysate. To verify the presence of the cadherin-Fc chimeras, an antibody that recognizes the Fc region of human IgG was used (Fig. 3.4E). In addition, antibodies that recognize the extracellular domain of human E-cadherin (Fig. 3.4F), human/chick N-cadherin (Fig. 3.4G) and human R-cadherin (Fig. 3.4H) confirmed the presence of each specific cadherin-Fc.
Figure 3.3. R-cadherin-Mediated Neurite Outgrowth is Blocked by N-cadherin Adhesion Blocking Antibodies. Retinal explants from E8 chick embryos were cultured on a laminin (A, E), E-cadherin (B, F), N-cadherin (C, G) or R-cadherin (D, H) substrate in the presence of adhesion blocking antibodies to chick N-cadherin (E, F, G, H) or antibody control (A, B, C, D). Antibodies against chick N-cadherin that recognize N-cadherin expressed on the surface of the chick retinal explant inhibited neurite outgrowth on a human N-cadherin (G) and a human R-cadherin (H) substrate, whereas they had no effect on neurite outgrowth on laminin (E) or a human E-cadherin (F) substrate. Scale bar, 200 µm.
Figure 3.4. NCD-2 Specificity. E-cadherin-Fc, N-cadherin-Fc, R-cadherin-Fc or E8 chick retina lysate were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies to chick N-cadherin (NCD-2) (A), intracellular human E-cadherin (B), intracellular human N-cadherin (C), chick R-cadherin (RCD-2) (D), Human Fc (E), extracellular human E-cadherin (F), extracellular human/chick N-cadherin (G) or extracellular human R-cadherin (H). E-cadherin protein migrates at ~120 kDa, N-cadherin migrates at ~130 kDa and R-cadherin migrates at ~125 kDa.
3.4.2 Unique Growth Cone Morphology on an E-cadherin, N-cadherin or R-cadherin Substrate

The growth cone is a dynamic structure located at the leading edge of a migrating axon (Wen and Zheng, 2006). Each growth cone interacts with various guidance cues in the surrounding extracellular environment. Subsequently, these interactions are transduced into the cell where intracellular signaling events induce cytoskeletal rearrangement of the axon, ultimately regulating axon growth and guidance. For that reason, the morphology of a growth cone serves as an external indicator of the signaling events occurring in the axon.

We previously observed distinct growth cone morphologies from retinal axons extending onto an E-cadherin substrate versus an N-cadherin substrate (Oblander et al., 2007). Based on our observation that R-cadherin mediated neurite outgrowth is dependent on heterophilic binding between N-cadherin and R-cadherin, we examined whether the morphology of growth cones on R-cadherin exhibited distinct characteristics. DiI, a strong lipophilic membrane dye, was placed directly onto E8 retinal explants and subsequently cultured for 24 hours. While diverse morphologies were present in each culture, overall, growth cones on an R-cadherin substrate had fairly broad lamellipodia with several short filopodia (Fig. 3.5D). Consistent with previously published work (Bixby and Zhang, 1990; Oblander et al., 2007; Payne et al., 1992), growth cones on a laminin substrate (Fig. 3.5A) had small, raised lamellipodia with few short filopodia. On an E-cadherin substrate (Fig. 3.5B), large, broad lamellipodia were observed with few, short filopodia. Growth cones on an N-cadherin substrate (Fig. 3.5C) had many filopodia processes with moderately sized lamellipodia.
Figure 3.5. Growth Cone Morphology. Dil labeling of growth cones on a laminin substrate (A) demonstrates a small lamellipodia morphology with few, short filopodia. Growth cones on an E-cadherin substrate (B) have very large, broad lamellipodia with few filopodial processes. On an N-cadherin substrate (C) growth cones have a larger lamellipodia compared to laminin but not as large as E-cadherin in addition to several filopodial processes. Growth cones on and R-cadherin substrate (D) have a hybrid morphology of moderately broad lamellipodia with several short filopodia. Scale bar, 10 µm.
The R-cadherin growth cone morphology is a hybrid between the E-cadherin and N-cadherin morphology. Taken together, R-cadherin growth cone morphology is distinct from E-cadherin or N-cadherin suggesting that all three cadherins may initiate distinct signaling pathways during neurite outgrowth.

3.4.3 PTPµ is Required for Classical Cadherin-mediated Neurite Outgrowth

Tyrosine phosphorylation of the cadherins and their associated molecules regulates cadherin function (Sallee et al., 2006; Yap et al., 2007). The Receptor Protein Tyrosine Phosphatases (RPTPs) are expressed in the chick visual system and mediate axon pathfinding (Beltran and Bixby, 2003; Brady-Kalnay, 2001; Ensslen-Craig and Brady-Kalnay, 2004; Johnson and Van Vactor, 2003). Specifically, RPTPmu (PTPµ) is expressed by chick RGCs and differentially regulates neurite outgrowth (Burden-Gulley et al., 2002). The catalytically active intracellular domain of PTPµ associates with E-cadherin, N-cadherin and R-cadherin (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995). Moreover, PTPµ expression and phosphatase activity is required for E-cadherin and N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007). Therefore, we hypothesized that PTPµ function plays a role in R-cadherin-mediated neurite outgrowth.

To investigate a functional role for endogenous PTPµ in R-cadherin-mediated neurite outgrowth, E8 chick retinal explants were cultured in the presence of a PTPµ wedge inhibitor peptide (WPTPµ-Tat) or scrambled control (SPTPµ-Tat) on a laminin, E-cadherin, N-cadherin or R-cadherin substrate for 24 hours (Fig. 3.6). The PTPµ specific WPTPµ-Tat peptide sequence corresponds to the HLH wedge shaped sequence
(Hoffmann et al., 1997), located in the juxtamembrane segment of PTPµ just N-terminal to the D1 catalytic domain (Xie et al., 2006). By mimicking PTPµ inter/intramolecular interactions that regulate catalytic activity of the phosphatase (Bixby, 2001; Brady-Kalnay, 2001; Ensslen-Craig and Brady-Kalnay, 2004), it has been proposed that the WPTPµ-Tat peptide regulates PTPµ function or catalytic activity. Each peptide includes a Tat-derived domain linked to the C terminus, which allows for uptake of the peptide into the cell. Incubation with WPTPµ-Tat had no effect on laminin-dependent neurite outgrowth (Fig. 3.6E) when compared to SPTPµ-Tat control (Fig. 3.6A). Similar to previously reported data (Oblander et al., 2007), inhibition of neurite outgrowth on an E-cadherin (Fig. 3.6F) and N-cadherin (Fig. 3.6G) substrate was observed in the presence of WPTPµ-Tat when compared to SPTPµ-Tat control (Fig. 3.6B, C). Quantitation of neurite outgrowth demonstrated a decrease in neurite length by 49% (Fig. 3.6I) and 65% in neurite density (Fig. 3.6J) on an E-cadherin substrate. On an N-cadherin substrate, neurite length decreased by 36% (Fig. 3.6I) and neurite density decreased by 62% (Fig. 3.6J). R-cadherin-mediated neurite outgrowth (Fig. 3.6H) was also inhibited in the presence of the WPTPµ-Tat when compared to SPTPµ-Tat control (Fig. 3.6D). Neurite length decreased by 50% (Fig. 3.6I) and neurite density decreased by 71% (Fig. 3.6J) on an R-cadherin substrate. The significant decrease in neurite length and density in the presence of WPTPµ-Tat observed on an R-cadherin substrate demonstrates the importance of PTPµ catalytic activity in R-cadherin-dependent neurite outgrowth.
Figure 3.6. PTPμ Wedge Peptide Inhibits Neurite Outgrowth on an E-, N- and R-cadherin Substrate. E8 chick retinal explants were cultured on laminin (A, E), E-cadherin (B, F), N-cadherin (C, G) or R-cadherin (D, H) substrates in the presence of 6.5 µM scrambled control (SPTPμ-Tat) (A, B, C, D) or PTPμ wedge peptide (WPTPμ-Tat) (E, F, G, H). Cultures incubated with WPTPμ-Tat had little to no effect on laminin-dependent neurite outgrowth (E) when compared to SPTPμ-Tat control (A), however neurite outgrowth decreased in the presence of WPTPμ-Tat on E-cadherin (F), N-cadherin (G) and R-cadherin (H) when compared to SPTPμ-Tat control (B, C, D). Neurite length (I) and neurite density (J) were quantitated in the presence of WPTPμ-Tat (black bars) or SPTPμ-Tat control (white bars). The asterisk denotes statistically significant changes in neurite length or density (p ≤ 0.001) compared to control. Scale bar, 200 µm.
3.4.4 Distinct Rho GTPases are Required for E-, N- and R-cadherin-mediated Neurite Outgrowth

One potential mechanism for the regulation of cadherin-mediated neurite outgrowth by PTP\(_\mu\) is through the Rho subfamily of small G-proteins. Cdc42, Rac1 and RhoA, play a central role in regulating cell adhesion through dynamic rearrangement of the cytoskeleton (reviewed in Heasman and Ridley, 2008). Activation of Cdc42 induces filopodia formation while Rac1 activation promotes lamellipodia formation in fibroblasts. The Rho GTPases cycle between GTP-bound “on/active” and GDP-bound “off/inactive” states, acting as molecular switches. Activity of each Rho GTPase is regulated by three factors, the GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs). The GAPs increase intrinsic GTPase activity, acting as a catalyst for the conversion of GTP bound Rho GTPases to GDP bound forms. Conversely, GEFs promote the exchange of GDP for GTP on Rho GTPases. GDIs bind to GDP-bound Rho GTPases and prevent the exchange of GDP for GTP. In addition to cell adhesion, Rho GTPases have been implicated in axon guidance and neurite outgrowth (Koh, 2006; Linseman and Loucks, 2008). Cdc42 and Rac1 are expressed in the chick retina and are involved in PTP\(_\mu\)-mediated growth cone rearrangement and neurite outgrowth (Major and Brady-Kalnay, 2007; Rosdahl et al., 2003).

In order to test a role for the Rho GTPases in cadherin-mediated neurite outgrowth, E8 chick retinal explants were infected with HSV encoding dominant negative Cdc42 (Cdc42 DN), Rac1 (Rac1 DN) or RhoA (RhoA DN) and cultured in the presence of the virus on a laminin, E-cadherin, N-cadherin or R-cadherin substrate for
24 hours. Previous studies have demonstrated a role for Cdc42 activity in laminin-mediated neurite outgrowth (Weston et al., 2000). A slight decrease in neurite density was observed on laminin in the presence of Cdc42 DN (Fig. 3.7D), but was not statistically significant. Expression of dominant negative Rac1 and RhoA had no effect on laminin-dependent neurite outgrowth (Fig. 3.7G, J) when compared to GFP control (Fig. 3.7A). On an E-cadherin-substrate, infection with Cdc42 DN had no effect on neurite outgrowth (Fig. 3.7E). A significant decrease in neurite outgrowth was observed when retinal explants were infected with Rac1 DN on an E-cadherin substrate (Fig. 3.7H) when compared to GFP control (Fig. 3.7B) suggesting that Rac1 activity is required for E-cadherin-mediated neurite outgrowth. Quantitation of these experiments demonstrated that neurite length decreased by 54% (Fig. 3.7M) and neurite density decreased by 63% (Fig. 3.7N). RhoA DN had no effect on E-cadherin-mediated neurite outgrowth (Fig. 3.7K). On an N-cadherin substrate, a significant decrease in neurite outgrowth was observed when explants were infected with Cdc42 DN (Fig. 3.7F) and Rac1 DN (Fig. 3.7I) when compared to GFP control (Fig. 3.7C), suggesting that Cdc42 and Rac1 activity are required for N-cadherin-mediated neurite outgrowth. Neurite length decreased by 60% (Fig. 7M) and neurite density decreased by 71% (Fig. 3.7N) when retinal explants were infected with Cdc42 DN and cultured on an N-cadherin substrate. When infected with Rac1 DN, neurite length decreased by 36% (Fig. 3.7M) and neurite density decreased by 45% (Fig. 3.7N) on an N-cadherin substrate. RhoA DN had no effect on N-cadherin-mediated neurite outgrowth (Fig. 3.7L). Taken together Rac1 activity appears to be critical for E-cadherin and N-cadherin-mediated neurite outgrowth while N-cadherin has a unique requirement for Cdc42 activity.
Figure 3.7. Rac1 Activity is Required for E-, and N-cadherin-Mediated Neurite Outgrowth While Cdc42 Activity is Only Required for N-cadherin-Mediated Neurite Outgrowth. E8 chick retinal explants were cultured on laminin (A, D, G, J), E-cadherin (B, E, H, K), or N-cadherin (C, F, I, L) substrates and infected with HSV encoding GFP control (A, B, C), Cdc42 DN (D, E, F), Rac1 DN (G, H I) or RhoA DN (J, K, L) for 24 hours. Infection with HSV encoding Cdc42 DN had no effect on laminin (D) or E-cadherin-mediated (E) neurite outgrowth. However, perturbation of neurite outgrowth was observed on N-cadherin (F). Infection with HSV encoding Rac1 DN had no effect on laminin (G), but demonstrated a dramatic decrease in neurite outgrowth on an E-cadherin (H) and N-cadherin (I) substrate. Infection with HSV encoding RhoA DN had no effect on laminin (J), E-cadherin (K) or N-cadherin-mediated (L) neurite outgrowth. Neurite length (M) and neurite density (N) were quantitated in the presence of GFP (white bars), Cdc42 DN (hatched bars), Rac1 DN (black bars) or RhoA DN (gray bars). The asterisk denotes statistically significant changes in neurite length (p ≤ 0.001) or density (p ≤ 0.001, p ≤ 0.01 for Rac1 DN on N-cadherin) compared to control. Scale bar, 200 µm.
Retinal explants cultured on an R-cadherin substrate when infected with HSV demonstrated slightly higher toxicity, therefore we could not determine the effects of individual Rho GTPases on R-cadherin-mediated neurite outgrowth (data not shown).

To test whether endogenous Rac1 activity is required for E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth, a cell-permeable Rac1-specific small molecule inhibitor was used. Rac1 interaction with guanine nucleotide exchange factors (GEFs) is required for GTP/GDP exchange and subsequent Rac1 activation (Linseman and Loucks, 2008). The Rac1-specific compound inhibits Rac1 GDP/GTP exchange by blocking Rac1 interaction with the Rac-specific GEFs Trio and Tiam1 (Gao et al., 2004). Furthermore, the Rac1-specific inhibitor has been shown not to affect the activity of Cdc42 or RhoA (Gao et al., 2004). E8 retinal explants were cultured in the presence of the Rac1 inhibitor or vehicle control and cultured on either a laminin, E-cadherin, N-cadherin or R-cadherin substrate for 24 hours (Fig. 3.8). Incubation with the Rac1 inhibitor had no effect on laminin-dependent neurite outgrowth (Fig. 3.8E) when compared to vehicle control (Fig. 3.8A). A decrease in neurite outgrowth was observed on an E-cadherin substrate (Fig. 3.8F) in the presence of the Rac1 inhibitor when compared to control (Fig. 3.8B). Quantitation of these experiments demonstrated that neurite length decreased by 69% (Fig. 3.8I) while neurite density decreased by 79% (Fig. 3.8J). Neurite outgrowth also decreased on an N-cadherin substrate (Fig. 3.8G) in the presence of the Rac1 inhibitor when compared to control (Fig. 3.8C). Neurite length decreased by 88% (Fig. 3.8I), while neurite density decreased by 96% (Fig. 3.8J) on an N-cadherin substrate. On an R-cadherin substrate, a decrease in neurite outgrowth was also observed (Fig. 3.8H) in the presence
Figure 3.8. Rac1 Specific Inhibitor Blocks Neurite Outgrowth on E-cadherin, N-cadherin and R-cadherin. E8 chick retinal explants were cultured on laminin (A, E), E-cadherin (B, F), N-cadherin (C, G), or R-cadherin (D, H) substrates for 24 hours in the presence of either control (A-D) or the Rac1 inhibitor (E-H) at a final concentration of 60 µM. Cultures incubated with Rac1 inhibitor had no effect on laminin-dependent neurite outgrowth (E) when compared to control (A). Perturbation of neurite outgrowth was observed on E-cadherin (F), N-cadherin (G) and R-cadherin (H) in the presence of the Rac1 inhibitor when compared to vehicle control (B, C, D). Neurite length (I) and neurite density (J) were quantitated in the presence of the Rac1 inhibitor (black bars) or control (white bars). The asterisk denotes statistically significant changes in neurite length or density (p ≤ 0.001) compared to control. Scale bar, 200 µm.
of the Rac1 inhibitor when compared to control (Fig. 3.8D). Neurite length decreased by 67% (Fig. 3.8I), while neurite density decreased by 84% (Fig. 3.8J). Perturbation of E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth using the Rac1-specific inhibitor confirms the importance of Rac1 activity in cadherin-dependent neurite outgrowth. This data also implicates a role for Trio and/or Tiam1 in regulating classical cadherin-dependent neurite outgrowth.

3.4.5 IQGAP1 Interaction with Cdc42 Contributes to N-cadherin and R-cadherin-mediated Neurite Outgrowth

IQGAP1 has been shown to bind activated Cdc42 and Rac1 (Hart et al., 1996; Swart-Mataraza et al., 2002; Zhang, 1997). IQGAP1 functions within the cell to recruit activated Rho GTPase family members to cytoskeletal proteins (Brown, 2006) and regulate cadherin-mediated adhesion (Noritake et al., 2005). Our laboratory has recently reported that PTPµ binds directly to IQGAP1 (Phillips-Mason et al., 2006). PTPµ may recruit activated Cdc42 via IQGAP1, which would in turn regulate the actin cytoskeleton and subsequently, neurite outgrowth.

To identify a role for IQGAP1 in cadherin-mediated neurite outgrowth, E8 chick retinal explants were cultured in the presence of a cell permeable, Tat-tagged IQGAP1 inhibitor peptide or scrambled control. The IQGAP1 inhibitor peptide competes for binding to the Cdc42 and Rac1 binding site on IQGAP1 (Mataraza et al., 2003). Robust neurite outgrowth was observed on laminin (Fig. 3.9E) and E-cadherin (Fig. 3.9F) substrate in the presence of the IQGAP1 inhibitor when compared to scrambled control (Fig. 3.9A, B), suggesting that the interaction of IQGAP1 with Cdc42 and Rac1 is not
Figure 3.9. IQGAP1 Specific Inhibitor Peptide Blocks N-cadherin and R-cadherin-Mediated Neurite Outgrowth. E8 chick retinal explants were cultured on laminin (A, E), E-cadherin (B, F), N-cadherin (C, G) or R-cadherin (D, H) substrates for 24 hours in the presence of either scrambled control (A, B, C, D) or the IQGAP1 inhibitor (E, F, G, H) at a final concentration of 12 µM. Cultures incubated with IQGAP1 inhibitor had no effect on laminin (E) or E-cadherin-dependent neurite outgrowth (F) when compared to scrambled control (A, B). Perturbation of neurite outgrowth was observed on an N-cadherin (G) and R-cadherin (H) substrate when compared to scrambled control (C, D). Neurite length (I) and neurite density (J) were quantitated in the presence of IQGAP1 Inhibitor (black bars) or scrambled control (white bars). The asterisk denotes statistically significant changes in neurite length ($p \leq 0.001$) or density ($p \leq 0.001, p \leq 0.01$ for R-cadherin) compared to control. Scale bar, 200 µm.
required for laminin or E-cadherin-mediated neurite outgrowth. Perturbation of neurite outgrowth on an N-cadherin substrate was observed in the presence of the IQGAP1 inhibitor peptide (Fig. 3.9G) when compared to scrambled control (Fig. 3.9C). Quantitation of these experiments demonstrated a decrease in neurite length of 31% (Fig. 3.9I) and neurite density decreased by 59% (Fig. 3.9J) on an N-cadherin substrate. Perturbation of neurite outgrowth on an R-cadherin substrate was observed in the presence of the IQGAP1 inhibitor peptide (Fig. 3.9H) when compared to scrambled control (Fig. 3.9D). Neurite length decreased by 36% (Fig. 3.9I) and neurite density decreased by 50% (Fig. 3.9J) on an R-cadherin substrate. Taken together, the Cdc42/Rac1 interaction with IQGAP1 contributes to N-cadherin and R-cadherin-mediated neurite outgrowth but not on an E-cadherin substrate further demonstrating that N-cadherin and R-cadherin signal through distinct pathways from E-cadherin.

3.4.6 PKCδ is Required for E-cadherin and R-cadherin-mediated Neurite Outgrowth

To gain further insight into the pathways mediated by the classical cadherins, we looked at the role of the serine/threonine kinase, Protein Kinase C delta (PKCδ) in cadherin-mediated neurite outgrowth. PKCδ is expressed in E8 chick RGC growth cones, and has been found in a complex with PTPµ in retinal neuroepithelial cells at high cell density (Rosdahl et al., 2002). In addition, perturbation of PKCδ has been shown to significantly decrease PTPµ-dependent neurite outgrowth (Ensslen-Craig and Brady-Kalnay, 2004; Rosdahl et al., 2002). To determine the role of PKCδ in cadherin-mediated neurite outgrowth we used the PKCδ specific inhibitor Rottlerin (Fig. 3.10). Treatment of E8 retinal explants with 0.6 μM Rottlerin or DMSO control had no effect
on laminin-mediated neurite outgrowth (Fig. 3.10A, E). A decrease in neurite outgrowth was observed on an E-cadherin substrate (Fig. 3.10F) in the presence of Rottlerin when compared to vehicle control (Fig. 3.10B). E-cadherin-mediated neurite outgrowth decreased in length by 41% (Fig. 3.10I) and neurite density decreased by 63% (Fig. 3.10J) in the presence of Rottlerin. No decrease in neurite outgrowth was observed on an N-cadherin substrate in the presence of Rottlerin (Fig. 3.10G), when compared to control (Fig. 3.10C). A slight decrease in neurite density on and N-cadherin substrate was observed (Fig. 3.10J), but was not statistically significant. Interestingly, neurite outgrowth decreased on an R-cadherin substrate in the presence of Rottlerin (Fig. 3.10H), when compared to vehicle control (Fig. 3.10D). Quantitation of these experiments demonstrated that neurite length on an R-cadherin substrate decreased by 50% (Fig. 3.10I) and neurite density decreased by 59% (Fig. 3.10J). While Rottlerin had little to no effect on N-cadherin-mediated neurite outgrowth, perturbation of R-cadherin-dependent neurite outgrowth was significant, suggesting that the signaling pathways required for N-cadherin/R-cadherin heterophilic binding-mediated neurite outgrowth are distinct from N-cadherin homophilic binding-mediated neurite outgrowth. We conclude that PKCδ serine-threonine kinase signaling is required for E-cadherin and R-cadherin-mediated neurite outgrowth.
Figure 3.10. PKCδ Inhibitor (Rottlerin) Blocks E-cadherin and R-cadherin-Mediated Neurite Outgrowth. E8 chick retinal explants were cultured on laminin (A, E), E-cadherin (B, F), N-cadherin (C, G) or R-cadherin (D, H) substrates for 24 hours in the presence of either DMSO control (A, B, C, D) or Rottlerin (E, F, G, H), a PKCδ inhibitor, at a final concentration of 0.6 µM. Cultures incubated with Rottlerin had no effect on laminin (E) neurite outgrowth when compared to DMSO control (A). Perturbation of neurite outgrowth was observed on an E-cadherin substrate (F) in the presence of the Rottlerin when compared to vehicle control (B). N-cadherin-mediated neurite outgrowth was unaffected by incubation with Rottlerin (G) when compared to vehicle control (C). However, neurite outgrowth on R-cadherin decreased in the presence of Rottlerin (H) when compared to DMSO control (D). Neurite length (I) and neurite density (J) were quantitated in the presence of Rottlerin (black bars) or DMSO control (white bars). The asterisk denotes statistically significant changes in neurite length (p ≤ 0.001) or density (p ≤ 0.001, p ≤ 0.05 for R-cadherin) compared to control. Scale bar, 200 µm.
3.5 Discussion

The classical cadherins function as essential cell adhesion molecules and within the visual system they have been characterized as growth promoting substrates for axon outgrowth. Three type I classical cadherins are expressed in the chick visual system, E-cadherin, N-cadherin and R-cadherin. Purified E-cadherin has been shown to promote RGC neurite outgrowth from all regions of the retina (Oblander et al., 2007). N-cadherin is expressed throughout the visual system and has been demonstrated to promote RGC neurite outgrowth as a purified substrate (Bixby and Zhang, 1990). R-cadherin is expressed by RGCs and has been shown to promote neurite outgrowth when expressed on the surface of neuroblastoma cells in vitro (Inuzuka et al., 1991a; Redies and Takeichi, 1993b). RGC axons extend onto R-cadherin-transfected neuroblastoma cells (Redies and Takeichi, 1993b), however a detailed analysis of R-cadherin-mediated RGC neurite outgrowth has not been undertaken. To confirm the ability of R-cadherin to promote neurite outgrowth, we utilized recombinant human R-cadherin-Fc as a substrate in a well-established in vitro RGC neurite outgrowth assay. In this study we demonstrate that purified R-cadherin has the ability to promote outgrowth from E6 to E10 in chick RGCs. R-cadherin-mediated neurite outgrowth is observed in all regions of the retina, implicating that neurite outgrowth is not dependent upon RGC body location.

Classical cadherins are characterized as homophilic cell-cell adhesion molecules (Halbleib and Nelson, 2006). However, heterophilic interactions between N-cadherin and R-cadherin have been observed. L-cells are one of a few cells lines that do not naturally aggregate. When L-cells, which do not express endogenous cadherins, are
transfected with cDNA encoding N-cadherin or R-cadherin and mixed together in an aggregation assay they form chimeric aggregates (Inuzuka et al., 1991a; Matsunami et al., 1993), demonstrating that R-cadherin can interact in trans with N-cadherin. This data suggests that R-cadherin expressed on the surface of one cell will recognize and bind to N-cadherin expressed on the surface of another cell. The N/R-cadherin heterophilic binding observed in the chimeric aggregates is attributed to their high sequence similarity (74%). We demonstrate that neurite outgrowth on an R-cadherin substrate was blocked by the addition of a chick-specific N-cadherin function blocking antibody, demonstrating that R-cadherin-mediated neurite outgrowth is a result of heterophilic R-cadherin/N-cadherin adhesion. N-cadherin and R-cadherin are differentially expressed within the chick visual system (Redies et al., 1993; Redies et al., 1992; Redies and Takeichi, 1993b), suggesting that they have distinctive roles in visual system development. N-cadherin is first to appear during development and correlates with the first wave of retinal differentiation required for organization and lamination of the retina while R-cadherin appears later during the second wave of differentiation when various retinal cell types emerge. At each stage of development, N-cadherin and R-cadherin appear to have a complimentary expression pattern. For example early in development of the optic nerve, RGC axons express N-cadherin while the cells of the rudimentary optic stalk express R-cadherin (Inuzuka et al., 1991b). These RGC axons are in close contact with neuroepithelial (glial) cells as they migrate through the optic nerve. It has been proposed that N-cadherin expressing retinal axons migrate within the retina by homophilic binding but then exit the eye by heterophilic binding along R-cadherin expressing neuroepithelial cells of the optic stalk (Silver and
Rutishauser, 1984), suggesting that heterophilic N-cadherin/R-cadherin binding can transmit distinct signals from homophilic binding to regulate RGC axon migration in vivo during development of the visual system. In vivo perturbation of R-cadherin in neuroepithelial cells has not been performed.

Within the N-cadherin and R-cadherin chimeric L-cell aggregates, N-cadherin and R-cadherin expressing cells segregate, implicating that there is a preference for homophilic binding over heterophilic binding (Inuzuka et al., 1991a; Matsunami et al., 1993). One example of preferential homophilic binding between N-cadherin and R-cadherin in vivo was demonstrated by Treubert-Zimmermann (Treubert-Zimmermann et al., 2002). In this study electroporation of chick stratum griseum centrale (SGC) axons with N-cadherin or R-cadherin caused them to selectively choose tectofugal axon pathways corresponding to the respective cadherin. Perturbation of R-cadherin in zebrafish results in a severe small eye phenotype, with defects in retinal lamination, proliferation and axon extension in vivo (Babb et al., 2005). When N-cadherin is perturbed in vivo, Xenopus retinal lamination and differentiation are normal (Riehl et al., 1996). However, axon initiation and extension is perturbed demonstrating a role for N-cadherin in early RGC axon formation (Riehl et al., 1996). RGC axons that do form, project out of the eye but prematurely terminate along the optic path suggesting that R-cadherin homophilic interactions can not compensate for the loss of N-cadherin. Taken together, perturbation of R-cadherin or N-cadherin in vivo demonstrates that R-cadherin and N-cadherin have distinct roles during visual system development and that one cadherin cannot compensate for the loss of the other cadherin.
In fact, our data and others suggest that homophilic R-cadherin-mediated neurite outgrowth is required for chick visual system development. In fact R-cadherin-dependent neurite outgrowth occurs in regions of the retina where N-cadherin-dependent neurite outgrowth is not observed. For example, little to no N-cadherin-mediated neurite outgrowth is observed in the dorsal-nasal region of the retina (Burden-Gulley et al., 2002), however neurite outgrowth is observed in the dorsal-nasal region of the retina when cultured on an R-cadherin substrate (Fig. 3.2). This observation suggests that dorsal-nasal RGC axon outgrowth is due to homophilic binding by R-cadherin.

Furthermore, the neurite outgrowth assay described was used to measure trans binding of cadherins expressed on the surface of the explant with the specific cadherin coated on the tissue culture dish. While trans binding is required for cadherin-mediated adhesion and migration, lateral cis binding where one cadherin monomer will dimerize with another cadherin monomer within the plane of the membrane on the same cell has also been suggested to contribute to cadherin-mediated adhesion (Leckband and Prakasam, 2006; Mege et al., 2006). They hypothesize that a cadherin monomer will first form lateral cis dimers, followed by trans binding to cadherin dimers on the surface of another cell. If cis binding does not occur, then the cadherin is recycled via endocytosis (Nelson, 2008; Yap et al., 2007). The NCD2 antibody used in the perturbation studies (Fig. 3.3) recognizes N-cadherin on the retinal explant. While the antibody is monoclonal and does not induce lateral dimerization of N-cadherin, it is possible that the NCD2 antibody could block N-cadherin/R-cadherin cis binding resulting in endocytosis of R-cadherin. Therefore cis interactions between N-cadherin
and R-cadherin on retinal ganglion cells may be required for R-cadherin-mediated neurite outgrowth.

The hybrid growth cone morphology observed on an R-cadherin substrate, suggests that R-cadherin homophilic or R/N-cadherin heterophilic interactions rely on distinct signaling pathways from E-cadherin and N-cadherin. Since the N-cadherin and R-cadherin expression pattern within the visual system is distinct and overlapping (Redies et al., 1993; Redies et al., 1992; Redies and Takeichi, 1993b), it is plausible that R/R-cadherin, N/N-cadherin and R/N-cadherin interactions with unique signaling pathways are required for proper formation of the visual system.

Reversible tyrosine phosphorylation of proteins serves as a key regulatory mechanism for cellular signaling. For example, tyrosine phosphorylation of the intracellular segment of the classical cadherins results in a loss of cadherin-mediated adhesion and destabilization of adherens junctions (Sallee et al., 2006; Yap et al., 2007). Dephosphorylation of the classical cadherins or their associated proteins has also been shown to regulate cell adhesion. The tyrosine phosphatase PTPµ associates with E-cadherin, N-cadherin and R-cadherin (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995) and is required for E-cadherin (Hellberg et al., 2002). Within the chick retina, RGCs express PTPµ during peak axon extension (Burden-Gulley and Brady-Kalnay, 1999). Our laboratory has previously demonstrated that PTPµ expression and catalytic activity are required for both E-cadherin and N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007). In the current study we demonstrate that PTPµ function is required for R-cadherin-mediated neurite outgrowth. Although PTPµ is required for all three cadherins, we hypothesize that
PTPµ alters cadherin function by recruiting additional regulatory molecules to the cadherin/catenin complex thus regulating cadherin-mediated adhesion/neurite outgrowth.

The Rho-GTPases Cdc42, Rac1 and RhoA are involved in many aspects of axon outgrowth and development (Koh, 2006). We have previously published a role for the Rho GTPases in PTPµ-mediated neurite outgrowth (Major and Brady-Kalnay, 2007). PTPµ promotes neurite outgrowth from nasal RGCs but is repulsive to temporal RGCs (Burden-Gulley et al., 2002). Cdc42 activity is required for PTPµ-mediated nasal outgrowth and temporal repulsion. Temporal repulsion of these neurites also requires inhibition of Rac1 activity (Major and Brady-Kalnay, 2007). By expressing dominant negative mutants of the Rho GTPases Cdc42, Rac1 and RhoA, we have identified overlapping and distinct requirements for the Rho GTPases in cadherin-mediated neurite outgrowth. Rac1 activity is required for E-cadherin, N-cadherin and R-cadherin mediated neurite outgrowth. We confirmed the requirement for Rac1 using a Rac1 specific inhibitor that blocks the interaction of Rac1 with Rac-specific GEFs Trio and Tiam1.

One regulatory pathway emerging for the regulation of E-cadherin-mediated neurite outgrowth is through the protein kinase C (PKC) family of lipid/dependent serine/threonine kinases. PKCs have been implicated in the regulation of E-cadherin-mediated adhesion and formation of adherens junctions (Hellberg et al., 2002; Lewis et al., 1994; Skoudy, 1995). Specifically, PKCδ inhibition is required for the restoration of E-cadherin mediated adhesion in LNCaP prostate carcinoma cells (Hellberg et al., 2002). Using the PKCδ specific inhibitor Rottlerin, we demonstrate that PKCδ serine-
threonine kinase signaling is required for E-cadherin and R-cadherin mediated neurite outgrowth but not N-cadherin-mediated neurite outgrowth. PKCδ is also required for PTPμ-mediated neurite outgrowth (Rosdahl et al., 2002). PTPμ is found in a complex with RACK1 (Mourton et al., 2001) and PKCδ in cultured retinal cells (RNEs) and in E8 retinal tissue (Rosdahl et al., 2002). RACK1, the receptor for activated protein kinase C, is a scaffolding protein known to regulate nervous system signaling pathways (Sklan, 2006). Activated PKC binds to RACK1 and is then recruited to the plasma membrane where it can phosphorylate substrates. We therefore speculate that PTPμ/RACK1/PKCδ is recruited to the cadherin/catenin complex at the cell membrane where PKCδ may regulate E-cadherin or R-cadherin function (Fig. 3.11).

It should be noted that RACK1 also binds to PKC substrates containing pleckstrin homology (PH) domains such as β-spectrin or dynamin-1 (Rodriguez et al., 1999). PH domains are also found in the Rac1-specific exchange factor Tiam1 (Habets et al., 1994). These PH domains are required for Tiam1 targeting to the plasma membrane (Stam et al., 1997) and regulation of Rac1 dependent membrane ruffling (Michiels et al., 1997). Tiam1-Rac1 signaling has also been shown to be required for the maintenance of E-cadherin-mediated cell-cell adhesion (Kraemer et al., 2007; Malliri et al., 2004). Trio also contains PH domains, which coordinate with Dbl homology (DH) domains to activate Rac1 (Chhatriwala et al., 2007). Trio was first identified as a leukocyte common antigen-related (LAR) receptor binding protein (Debant et al., 1996). Specifically Trio binds to the catalytically inactive D2 PTPase domain of LAR. The receptor tyrosine phosphatase LAR belongs to the type II group of RPTPs that contain immunoglobulin domains, which include the PTPμ subfamily
and the LAR subfamily (Brady-Kalnay and Tonks, 1995; Ensslen-Craig and Brady-Kalnay, 2004). Trio and LAR are expressed by neurons and regulate neurite outgrowth and axon pathfinding (Bateman et al., 2000; Maurel-Zaffran et al., 2001).

Our data identifies a unique requirement for Cdc42 activity in N-cadherin and R-cadherin-mediated neurite outgrowth. Using an IQGAP1 inhibitor peptide that competes for binding to the Cdc42 and Rac1 binding site on IQGAP1, we show that the Cdc42/Rac1 interaction with IQGAP1 contributes to N-cadherin and R-cadherin-mediated neurite outgrowth. IQGAP1 has homology to a RasGAP, however IQGAP1 does not display RasGAP activity and does not bind to Ras (Weissbach et al., 1994). Instead, IQGAP1 interacts with activated Rac1 and Cdc42 (Briggs and Sacks, 2003). IQGAP1 has a higher affinity for Cdc42 than Rac1 and acts to stabilize GTP-bound Cdc42 (Erickson et al., 1997; Hart et al., 1996; Kuroda et al., 1998; Swart-Mataraza et al., 2002; Zhang, 1997). IQGAP1 also binds to PTPµ and the IQGAP1/PTPµ association increases in the presence of activated Cdc42 (Phillips-Mason et al., 2006). In addition, an association with IQGAP1 and Cdc42/Rac1 is required for PTPµ-mediated neurite outgrowth (Phillips-Mason et al., 2006). We therefore speculate that PTPµ recruits IQGAP1/Cdc42 to the cadherin/catenin complex at the cell membrane and subsequently regulates N-cadherin and/or R-cadherin function.
**Figure 3.11.** Diagram Summarizing the Potential Signaling Pathways Involved in E-cadherin, N-cadherin and R-cadherin-Mediated Neurite Outgrowth.
3.6 Acknowledgements

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CHAPTER 4

Conclusions and Future Directions
4.1 Conclusions

It is exciting to find that after 22 years of investigating cadherin function in the visual system, new roles continue to emerge. Certainly this points to the complexity required for visual system development. The classical cadherins, E-cadherin, N-cadherin and R-cadherin are expressed in the chick visual system (Suzuki and Takeichi, 2008; Takeichi, 2007). Numerous studies have demonstrated a functional role for N-cadherin and to a lesser extent R-cadherin in RGC axon growth (Bixby and Zhang, 1990; Redies and Takeichi, 1993a). In this dissertation, a novel function for E-cadherin in promoting RGC axon outgrowth was identified. E-cadherin function is classically associated with stable cell-cell adhesion. This data contributes to the mounting evidence for the importance of E-cadherin in dynamic cell-cell adhesion during cell migration. Furthermore, identification of E-cadherin as a growth promoting substrate for RGC axon outgrowth allows for the determination of unique signaling pathways required for each classical cadherin in axon outgrowth.

One principle area addressed in this dissertation is that of classical cadherin binding and its relationship to neurite outgrowth. This was examined by culturing chick retinal explants on purified human E-cadherin, N-cadherin or R-cadherin substrate in the presence of adhesion blocking antibodies to chick-specific cadherins as discussed in Chapter 2 and Chapter 3. Antibodies raised against chick E-cadherin perturbed neurite outgrowth on an E-cadherin substrate but not N-cadherin demonstrating that E-cadherin-mediated neurite outgrowth requires E-cadherin homophilic binding. N-cadherin-mediated neurite outgrowth requires homophilic binding. This has been demonstrated previously (Bixby and Zhang, 1990; Burden-Gulley and Brady-Kalnay,
1999) and is reiterated in Chapter 2 and Chapter 3. Antibodies raised against chick N-
cadherin significantly decreased R-cadherin-mediated neurite outgrowth. This data
demonstrates the ability of R-cadherin to mediated neurite outgrowth through
heterophilic binding to N-cadherin. Experiments using function blocking antibodies
specific to chick R-cadherin or infection of chick retinal explants with antisense R-
cadherin in the retinal explant model system will clarify the role of R-cadherin
homophilic binding in neurite outgrowth.

Tyrosine phosphorylation and subsequent dephosphorylation has emerged as a
major regulatory mechanism of cadherin function (McLachlan and Yap, 2007; Nelson,
2008). The RPTP, PTPµ is of interest in cadherin-mediated axon growth and guidance
due to its expression pattern in the chick visual system (Burden-Gulley et al., 2002), its
association with the classical cadherins (Brady-Kalnay et al., 1998; Brady-Kalnay et al.,
1995; Hiscox and Jiang, 1998) and its ability to regulate N-cadherin-mediated neurite
outgrowth (Burden-Gulley and Brady-Kalnay, 1999). In this dissertation, the
importance of PTPµ function was demonstrated for E-cadherin, N-cadherin and R-
cadherin-mediated neurite outgrowth. Infection of retinal explants with herpes simplex
virus encoding antisense PTPµ or catalytically inactive PTPµ resulted in a significant
decrease in neurite outgrowth on an E-cadherin or N-cadherin substrate. Incubation
with a PTPµ specific peptide, known to regulate PTPµ function, also resulted in
perturbation of neurite outgrowth on all three classical cadherin substrates reiterating
the importance of PTPµ function in cadherin-mediated neurite outgrowth.

Finally, distinct PTPµ associated proteins were required for E-cadherin, N-
cadherin and R-cadherin-mediated neurite outgrowth. Using dominant negative Rho
GTPases and a Rac1 specific inhibitor, the requirement for Rac1 activity in E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth as well as a role for Cdc42 activity in N-cadherin-mediated neurite outgrowth was demonstrated. Perturbation of the interaction between IQGAP1, a scaffolding protein known to bind to PTPµ and Cdc42, also resulted in a significant decrease in N-cadherin and R-cadherin-mediated neurite outgrowth. This data reiterates the fundamental importance of Rho GTPases in cadherin-mediated neurite outgrowth and identifies IQGAP1 as a key regulator of N-cadherin and R-cadherin-mediated neurite outgrowth. A requirement for the serine-threonine kinase PKCδ is known to regulate PTPµ-mediated neurite outgrowth (Ensslen and Brady-Kalnay, 2004). Perturbation of PKCδ resulted in a significant decrease in E-cadherin and R-cadherin-mediated neurite outgrowth, demonstrating a requirement for PKCδ in E-cadherin and R-cadherin-mediated neurite outgrowth. The experiments described in this dissertation demonstrate the requirement for unique signaling pathways in E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth. Whether these signaling pathways regulate cadherin-dependent adhesion via changes in confirmation or trans binding affinity (inside-out) or are activated in response to cadherin-dependent adhesion via trans binding (outside-in) is not clear. Thus, further studies on the mechanisms by which E-cadherin, N-cadherin and R-cadherin-mediated adhesion regulate axon outgrowth are needed.

Based on the studies presented in this dissertation and previously published work, I hypothesize the following in regard to PTPµ function in classical-cadherin-mediated neurite outgrowth. Since PTPµ catalytic activity is required for cadherin-mediated neurite outgrowth, PTPµ must be regulating the phosphorylation state of the
cadherins themselves or their associated proteins. The requirement for IQGAP1 in N-cadherin and R-cadherin-mediated neurite outgrowth also suggests that PTPµ binding partners indirectly regulate classical cadherin-mediated function. Inhibition of IQGAP1 association with Rac1 and Cdc42 does not completely abolish N-cadherin or R-cadherin-mediated neurite outgrowth. Therefore, other regulatory proteins not yet identified must be contributing to N-cadherin and R-cadherin-mediated neurite outgrowth. The identification of distinct signaling molecules in the regulation of classical cadherin-mediated neurite outgrowth and their distinct expression patterns in the visual system implies separate roles for E-cadherin, N-cadherin and R-cadherin in visual system development. Futures studies investigating these questions will surely make for at least another 22 years of stimulating findings!

4.2 Future Directions

4.2.1 The Cadherins and their Associated Proteins as Substrates of PTPµ

What is not clear from this dissertation and previous work is if PTPµ tyrosine phosphatase activity is acting directly on the cadherins or their associated proteins. A role for E-cadherin, N-cadherin and R-cadherin as direct substrates for PTPµ has not been identified, yet several studies suggest they are bona fide substrates. PTPµ associates with E-cadherin, N-cadherin and R-cadherin (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995). In addition, PTPµ binds directly to the 38 COOH-terminal residues of the E-cadherin cytoplasmic tail (Brady-Kalnay et al., 1998). Alignment of the cytoplasmic segment of E-cadherin, N-cadherin and R-cadherin reveals that the 38 amino acids of E-cadherin that are required for binding to PTPµ are 74% identical to the
corresponding N-cadherin and R-cadherin sequence, suggesting that PTPµ can bind directly to N-cadherin and R-cadherin in addition to E-cadherin. In fact, PTPµ is observed in a complex with N-cadherin and R-cadherin (Brady-Kalnay et al., 1998; Burden-Gulley and Brady-Kalnay, 1999; Phillips-Mason et al., 2006).

Six conserved tyrosine residues can be phosphorylated in the cytoplasmic tail of the classical cadherins. Of these, Tyr851 and Tyr883 reside in the 38 COOH terminal residues required for cadherin binding to PTPµ. Tyr883 has been identified as the major Src tyrosine phosphorylation site (Xu and Carpenter, 1999), making it an ideal candidate for dephosphorylation by PTPµ. We have previously reported that the association between PTPµ and the cadherins is regulated by changes in cadherin tyrosine phosphorylation (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995). For example, when E-cadherin tyrosine phosphorylation increases, the association between PTPµ and the E-cadherin decreases (Brady-Kalnay et al., 1998). The reverse also holds true, demonstrating that the phosphorylation state of E-cadherin regulates its association with PTPµ. To investigate the role of cadherin tyrosine phosphorylation in cadherin-mediated neurite outgrowth, expression of cadherins containing single point mutations of their cytoplasmic segment Tyr851 or Tyr883 residues in the retinal explant model system is required. Generation of phospho-specific cadherin antibodies to Tyr851 or Tyr883 would also aid in visualizing the changes in tyrosine phosphorylation of cadherin-mediated neurite outgrowth. Further biochemical analysis of the ability of PTPµ to directly dephosphorylate cadherins is also necessary.

A role for direct tyrosine phosphorylation in the regulation of cadherin function has been demonstrated. In MDCK cells, tyrosine phosphorylation of E-cadherin by the
Src family kinase, v-Src, disrupts E-cadherin function by ubiquination, endocytosis and degradation of E-cadherin in the lysosome (Fujita et al., 2002; Palacios et al., 2005; Shen et al., 2008). Interestingly, expression of constitutively active or dominant negative Src also disrupts E-cadherin-mediated adhesion (McLachlan and Yap, 2007), suggesting that tyrosine phosphorylation of E-cadherin at low levels is required for E-cadherin-mediated cell adhesion. This delicate balance of cadherin phosphorylation suggests tight regulation of kinase and phosphatase activity.

PTPµ binds to RACK1 and co-localizes to cell-cell contacts at high cell density (Mourton et al., 2001). RACK1 also binds to activated PKC (Mochly-Rosen and Gordon, 1998). Our lab has demonstrated that constitutively active Src disrupts PTPµ binding to RACK1 (Mourton et al., 2001). RACK1 is tyrosine phosphorylated by Src (Chang et al., 2002), and is a potential substrate for PTPµ. RACK1 association with Src is increased by active PKC and tyrosine phosphorylation of RACK1 (Chang et al., 2001). Because the association between PTPµ and RACK1 is lost in the presence of Src, the ability of RACK1 to recruit PKC to the cadherin/catenin complex may also be lost. Investigating the interplay between Src family kinases and RPTPs in cadherin-mediated neurite outgrowth would be a noteworthy next step.

PKCδ is one member of the PKC family of serine/threonine kinases and is required for E-cadherin and R-cadherin-mediated neurite outgrowth as demonstrated in Chapter 3. Tyrosine phosphorylation of PKCδ is proposed to regulate its activation and subsequent kinase activity (Steinberg, 2008). PKCδ activation is classically attributed to second messenger systems such as calcium and DAG, followed by PKCδ recruitment to its specific target via lipid cofactors (Steinberg, 2008). More recently,
phosphorylation of PKCδ has been demonstrated to regulate PKCδ activation (Steinberg, 2004; Steinberg, 2008). PKCδ has multiple tyrosine residues in the catalytic domain (Tyr512 and Tyr523), regulatory domain (Tyr52, Tyr64, Tyr155 and Tyr187) and hinge region (Tyr311 and Tyr332), each of which are independently regulated (Steinberg, 2008). PKCδ is a substrate for Src and Tyr311 is proposed to be the major tyrosine phosphorylation site (Rybin et al., 2007; Rybin et al., 2004; Rybin et al., 2008). PKCδ associates with PTPµ in E8 retina and retinal neuroepithelial cells (Rosdahl et al., 2002) and is a potential substrate for PTPµ. Furthermore, our lab has demonstrated a requirement for PKCδ activity in PTPµ-mediated neurite outgrowth (Ensslen and Brady-Kalnay, 2004; Rosdahl et al., 2002). Dephosphorylation of PKCδ may alter its subcellular location, which may alter PKCδ substrate availability and subsequent signaling. GAP-43, CAP-23 and MARCKS are three PKCδ substrates required for neurite outgrowth (Aigner and Caroni, 1993; Frey et al., 2000; Laux et al., 2000; Meiri et al., 1998). Preliminary data from our laboratory suggests that Tyr311 is dephosphorylated by PTPµ. Expression of PKCδ containing single point mutations at Tyr311 mimicking either the phosphorylated or dephosphorylated forms of PKCδ in the retinal explant model system would test the significance of Tyr311 phosphorylation in cadherin-mediated neurite outgrowth. I hypothesize that the PKCδ mutants would alter E-cadherin and R-cadherin-dependent neurite outgrowth but not N-cadherin-dependent neurite outgrowth.

An alternative mechanism for the regulation of cadherin-mediated neurite outgrowth via PTPµ is through tyrosine dephosphorylation of cadherin associated proteins. For example, the tyrosine phosphorylation of the cadherin associated protein
p120 regulates cadherin function (Alema and Salvatore, 2007). P120-catenin (p120), first identified as a Src tyrosine kinase substrate, binds directly to the cytoplasmic segment of the classical cadherins and has been shown to regulate cadherin turnover at the cell surface (Reynolds, 2007). For example, p120 blocks clathrin-mediated endocytosis of cadherins (Miyashita and Ozawa, 2007; Xiao et al., 2005). Although tyrosine phosphorylated p120 preferentially binds to cadherins in vivo (Kinch et al., 1995; Papkoff, 1997), tyrosine-phosphorylation-defective p120 mutants bind to and stabilize cadherins at the cell surface (Mariner et al., 2004; Xia et al., 2003). PTPµ binds to and dephosphorylates p120-catenin (Zondag et al., 2000). Therefore, PTPµ may keep p120 in a dephosphorylated state at the cadherin/catenin complex to regulate cadherin function. Expression of tyrosine-phosphorylation-defective p120 mutants in the chick visual system would demonstrate the requirement for p120 tyrosine phosphorylation in cadherin-mediated neurite outgrowth.

The potential for overlapping function between RPTP family members in visual system development cannot be ignored. Four other RPTPs have been identified in the chick visual system, PTPα, PTPγ, CRYP-2 and CRYPα also known as PTPσ (Ledig et al., 1999b). CRYP-2 is a growth inhibitory repulsive cue for RGC neurites resulting in growth cone collapse (Stepanek et al., 2001). PTPγ is also a growth inhibitory cue for NGF-induced neurite outgrowth of PC12 cells (Shintani et al., 2001). PTP1B, a non-receptor PTP binds directly to N-cadherin (Balsamo, 1998; Rhee et al., 2001) and is required for N-cadherin-mediated neurite outgrowth from chick RGCs (Pathre et al., 2001). LAR also associates with N-cadherin but requires the amino-terminal domain of β-catenin for this association (Kypta et al., 1996). Another RPTP, CRYPα is expressed
in chick RGCs and is required for intraretinal RGC axon outgrowth (Ledig et al., 1999a; Ledig et al., 1999b), growth cone morphology (Ledig et al., 1999a; Mueller, 2000) and axon targeting in the optic tectum (Rashid-Doubell et al., 2002). Recent studies demonstrate that CRYPα binds directly to and dephosphorylates N-cadherin *in vivo* and *in vitro* (Siu et al., 2007). In the absence of CRYPα, N-cadherin tyrosine phosphorylation is increased resulting in N-cadherin-mediated dorsal root ganglia (DRG) neurite outgrowth defects (Siu et al., 2007), demonstrating a role for tyrosine phosphatase activity in cadherin-mediated neurite outgrowth. *In vivo* injection of RPTP specific inhibitors, function blocking antibodies or antisense oligonucleotides alone or in combination in the chick visual system is needed. Characterizing the distinct and overlapping function of RPTPs in axon growth and guidance will be an important contribution to visual system development.

4.2.2 Other PTPµ Signaling Pathways and Associated Proteins

It is important to note that PTPµ binding partners are not necessarily tyrosine phosphatase substrates, and that PTPµ can regulate cadherin-mediated function through secondary effects such as recruiting other signaling molecules to the cadherin/catenin complex. For example, PTPµ binds directly to IQGAP1 independent of its phosphorylation state (Phillips-Mason et al., 2006) suggesting that IQGAP1 is not a substrate of PTPµ. IQGAP1 is a substrate for PKC and phosphorylation of IQGAP1 increases IQGAP1-mediated neurite outgrowth (Li et al., 2005). Therefore, regulation of IQGAP1 by PKC may alter the ability of IQGAP1 to associate with PTPµ subsequently regulating either N-cadherin or R-cadherin-mediated neurite outgrowth.
IQGAP1 can also regulate cadherin-mediated neurite outgrowth through its associated proteins. IQGAP1 functions to bind activated Cdc42 and to a lesser extent Rac1 (Hart et al., 1996; Joyal et al., 1997). As demonstrated in Chapter 3, IQGAP1/Cdc42 is required for N-cadherin and R-cadherin-mediated neurite outgrowth. Rac1 activity is also required for E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth. IQGAP1/Cdc42/Rac1 has been shown to be required for the inhibition of cadherin endocytosis (Akhtar and Hotchin, 2001; Izumi et al., 2004), suggesting that the Rho GTPases are not only required for regulation of the actin cytoskeleton but also cadherin stability at the cell surface in cadherin-mediated neurite outgrowth. What is not clear from Chapter 3 is how PTPµ mediates the Rho GTPases in cadherin-mediated neurite outgrowth. Two possible mechanisms are discussed below.

We observed inhibition of neurite outgrowth on an E-cadherin, N-cadherin and R-cadherin substrate in the presence of a cell permeable Rac1 inhibitor peptide (Chapter 3, Fig 3.8). Rac1 activity is regulated by the GEFs, GAPs and GDIs (Heasman and Ridley, 2008; Linseman and Loucks, 2008). The Rac1-specific compound inhibits Rac1 GDP/GTP exchange by blocking Rac1 interaction with the Rac-specific GEFs Trio and Tiam1 (Gao et al., 2004). In relation to cadherin function, Tiam1 stimulates E-cadherin-mediated cell-cell adhesion by activating Rac1 (Hordijk et al., 1997), demonstrating a role for Tiam1 in Rac1/E-cadherin-mediated cell-cell adhesion. Tiam1 tyrosine phosphorylation leads to Rac1 activation and formation of lamellipodia in fibroblasts (Miyamoto et al., 2006), as a result Tiam1 is a potential substrate for PTPµ. If this hypothesis were true, then PTPµ would inhibit Rac1 via Tiam1. This is
contradictory to the hypothesis that Rac1 activity is required for cadherin-mediated neurite outgrowth. One explanation is that the transient cadherin-mediated cell contacts formed during cell migration require constant cycling of Rac1 activity. Thus PTP\(\mu\) dephosphorylation of Tiam1 would contribute to the tight regulation of Rac1. Immunoprecipitation of tyrosine phosphorylated Tiam1, PTP\(\mu\) and the cadherins along with co-localization studies of PTP\(\mu\) with Tiam1 would support this hypothesis.

The Rac1-specific compound also blocks the interaction between Rac1 and the GEF Trio. Trio activates Rac1 through its D1 GEF domain (Debant et al., 1996; Estrach et al., 2002). Trio activation of Rac1 regulates netrin-1-mediated neurite outgrowth (Briancon-Marjollet et al., 2008). No known function for Trio in classical cadherin-mediated neurite outgrowth has been established. Trio may also be a substrate for PTP\(\mu\) given that Trio binds to another RPTP, LAR in the D2 domain (Debant et al., 1996). A high degree of sequence homology exists between RPTP D2 domains, therefore PTP\(\mu\) function may regulate classical cadherin-mediated neurite outgrowth through modulation of Trio and thus Rho GTPase activity. Expression of a Trio deletion mutant lacking the D1 GEF domain required for Rac1 activation in the chick retinal explant model system would demonstrate a specific role for Trio in cadherin-mediated neurite outgrowth.

4.2.3 *In vivo* Relevance of PTP\(\mu\) and Cadherin-Dependent Regulation of Axon Growth and Guidance in the Visual System

The complexity required for visual system development makes it a fascinating yet challenging system to work in. Based on the *in vitro* experiments described in this
dissertation, it is clear that PTP\(\mu\) and its associated proteins are required for classical cadherin-mediated chick RGC neurite outgrowth. But does this theory hold true in vivo? In the in vitro chick retinal explant model system, RGC axons are cultured on one specific growth promoting substrate. During development of the visual system, RGC axons are exposed to countless growth promoting or inhibitory guidance molecules with distinct and overlapping functions (Bao, 2008; Kamiguchi, 2007). Still, distinct defects in axon guidance are observed after in vivo perturbation of E-cadherin (Dumstrei et al., 2003a), N-cadherin (Iwai et al., 1997; Riehl et al., 1996; Treubert-Zimmermann et al., 2002) and R-cadherin (Babb et al., 2005). Perturbation of E-cadherin results in the abnormal trajectory of proximal axons during Drosophila neurogenesis (Dumstrei et al., 2003a). Expression of dominant negative N-cadherin in Xenopus resulted in RGC axon initiation elongation, and targeting defects (Riehl et al., 1996). Drosophila loss of function mutants for N-cadherin also resulted in axon pathfinding errors (Iwai et al., 1997). In zebrafish, knockdown of R-cadherin results in a small eye phenotype with defects in retinal lamination, neuroepithelial cell differentiation and axon extension (Babb et al., 2005). Axons that do extend out of the eye project to the contralateral tectum but do not properly arborize (Babb et al., 2005). Perturbation of E-cadherin or R-cadherin has not been demonstrated in the chick or higher vertebrate visual systems. Antibody inhibition, knockdown or conditional knockout models of E-cadherin, N-cadherin and R-cadherin in the visual system alone or in combination will demonstrate a functional role for the classical cadherins in axon outgrowth.

Furthermore, a functional role of PTP\(\mu\) in RGC axon guidance in vivo is lacking. PTP\(\mu\) is expressed in a gradient within the retina and optic tectum of the chick (Burden-
Gulley et al., 2002), suggesting that PTPμ functions as a guidance cue in vivo. PTPμ deficient mice do not demonstrate visual system defects (Koop et al., 2003), however axon guidance was not studied in detail. The lack of significant defects observed in visual system development of PTPμ null mice suggests that other RPTPs may be compensating for the loss of PTPμ in vivo. Therefore antibody inhibition, knockdown or RPTP double and triple knockout models are required to investigate the role of PTPμ in RGC axon growth and guidance.

4.3 Summary

In closing, the studies described in this dissertation establish a novel role for E-cadherin in chick RGC neurite outgrowth and characterize the cadherin binding specificity required for E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth. In addition, these studies demonstrate that PTPμ and its associated proteins differentially regulate E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth in vitro. Future studies characterizing the PTPμ signaling pathway-dependent regulation of the classical cadherins will contribute to a greater understanding of the basic question “How does and axon find its target?”


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