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Distinct PTPmu-associated signaling molecules differentially regulate neurite outgrowth on E-, N-, and R-cadherin

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Abstract

Classical cadherins play distinct roles in axon growth and guidance in the visual system, however, the signaling pathways they activate remain unclear. Growth cones on each cadherin substrate have a unique morphology suggesting that distinct signals are activated by neurite outgrowth on E-, N-, and R-cadherin. We previously demonstrated that receptor protein tyrosine phosphatase-mu (PTPmu) is required for E- and N-cadherin-dependent neurite outgrowth. In this manuscript, we demonstrate that PTPmu regulates R-cadherin-mediated neurite outgrowth. Furthermore, we evaluated whether known PTPmu-associated signaling proteins, Rac1, Cdc42, IQGAP1 and PKC δ , regulate neurite outgrowth mediated by these cadherins. While Rac1 activity is required for neurite outgrowth on all three cadherins, Cdc42/IQGAP1 are required only for N- and R-cadherin-mediated neurite outgrowth. In addition, we determined that PKC activity is required for E- and R-cadherin-mediated, but not N-cadherin-mediated neurite outgrowth. In summary, distinct PTP μ -associated signaling proteins are required to promote neurite outgrowth on cadherins.

Keywords

neurite outgrowth; cadherin; retina; chick; axon guidance; protein tyrosine phosphatase; cell-cell adhesion

Introduction

The classical cadherin subfamily of cell adhesion molecules, E-, N- and R-cadherin, are expressed in restricted and complimentary patterns throughout the retina and retinofugal pathway (Inuzuka et al., 1991a; Inuzuka et al., 1991b; Redies et al., 1993; Redies and Takeichi, 1993; Wohrn et al., 1998). While all three cadherins promote retinal ganglion cell (RGC) axon outgrowth *in vitro* (Bixby and Zhang, 1990; Oblander et al., 2007; Redies and Takeichi, 1993), their unique pattern of expression during development suggests that they play distinct roles in 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

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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; Liu et al., 1999; 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-Jones et al., 1999; Oblander et al., 2007; Xu et al., 2002), however *in vivo* perturbation of E-cadherin in the visual system has not been carried out. While it is evident that the classical cadherins are required for axon growth and guidance, the precise signaling cascades activated in response to E-, N- and R-cadherin-mediated axon outgrowth remain unclear.

Classical cadherins 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 (Halbleib and Nelson, 2006; Leckband and Prakasam, 2006; Nelson, 2008; Yap et al., 2007). Previous studies have evaluated the ability of purified N- and E-cadherin to function as substrates for neurite outgrowth (Bixby and Zhang, 1990; Burden-Gulley and Brady-Kalnay, 1999; Burden-Gulley et al., 2002; Oblander et al., 2007). These studies demonstrate that both N- and E-cadherin-mediated outgrowth require homophilic binding between identical cadherin molecules on the neurite and on the substrate (Oblander et al., 2007; Redies and Takeichi, 1993). The only previous study of R-cadherin-mediated neurite outgrowth suggested a heterophilic mechanism of action whereby N-cadherin on the cell surface binds to R-cadherin-transfected neuroblastoma cells (Redies and Takeichi, 1993), which express little if any endogenous cadherins (Matsunaga et al., 1988).

Intracellularly, the highly conserved cytoplasmic segment of the classical cadherins tethers the cadherins to the actin cytoskeleton via an association with the catenin family of proteins, α -catenin, β -catenin, γ -catenin/plakoglobin and p120 (see Fig. 11) as well as their associated proteins α -actinin, afadin, ajuba, formin, vinculin and ZO1 (Weis and Nelson, 2006). Association of cadherin with the cytoskeleton is required for cadherin-mediated cell-cell adhesion (Nelson, 2008; Yap et al., 2007). Since all classical cadherins bind catenins, distinct signal transduction would require additional binding partners to generate a unique signal.

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

In order to examine the intracellular signaling pathways required for E-, N- 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). We demonstrate a role for PTP μ in R-cadherin-mediated neurite outgrowth. We observe unique growth cone morphologies, suggesting that distinct signaling mechanisms underlie neurite outgrowth on a particular cadherin substrate. We present evidence that E-, N- and R-cadherin-mediated neurite outgrowth requires PTP μ associated signaling proteins including PKC δ , IQGAP1, Rac1 and Cdc42. E-cadherin mediated neurite outgrowth requires both Rac1 and PKC δ serine-threonine kinase. N-cadherin-mediated neurite outgrowth requires Rac1 and IQGAP1/Cdc42 activity. R-cadherin-mediated neurite outgrowth requires Rac1 and IQGAP1/Cdc42 activity but also

requires PKC serine-threonine kinases. Our results demonstrate that while PTP μ function is required for neurite outgrowth mediated by all three classical cadherins, each cadherin signals through distinct PTP μ associated pathways.

Results

Purified R-cadherin promotes neurite outgrowth

In order to determine whether purified R-cadherin can 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 an R-cadherin substrate after 24 hours in culture (Fig 1A). 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 an R-cadherin substrate (length = 206 ± 46 μm ; density = 8705 ± 3899 μm^2). Longer, more fasciculated neurites were observed at E7 (length = 552 ± 91 μm ; density = 15566 ± 2734 μm^2). Similar to E- and N-cadherin-mediated neurite outgrowth (Burden-Gulley et al., 2002; Oblander et al., 2007) maximal neurite outgrowth on R-cadherin was observed at E8 (length = 1070 ± 37 μm ; density = 25180 ± 3827 μm^2). At E9 (length = 703 ± 44 μm ; density = 19907 ± 3968 μm^2) and E10 (length = 754 ± 72 μm ; density = 20695 ± 3512 μm^2) neurite outgrowth is still observed on an R-cadherin substrate although outgrowth is less robust than at E8.

Observations of chick RGC neurite-outgrowth on an E- 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 (Burden-Gulley et al., 2002; Oblander et al., 2007). Since robust neurite outgrowth was observed at E8, we next examined whether the RGC 3-dimensional position within the retina also influenced R-cadherin-mediated neurite outgrowth. Substantial R-cadherin-mediated neurite outgrowth was observed from all regions of the retina (Fig. 1B), unlike N-cadherin (Fig. 1C). The most robust RGC neurite outgrowth on R-cadherin was observed primarily in the ventral region of the retina [ventral-nasal (length = 1113 ± 34 μm ; density = 34241 ± 3860 μm^2) and ventral-temporal (length = 1205 ± 42 μm ; density = 36526 ± 2904 μm^2)], while dorsal-temporal (length = 1003 ± 37 μm ; density = 31041 ± 1975 μm^2) and dorsal-nasal (length = 1011 ± 62 μm ; density = 33151 ± 4876 μm^2) regions had slightly lower levels of neurite outgrowth on R-cadherin. Neurites from the dorsal-nasal region of the retina were shorter (980 ± 90 μm versus 1245 ± 57 μm) and less dense (17311 ± 3313 μm^2 versus 54751 ± 7149 μm^2) than those in the ventral-nasal region on N-cadherin. 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.

To confirm that R-cadherin-mediated neurite outgrowth requires N-cadherin expression in RGCs, suggestive that heterophilic binding is required, E8 retinal explants were cultured on laminin, E-, N- or R-cadherin substrate in the absence (Fig. 2A–D) or presence (Fig. 2E–H) of a function-blocking monoclonal antibody to chick N-cadherin (NCD-2). The NCD-2 antibody is N-cadherin chick specific (Hatta and Takeichi, 1986) 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. 2E, F). NCD-2 blocked neurite outgrowth on an N-cadherin substrate (Fig. 2G; (Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007). NCD-2 also blocked neurite outgrowth on an R-cadherin substrate (Fig. 2H). These results concur with previous studies (Redies and Takeichi, 1993) that R-cadherin-mediated neurite outgrowth can occur via a heterophilic interaction with N-cadherin. At present, chick-specific adhesion-blocking R-cadherin antibodies are not available.

We confirmed the specificity of the NCD-2 antibody to chick N-cadherin by immunoblotting the purified human E-cadherin-Fc, N-cadherin-Fc, R-cadherin-Fc and E8 chick retina lysate

with the NCD-2 antibody. The NCD-2 antibody recognized one band in the E8 chick retina lysate corresponding to N-cadherin but not to the human N-cadherin-Fc (Fig. 2I), confirming that NCD-2 is specific to chick N-cadherin. Antibodies that recognize the intracellular domains of E- or N-cadherin, and a chick specific antibody to the extracellular segment of R-cadherin (RCD-2) were used to confirm the presence of all three classical cadherins in the E8 chick retina lysate. The RCD-2 antibody does not block R-cadherin-mediated adhesion (Redies et al., 1993). Antibodies that recognize the extracellular domain of human E-cadherin, human/chick N-cadherin and human R-cadherin confirmed the presence of each specific cadherin-Fc.

It is interesting that neurite outgrowth is observed in the dorsal-nasal region of the retina on an R-cadherin substrate (Fig. 1B) but to a far lesser extent on an N-cadherin substrate (Fig. 1C), suggesting that R-cadherin is recognized by dorsal-nasal RGCs as a distinct substrate. In order to determine if the dorsal-nasal neurite outgrowth observed on an R-cadherin substrate involves heterophilic binding of N-cadherin, E8 retinal explants from the nasal region of the retina were cultured in the presence of NCD-2. NCD-2 had no effect on laminin-mediated neurite outgrowth (Fig. 3A). Any neurite outgrowth observed in the dorsal-nasal region of the retina on an N-cadherin substrate was blocked in the presence of NCD-2 (Fig. 3B). In addition, NCD-2 blocked neurite outgrowth from the ventral-nasal region of the retina on N-cadherin (Fig. 3B) as previously demonstrated in Figure 2. Neurite outgrowth observed in the dorsal-nasal region of the retina on an R-cadherin substrate was blocked by the addition of NCD-2 (Fig. 3C), confirming that the neurite outgrowth observed on an R-cadherin substrate likely involves heterophilic binding of N-cadherin. Furthermore the ability of R-cadherin, and to a lesser extent N-cadherin, to promote neurite outgrowth from the dorsal-nasal region of the retina suggests that the N/R-cadherin binding is a unique signal.

Unique growth cone morphology on E-, N- or R-cadherin substrates

We previously observed distinct growth cone morphologies from retinal axons extending onto an E-cadherin substrate versus an N-cadherin substrate, suggesting that unique signaling pathways occur in these structures upon binding to the different substrates (Oblander et al., 2007). To examine the morphology of growth cones on R-cadherin, we used DiI, a lipophilic membrane dye, to highlight individual growth cones. While diverse morphologies were present in each culture, overall, growth cones on a laminin substrate were small with few filopodia (Fig. 4A), consistent with previously published work (Bixby and Zhang, 1990; Oblander et al., 2007; Payne et al., 1992). On an E-cadherin substrate, very broad growth cones were observed with few filopodia (Fig. 4B), as previously reported (Oblander et al., 2007). Growth cones on an N-cadherin substrate had many filopodial processes (arrowheads) with a moderate growth cone area (Fig. 4C; Burden-Gulley and Brady-Kalnay, 1999). The R-cadherin growth cone morphology is a hybrid between the E-cadherin and N-cadherin morphology (Fig. 4D). Growth cones on an R-cadherin substrate were fairly broad with several filopodia (Fig. 4D). Quantitation of the average number of filopodia per growth cone demonstrates that R-cadherin growth cones have significantly more filopodia compared to E-cadherin ($p \leq 0.01$) but significantly less than N-cadherin ($p \leq 0.0001$) (Fig. 4E). Furthermore, the average area of a growth cone on an R-cadherin substrate is $152 \mu\text{m}^2$, slightly less than E-cadherin ($184 \mu\text{m}^2$) but not significant. The growth cone area on R-cadherin is greater than N-cadherin ($126 \mu\text{m}^2$) ($p \leq 0.05$) (Fig. 4F) suggesting that growth cone area of R-cadherin is more similar to E-cadherin. Taken together, R-cadherin growth cone morphology is distinct from E- or N-cadherin suggesting that all three cadherins activate distinct signaling pathways to mediate neurite outgrowth.

PTP μ is required for R-cadherin-mediated neurite outgrowth

To investigate a functional role for endogenous PTP μ in R-cadherin-mediated neurite outgrowth, E8 chick retinal explants were cultured in the presence of scrambled control

(SPTP μ -Tat) (Fig. 5A–D) or a PTP μ wedge inhibitor peptide (WPTP μ -Tat) (Fig. 5E–H) on a laminin (Fig. 5A, E), E- (Fig. 5B, F), N- (Fig. 5C, G) or R-cadherin (Fig. 5D, H) substrate for 24 hours. The PTP μ specific WPTP μ -Tat peptide sequence (Xie et al., 2006) corresponds to the HLH wedge shaped sequence (Hoffmann et al., 1997), located in the juxtamembrane domain of PTP μ near the D1 catalytic domain. By mimicking PTP μ 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 WPTP μ -Tat peptide regulates PTP μ function or catalytic activity (Xie et al., 2006). Each peptide includes a Tat-derived domain linked to the C terminus, which allows for uptake of the peptide into the cell. Incubation with WPTP μ -Tat had no effect on laminin-dependent neurite outgrowth (Fig. 5E) when compared to SPTP μ -Tat control (Fig. 5A). Similar to previously reported data (Oblander et al., 2007), inhibition of neurite outgrowth on an E-cadherin (Fig. 5F) and N-cadherin (Fig. 5G) substrate was observed in the presence of WPTP μ -Tat when compared to SPTP μ -Tat control (Fig. 5B, C). Quantitation of neurite outgrowth demonstrated a decrease in neurite length by 49% (Fig. 5I) and 65% in neurite density (Fig. 5J) on an E-cadherin substrate. On an N-cadherin substrate, neurite length decreased by 36% (Fig. 5I) and neurite density decreased by 62% (Fig. 5J). R-cadherin-mediated neurite outgrowth (Fig. 5H) was also inhibited in the presence of the WPTP μ -Tat when compared to SPTP μ -Tat control (Fig. 5D). On an R-cadherin substrate, neurite length decreased by 50% (Fig. 5I) and neurite density decreased by 71% (Fig. 5J). The significant decrease in neurite length and density in the presence of WPTP μ -Tat observed on an R-cadherin substrate demonstrates the importance of PTP μ catalytic activity in R-cadherin-dependent neurite outgrowth.

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 μ is through the Rho subfamily of small G-proteins. Rac1 and Cdc42 regulate neurite outgrowth and growth cone morphology of most neurons including on a PTP μ substrate (Major and Brady-Kalnay, 2007). In order to test a role for the Rho GTPases in cadherin-mediated neurite outgrowth, E8 chick retinal explants were infected with HSV encoding GFP control (Fig. 6A–C), dominant negative Cdc42 (Cdc42 DN) (Fig. 6D–F), Rac1 (Rac1 DN) (Fig. 6G–I), or RhoA (RhoA DN) (Fig. 6J–L) and cultured in the presence of the virus on different substrates. Previous studies have demonstrated a role for Cdc42 activity in laminin-mediated neurite outgrowth in PC12 cells (Weston et al., 2000). A slight decrease in neurite density was observed on laminin in retinal neurons in the presence of Cdc42 DN (Fig. 6D), but was not statistically significant (Fig. 6M, N). Likewise, dominant negative Rac1 or RhoA had no effect on laminin-dependent neurite outgrowth (Fig. 6G, J) when compared to GFP control (Fig. 6A). A decrease in neurite outgrowth was observed when retinal explants were infected with Rac1 DN on an E-cadherin substrate (Fig. 6H) but not with Cdc42 DN (Fig. 6E) or RhoA DN (Fig. 6K). Quantitation of these experiments demonstrated that neurite length and density were significantly reduced on E-cadherin by 54% (Fig. 6M) and 63% (Fig. 6N), respectively, in the presence of Rac1 DN virus. On an N-cadherin substrate, a decrease in neurite outgrowth was observed when explants were infected with Cdc42 DN (Fig. 6F) and Rac1 DN (Fig. 6I) but not RhoA DN (Fig. 6L). Neurite length and density were significantly reduced by 60% (Fig. 6M) and 71% (Fig. 6N), respectively, 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. 6M) and neurite density decreased by 45% (Fig. 6N) on an N-cadherin substrate.

Infection of retinal explants with control HSV encoding GFP had nonspecific effects on neurites cultured on an R-cadherin substrate, therefore we utilized the protein transduction reagent Chariot to deliver control (Fig. 7A, B), dominant negative Cdc42 (Fig. 7C, D) or Rac1 (Fig. 7E, F) recombinant proteins or the RhoA inhibitor C3 transferase (Fig. 7G, H) into the RGCs and cultured for 24 hours. Transduction with Cdc42, Rac1 or C3 transferase had no

effect on laminin-dependent neurite outgrowth (Fig. 7C, E, G) when compared to vehicle control (Fig. 7A). On an R-cadherin substrate, a decrease in neurite outgrowth was observed when explants were transduced with Cdc42 DN (Fig. 7D) and Rac1 DN (Fig. 7F), but not when treated with C3 transferase that inhibits RhoA (Fig. 7H). Neurite length was significantly decreased by 63% (Fig. 7I) and neurite density was significantly decreased by 74% (Fig. 7J) when retinal explants were transduced with Cdc42 DN. When transduced with Rac1 DN, neurite length and density were also significantly decreased by 48% (Fig. 7I) and 81% (Fig. 7J), respectively. These data demonstrate that only Rac1 activity is required for E-cadherin-mediated neurite outgrowth, while both Cdc42 and Rac1 activities are required for N- and R-cadherin-mediated neurite outgrowth.

To test whether endogenous Rac1 activity is required for neurite outgrowth on the three classical cadherins, a cell-permeable Rac1-specific small molecule inhibitor was used in outgrowth experiments. 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). The Rac1-specific inhibitor does not affect the activity of Cdc42 or RhoA (Gao et al., 2004). Incubation with control (Fig. 8A–D) or the Rac1 inhibitor (Fig. 8E–H) had no effect on laminin-dependent neurite outgrowth (Fig. 8E) when compared to vehicle control (Fig. 8A). A decrease in neurite outgrowth was observed on E- (Fig. 8F), N- (Fig. 8G), and R-cadherin (Fig. 8H) substrates in the presence of the Rac1 inhibitor. Quantitation of these experiments demonstrated that neurite length decreased by 69%, 88%, and 67%, respectively, on the three substrates (Fig. 8I). Neurite density was decreased by 79%, 96%, and 84%, respectively, on the three substrates (Fig. 8J). These data confirm the importance of Rac1 activity in cadherin-dependent neurite outgrowth.

IQGAP1 interaction with Cdc42 contributes to N- 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 et al., 1997), and stabilizes their active conformation. In addition, IQGAP1 and PTP μ bind directly (Phillips-Mason et al., 2006). To determine whether IQGAP1 activity is also required for cadherin-mediated neurite outgrowth, retinal explants were cultured in the presence of a cell-permeable, Tat-tagged IQGAP1 inhibitor peptide (Fig. 9E–H) or scrambled control (Fig. 9A–D). The IQGAP1 inhibitor peptide competes for binding to the Cdc42 and Rac1 binding site on IQGAP1 (Mataraza et al., 2003). Neurite outgrowth on laminin (Fig. 9E) and E-cadherin (Fig. 9F) was unaffected in the presence of the IQGAP1 inhibitor when compared to scrambled control (Fig. 9A, B). A decrease in neurite outgrowth on an N-cadherin substrate was observed in the presence of the IQGAP1 inhibitor peptide (Fig. 9G) when compared to scrambled control (Fig. 9C). Neurite outgrowth on an R-cadherin substrate was also reduced in the presence of the IQGAP1 inhibitor peptide (Fig. 9H) when compared to scrambled control (Fig. 9D). Quantitation of these experiments demonstrated a decrease in neurite length by 31% (Fig. 9I) and neurite density decreased by 59% (Fig. 9J) on an N-cadherin substrate. On an R-cadherin substrate, neurite length decreased by 36% (Fig. 9I) and neurite density decreased by 50% (Fig. 9J). This data suggests that the Cdc42/Rac1 interaction with IQGAP1 contributes to N- and R-cadherin-mediated neurite outgrowth but is not necessary for E-cadherin-mediated outgrowth.

PKC δ is required for E- and R-cadherin-mediated neurite outgrowth

The PTP μ binding protein RACK1 interacts with PKC δ and PKC δ activity is required for PTP μ -dependent outgrowth (Rosdahl et al., 2002; Ensslen and Brady-Kalnay, 2004). To evaluate whether PKC signaling is also required for neurite outgrowth on classical cadherins, likely downstream of PTP μ , we used the PKC δ inhibitor Rottlerin (Fig. 10). Treatment of E8 retinal explants with DMSO control (Fig. 10A–D) or 0.6 μ M Rottlerin (Fig. 10E–H) had no

effect on laminin- (Fig. 10A, E) or N-cadherin- (Fig. 10C, G) mediated neurite outgrowth. Decreased neurite outgrowth was observed on E-cadherin (Fig. 10F) and R-cadherin (Fig. 10H) in the presence of Rottlerin. E-cadherin-mediated neurite outgrowth decreased in length by 41% (Fig. 10Q) and neurite density decreased by 63% (Fig. 10R) in the presence of Rottlerin. Neurite length and density significantly decreased on an R-cadherin substrate by 50% (Fig. 10Q) and 59% (Fig. 10R), respectively.

Since Rottlerin can affect the activity of other kinases (Gschwendt et al., 1994; Soltoff, 2007), we wanted to confirm a role for PKC δ and potentially implicate other PKCs in cadherin-mediated neurite outgrowth. We previously identified six different PKC isoforms in E8 chick retina in addition to PKC δ (Rosdahl et al., 2002). We cultured E8 retinal explants in the presence of two other PKC chemical inhibitors Gö6976 or Gö6983. Gö6983 is a cell-permeable inhibitor that blocks the kinase activity of PKC α , PKC β , PKC δ , PKC γ and PKC ζ (Gschwendt et al., 1996; Gschwendt et al., 1998). Gö6976 is a cell permeable inhibitor that selectively blocks the Ca²⁺ dependent PKCs, PKC α and PKC β_1 but does not block the kinase activity of PKC δ (Gschwendt et al., 1998; Martiny-Baron et al., 1993). Rottlerin does not affect the activity of PKC γ and PKC ζ at this concentration (Gschwendt et al., 1994). Therefore, by comparing the inhibition of neurite outgrowth in the presence of each PKC inhibitor we can confirm the requirement for PKC δ in cadherin-mediated neurite outgrowth.

E8 retinal explants cultured in the presence of either 0.4 μ M Gö6976 (Fig. 10I-L) or 1.2 μ M Gö6983 (Fig. 10M-P) had no effect on laminin-mediated neurite outgrowth (Fig. 10I, M) or N-cadherin-mediated neurite outgrowth (Fig. 10K, O). A decrease in neurite outgrowth was observed on an E-cadherin substrate in the presence of Gö6983 (Fig. 10N) but not Gö6976 (Fig. 10J), suggesting that neither PKC α nor PKC β_1 is required for E-cadherin-mediated neurite outgrowth. E-cadherin-mediated neurite outgrowth decreased in length by 58% (Fig. 10Q) and neurite density decreased by 69% (Fig. 10R) in the presence of Gö6983. Neurite outgrowth decreased on an R-cadherin substrate in the presence of both Gö6976 and Gö6983 (Fig. 10L, P), when compared to vehicle control (Fig. 10D). Quantitation of these experiments demonstrated that neurite length on an R-cadherin substrate decreased by 33% (Fig. 10Q) and neurite density decreased by 63% (Fig. 10R) in the presence of Gö6976. In the presence of Gö6983, neurite length on an R-cadherin substrate decreased by 72% (Fig. 10Q) and neurite density decreased by 83% (Fig. 10R). While Rottlerin, Gö6976 and Gö6983 had 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-dependent neurite outgrowth are distinct from N-cadherin homophilic binding-mediated neurite outgrowth. We conclude that PKC δ serine-threonine kinase signaling is required for E-cadherin-mediated neurite outgrowth and that there is a unique requirement for PKC δ and additional PKCs, potentially PKC α or β_1 , in R-cadherin-mediated neurite outgrowth.

Discussion

The studies presented in this paper demonstrate that distinct signals are required to mediate RGC neurite outgrowth on the classical cadherins E-, N-, and R-cadherin. Our results are summarized in a model presented in Figure 11. The first aspect of the model that should be considered is the mode of recognition between the cadherin molecules on the substrate and the RGC neurites. In general, classical cadherins are homophilic cell-cell adhesion molecules (Halbleib and Nelson, 2006). However, heterophilic interactions of cadherins have been observed (Duguay et al., 2003; Foty and Steinberg, 2005; Niessen and Gumbiner, 2002). In our model, we suggest that both N- and E-cadherin interact homophilically to mediate outgrowth, but that R-cadherin may interact heterophilically with N-cadherin to mediate outgrowth (Fig. 11). This assertion is supported by the fact that when L-cells, which do not

express endogenous cadherins and do not spontaneously aggregate, are transfected with cDNA encoding N-cadherin or R-cadherin and are 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. Our experiments show that R-cadherin-mediated outgrowth is blocked by the addition of the chick-specific N-cadherin function-blocking antibody, NCD-2. This demonstrates that R-cadherin-mediated neurite outgrowth requires N-cadherin molecules on the RGC neurite, potentially implicating a heterophilic R-cadherin/N-cadherin interaction (Fig. 11). It is also possible that R-cadherin-mediated neurite outgrowth is homophilic but requires *cis* interactions with N-cadherin on the RGC neurite (Shan et al., 2000) (see Figure 11).

Reversible tyrosine phosphorylation of cadherins serves as a key regulatory mechanism for cadherin-mediated adhesion. 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 PTP μ associates with E-, N- and R-cadherin (Brady-Kalnay et al., 1998; Brady-Kalnay et al., 1995) and is required for E-cadherin-mediated cell adhesion (Hellberg et al., 2002). Within the chick retina, RGCs express PTP μ during axon extension (Burden-Gulley and Brady-Kalnay, 1999). Our laboratory has previously demonstrated that PTP μ expression and catalytic activity are required for both E- and N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007). In the current study, we demonstrate that PTP μ activity is also required for R-cadherin-mediated neurite outgrowth. We hypothesize that PTP μ alters cadherin function by recruiting different regulatory molecules to the cadherin/catenin complex thus distinctly regulating cadherin-mediated adhesion/neurite outgrowth (Fig. 11).

The Rho GTPase subfamily members are likely candidates for signaling molecules that may be recruited by PTP μ to regulate cadherin-mediated outgrowth. Cdc42, Rac1 and RhoA play a central role in regulating cell adhesion through dynamic rearrangement of the cytoskeleton, reviewed in (Heasman and Ridley (2008)). Rho GTPases have also been implicated in regulating axon guidance and neurite outgrowth (Koh, 2006; Linseman and Loucks, 2008). Activation of Cdc42 induces filopodia formation while Rac1 activation promotes lamellipodia formation. One means of regulating the Rho GTPases is through binding to cytoplasmic proteins that stabilize the active forms of the proteins or that recruit GTPases to their substrates. IQGAP1 is an example of one such protein. 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 et al., 1997).

Cdc42, Rac1 and RhoA are expressed in the chick retina and are involved in PTP μ -mediated growth cone rearrangement and neurite outgrowth (Major and Brady-Kalnay, 2007; Rosdahl et al., 2003). PTP μ promotes neurite outgrowth from nasal RGCs but is repulsive to temporal RGCs (Burden-Gulley et al., 2002). Cdc42 activity is required for PTP μ -mediated nasal outgrowth and temporal repulsion. Temporal repulsion of these neurites also requires inhibition of Rac1 activity (Major and Brady-Kalnay, 2007). We have also previously shown that IQGAP1 binds directly to PTP μ and that the IQGAP1/PTP μ association increases in the presence of activated Cdc42 (Phillips-Mason et al., 2006), which is required for PTP μ -mediated neurite outgrowth (Phillips-Mason et al., 2006).

In this manuscript, we determined that Rac1 activity is required for E-, N-, and R-cadherin-mediated neurite outgrowth. The Rac1 inhibitor that was used blocks the interaction of Rac1 with its GEFs Trio and Tiam-1 (Gao et al., 2004). Trio is a GEF that binds the RPTP LAR

(Debant et al., 1996). Tiam-1 is known to regulate cadherin-dependent adhesion (Kraemer et al., 2007; Malliri et al., 2004). Future studies will investigate Trio and Tiam-1 binding to PTP μ and the cadherins in the retina. Our results also identified a requirement for Cdc42 activity in N- 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 demonstrated that an interaction between Cdc42/Rac1 and IQGAP1 likely contributes to N- and R-cadherin-mediated neurite outgrowth. We hypothesize that PTP μ recruits IQGAP1/Cdc42 to N- and R-cadherin to regulate neurite outgrowth (Fig. 11). IQGAP1 binds β -catenin, which displaces cadherin from binding β -catenin and downregulates cell adhesion (Fukata et al., 1999). Our hypothesis is that IQGAP1 binding to PTP μ would prevent IQGAP1 binding to β -catenin, which may stabilize cadherin-dependent cell adhesion. IQGAP1 binding to PTP μ is enhanced in the presence of Cdc42 (Phillips-Mason et al., 2006), therefore, IQGAP1 would likely couple PTP μ to Cdc42. Because IQGAP1 was not required for E-cadherin-mediated retinal neurite outgrowth we suggest that Rac1 is recruited to E-cadherin via an alternative mechanism (Fig. 11).

It is important to consider whether cross-talk between the Rho GTPases may be occurring in our study of the regulation of cadherin-mediated neurite outgrowth. We previously examined whether inhibiting one Rho GTPase in neurite outgrowth assays may be having unintended secondary effects on other Rho GTPases (Major and Brady-Kalnay, 2007). Using different combinations of DN-Rho GTPases we demonstrated that there is no effect of combination therapy versus single treatment of a DN-Rho GTPase on PTP μ -mediated neurite outgrowth (Major and Brady-Kalnay, 2007). We predict therefore that alteration of neurite outgrowth observed in our present study is due to the addition of the DN-Rho GTPases and not secondary effects caused by changes in activation of other Rho GTPases. In addition, the Rac1 specific inhibitor has no effect on Cdc42 or RhoA (Gao et al., 2004). Furthermore, each Rho GTPase exhibited distinct effects on each cadherin substrate.

We hypothesize that PTP μ also plays a role in recruiting the protein kinase C (PKC) family of lipid-dependent serine-threonine kinases to regulate cadherin-mediated neurite outgrowth. 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 and Garcia de Herreros, 1995). Using the PKC δ inhibitor Rottlerin, we demonstrate here that PKC δ serine-threonine kinase signaling is required for E- and R-cadherin-mediated neurite outgrowth but not N-cadherin-mediated neurite outgrowth. Furthermore, we show that R-cadherin-mediated neurite outgrowth may require additional PKCs.

Rottlerin was originally described as a PKC inhibitor with specificity for PKC δ (Gschwendt et al., 1994). After this initial publication, it was reported that Rottlerin is ineffective at directly inhibiting PKC δ activity but likely inhibits the enzyme by an indirect mechanism via ATP depletion, reactive oxygen species generation and altering PKC δ tyrosine phosphorylation (Soltoff, 2007). Additional studies have demonstrated, however, that Rottlerin does decrease PKC δ activity (Chaudhuri et al., 2005; Fan et al., 2006; Fan et al., 2009). In addition, treatment of various neuronal cell types with Rottlerin results in data comparable to that of the same neuronal cell types treated with dominant negative PKC δ (Zhang et al., 2007), neurons from PKC δ knockout mice (Zhang et al., 2007; Zhang et al., 2009), PKC δ -small interfering RNA (Zhang et al., 2007) or catalytically inactive PKC δ K376R (Kanthasamy et al., 2008) supporting the assertion that Rottlerin inhibits PKC δ activity.

Activated PKC binds to the scaffolding protein RACK1 and is then recruited to the plasma membrane where it can phosphorylate its substrates (Sklan et al., 2006). PTP μ is found in a complex with RACK1 (Mourton et al., 2001) and PKC δ in cultured retinal cells (RNEs) and in E8 retinal tissue (Rosdahl et al., 2002). PKC δ activity and the interaction between PKC δ

and RACK1 are required for PTP μ -mediated neurite outgrowth (Ensslen and Brady-Kalnay, 2004; Rosdahl et al., 2002). Furthermore, a multimolecular complex consisting of PKC β_{II} , RACK1, PTP μ , β -catenin, and E-cadherin among other proteins was identified in mouse kidney collecting duct epithelial cells (Chattopadhyay et al., 2003). Disruption of the PKC β_{II} /RACK1 interaction blocked the association of the multimolecular complex with α -actinin and resulted in a decrease in cell-cell adhesion, demonstrating that PKC β_{II} is required to stabilize the PTP μ /E-cadherin/ β -catenin complex with the actin cytoskeleton. We therefore hypothesize that PTP μ /RACK1/PKC δ is recruited to the cadherin/catenin complex at the cell membrane where PKC δ may regulate E- or R-cadherin-dependent processes, but not N-cadherin-dependent neurite outgrowth (Fig. 11).

The difference in the ability of retinal neurites from the dorsal-nasal quadrant of the retina to grow on a substrate of R- but not N-cadherin warrants further examination. We speculate that in this retinal quadrant the signals required for R-cadherin outgrowth are present whereas those required for N-cadherin outgrowth are missing. Those signals could be at the level of the receptor or within the neurites. Considering the former, perhaps the levels of N-cadherin receptor expressed on the surface of the RGCs are too low in this retinal quadrant for homophilic *trans* interactions to mediate N-cadherin-dependent neurite outgrowth, but is sufficient to act in concert either in *cis* or *trans* with R-cadherin on RGCs to mediate outgrowth on R-cadherin. The absence of a chick R-cadherin specific function-blocking antibody precludes us from testing this hypothesis at the moment. Alternatively, yet unidentified signals downstream of N-cadherin may be lacking in the dorsal-nasal retina that are not required for R- and E-cadherin-mediated outgrowth. Our current study supports this hypothesis given that the signals required for neurite outgrowth are not the same for all three cadherins. Furthermore, differential signaling via PKC δ in the nasal and temporal retina has been demonstrated to explain the differential responses of RGC neurites to a substrate of PTP μ (Ensslen and Brady-Kalnay, 2004). Future studies that identify distinct N-cadherin signals may help test these hypotheses.

To summarize, the studies we present in this manuscript demonstrate that distinct signal transduction pathways are required for neurite outgrowth mediated by individual classical cadherins. We hypothesize that PTP μ recruits different adapter proteins to distinct cadherin complexes to transduce signals on that particular substrate. For E-cadherin-mediated neurite outgrowth, PTP μ , PKC δ , and Rac1 are required (Fig. 11). For N-cadherin-mediated neurite outgrowth, PTP μ , Rac1, and Cdc42 activities, but not PKC activity are required (Fig. 11). Finally, for R-cadherin-mediated neurite outgrowth, PTP μ , Rac1, Cdc42, and PKCs are required (Fig. 11). We hypothesize that the differences we observed in growth cone morphology on the purified cadherins can be at least partially explained by these distinct signaling pathways. Future studies will also determine whether the above-mentioned signaling pathways regulate cadherin-dependent outgrowth via inside-out or outside-in mechanisms.

Experimental Methods

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). A mouse monoclonal to E-cadherin (HECD-1), a generous gift from Margaret Wheelock at the University of Nebraska Medical Center, was used as an antibody control. 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).

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. A dose response curve was performed for each substrate and the minimum concentration of substrate required to observe robust neurite outgrowth on that particular cell adhesion molecule was used in all subsequent experiments. 0.35 μ g of E-cadherin-Fc, 0.065 μ g of N-cadherin-Fc, 0.35 μ g R-cadherin-Fc, 1 μ g of BTI laminin or 4 μ g 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 (1992))) 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 and Lemmon, 1990; Halfter et al., 1983). Briefly, neural retinas were flattened on concanavalin-coated nitrocellulose filters and cut into 350 μ m-wide explants (either parallel or perpendicular to the optic fissure). 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, and 0.025 μ g/ml amphotericin (Sigma). All explants were incubated at 37°C for 24 hours, fixed in 4% paraformaldehyde, 0.1% glutaraldehyde and imaged using a SPOT-RT digital camera and image acquisition software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Antibody inhibition studies

Ascites for the function blocking rat monoclonal anti-chicken N-cadherin blocking antibody, NCD-2 (Hatta and Takeichi, 1986), was used at 1:100. An isotype matched mouse IgG2a 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.

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 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.

PTP μ inhibitor peptide studies

6.5 μ M of the PTP μ wedge peptide (WPTP μ -Tat) or scrambled control (SPTP μ -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).

Rho GTPase studies

We utilized the HSV-1 vector pHSV-IRES-GFP-MCS previously described (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). 7 μ l 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.

E8 retinal explants were transduced with 8 μ g of dominant negative Cdc42, dominant negative Rac1 or exoenzyme C3 transferase using the protein transduction reagent Chariot (Active Motif, Carlsbad, CA) at the time of plating. Dominant negative Cdc42 and Rac1 recombinant proteins are GST tagged and have a single point mutation (T17N) that abolishes the protein's affinity for GTP and reduces its affinity for GDP (Catalog #C17G01 and #R17G01, Cytoskeleton, Denver, CO). Exoenzyme C3 transferase is an ADP ribosyl transferase that inhibits RhoA via ribosylation at asparagine residue 41 (Catalog #CT03, Cytoskeleton).

The cell permeable small molecule NSC23766 specifically inhibits Rac1 GDP/GTP exchange activity by blocking the Rac1 interaction with Rac-specific GEFs Trio and Tiam1 ($IC_{50} \sim 50$ μ M) (Catalog #553502; Calbiochem, San Diego, CA). A final concentration of 60 μ M Rac1 inhibitor was added at the time of plating.

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 (Mataraza et al., 2003; Phillips-Mason et al., 2006). In brief, the IQGAP1 peptide corresponds to amino acids 1054–1077 of IQGAP1 plus the N-terminal TAT sequence (GRKKRRQRRRMVVSFNRGARGQNALRQILAPVVK), which was originally developed by Dr. David Sacks (Mataraza et al., 2003) and synthesized by Genemed Synthesis, San Francisco, CA). A final concentration of 12 μ M peptide was added at the time of plating. Both peptides include a membrane-penetrant Tat-derived sequence, which promotes cellular uptake of the peptide (Wadia and Dowdy, 2002).

PKC δ inhibition studies

The cell-permeable protein kinase C inhibitor Rottlerin (Calbiochem), that exhibits selectivity for PKC δ (Gschwendt et al., 1994), was added at the time of plating at a final concentration of 0.6 μ M. The PKC chemical inhibitors Gö6976 and Gö6983 (Calbiochem) were added at 0.4 μ M and 1.2 μ M respectively. These concentrations were used in proportion to the IC_{50} values, as the IC_{50} of Gö6976 is one third that of Gö6983 (Gschwendt et al., 1996; Martiny-Baron et al., 1993). Gö6976 is a potent protein kinase C (PKC) inhibitor that discriminates between Ca²⁺-dependent and -independent isoforms of PKC selectively inhibits PKC α and PKC β_1 but does not inhibit the activity of, PKC δ - ϵ , or - ζ . The broad spectrum protein kinase C (PKC) inhibitor Gö6983 inhibits PKC α , PKC β , PKC γ , PKC δ , and PKC ζ . DMSO at an equivalent concentration was added to control dishes at the time of plating.

Quantitation

Growth cone area was analyzed using Metamorph software version 6.3r4 (Universal Imaging, Downingtown, PA). In brief, images were taken at 40 $\times$ magnification and fifteen random growth

cones were selected from each substrate. The growth cone, including the transitional zone and central domain, was traced using a freehand tool. The area in square pixels was calculated and then converted to square microns. The number of filopodia per growth cone was analyzed visually. Any protrusion from the leading edge of the growth cone longer than 4 μm from the leading edge of the growth cone and wider than 2 μm was counted as a filopodia. The average number of filopodia was determined from fifteen growth cones per substrate.

Neurite outgrowth was analyzed using SPOT-RT digital camera and image acquisition software (Diagnostic Instruments, Inc., Sterling Heights, MI). Neurite length was calculated using a well-established system (Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007). In short, the length of the five longest neurites in the field of view was measured perpendicular to the explant tissue. Neurite density was calculated by quantitating the area of the image occupied by neurites (Burden-Gulley and Brady-Kalnay, 1999; Oblander et al., 2007). Images were analyzed using Metamorph software. The data from all similar experiments were combined, analyzed by Student's *t* test and graphed (Microsoft Excel, 10.0.0, 2001).

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 $\mu\text{g/ml}$ aprotinin, 5 $\mu\text{g/ml}$ leupeptin and 1 $\mu\text{g/ml}$ pepstatin), dounced and incubated on ice for 30 minutes. The triton insoluble material was removed by centrifugation (10,000 rpm for 3 minutes), 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.

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A: Neurite outgrowth during development **B: R-cadherin neurite outgrowth** **C: N-cadherin neurite outgrowth**

Figure 1. R-cadherin promotes RGC neurite outgrowth at various stages of development and is independent of RGC cell body origin

Chick retinal explants from E6, E7, E8, E9 and E10 were isolated and cultured on an R-cadherin substrate (A). E8 chick retina explants were also sectioned parallel to the optic fissure and explants from each region of the retina were cultured on R-cadherin (B) or N-cadherin (C) substrates. Images were acquired 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 μ m.

Figure 2. R-cadherin-mediated neurite outgrowth is blocked by N-cadherin adhesion blocking antibodies

Retinal explants from E8 chick embryos were cultured on laminin (A, E), E-cadherin (B, F), N-cadherin (C, G) or R-cadherin (D, H) substrates in the presence of antibody control (A–D) or adhesion blocking antibodies to chick N-cadherin (E–H). Antibodies against chick N-cadherin that recognize N-cadherin expressed on the surface of the chick retinal explant inhibited neurite outgrowth on human N-cadherin (G) and human R-cadherin (H) substrates, whereas they had no effect on neurite outgrowth on laminin (E) or human E-cadherin (F) substrate. Scale bar, 200 μ m. (I) E-cadherin-Fc, N-cadherin-Fc, R-cadherin-Fc or E8 chick retina lysate was separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies to chick N-cadherin (NCD-2), intracellular human E-cadherin, intracellular human N-cadherin, chick R-cadherin (RCD-2), human Fc, extracellular human E-cadherin, extracellular human/chick N-cadherin or extracellular human R-cadherin. E-cadherin, N-cadherin and R-cadherin protein migrates at ~120 kDa, ~130 kDa or ~125 kDa respectively.

Figure 3. NCD-2 blocks neurite outgrowth from the dorsal-nasal region of the retina on an R-cadherin substrate

E8 retinal explants from the dorsal-nasal or ventral-nasal region of the retina were cultured on laminin (A), N-cadherin (B) or R-cadherin (C) substrates in the presence of a control antibody or NCD-2. NCD-2 had no effect on laminin (A). Neurite outgrowth from both the dorsal-nasal region and ventral nasal region of the retina was blocked in the presence of the chick specific NCD-2 antibody on an R-cadherin substrate. On an N-cadherin substrate, NCD-2 blocked neurite outgrowth from the dorsal-nasal and ventral-nasal region of the retina. Scale bar, 200 μ m.

Figure 4. Growth cone morphology varies on different classical cadherin substrates

DiI labeling of neurites on a laminin substrate (white bars) demonstrates the morphology of a small growth cone with few filopodia (A). Growth cones on an E-cadherin substrate (black bars) were very large and broad with few filopodial processes (B). On an N-cadherin substrate (hatched bars) growth cones were moderate with more filopodial processes (C). Growth cones on an R-cadherin substrate (gray bars) have hybrid morphology of broad growth cones with several filopodia (D). Representative images are shown. Arrowheads indicate the location of filopodia that were counted. The average number of filopodia per growth cone (E) and growth cone area (F) was quantitated for each substrate. Scale bar, 10 μm .

Figure 5. 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–D) or PTPμ wedge peptide (WPTPμ-Tat) (E–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–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 ($p \leq 0.001$) compared to control. Scale bar, 200 μm.

Figure 6. 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–C), Cdc42 DN (D–F), Rac1 DN (G–I) or RhoA DN (J–L). Infection Cdc42 DN, Rac1 DN or RhoA DN had no effect on laminin (D, G, J) when compared to control (A). Infection with Cdc42 DN and RhoA DN also had no effect on E-cadherin-mediated neurite outgrowth (E, K). However perturbation of E-cadherin-mediated neurite outgrowth was observed when infected with Rac1 DN (H) as compared to control (B). Infection with Cdc42 DN or Rac1 DN decreased neurite outgrowth on an N-cadherin substrate (F, I) when compared to control (C). Infection with RhoA DN had no effect on N-cadherin-mediated neurite outgrowth (L). Neurite length (M) and neurite density (N) were quantitated in the presence of GFP (white bars), Cdc42 DN (black bars), Rac1 DN (hatched bars) or RhoA DN (gray 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 Rac1 DN on N-cadherin) compared to control. Scale bar, 200 μm .

Figure 8. Rac1 specific inhibitor blocks neurite outgrowth on E-, N- 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) in the presence of control (A–D) or Rac1 inhibitor (E–H). 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–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 ($p \leq 0.001$) compared to control. Scale bar, 200 μm .

Figure 9. IQGAP1 specific inhibitor peptide blocks N- 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) in the presence of scrambled control (A–D) or IQGAP1 inhibitor (E–H). 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). Decreased 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.

Figure 10. PKC δ inhibitors block E- and R-cadherin-mediated neurite outgrowth

E8 chick retinal explants were cultured on laminin (A, E, I, M), E-cadherin (B, F, J, N), N-cadherin (C, G, K, O) or R-cadherin (D, H, L, P) in the presence of either DMSO control (A–D), Rottlerin (E–H), Gö6976 (I–L) or Gö6983 (M–P). No effect on laminin-dependent neurite outgrowth was observed in the presence of any inhibitor (E, I, M) when compared to DMSO control (A). Perturbation of neurite outgrowth was observed on an E-cadherin substrate when cultures were incubated in the presence of Rottlerin (F) or Gö6983 (N), compared to DMSO control (B). Incubation with Gö6976 (J) had no effect on E-cadherin-mediated neurite outgrowth. On an N-cadherin substrate Rottlerin (G), Gö6976 (K), or Gö6983 (O) had no effect on neurite outgrowth when compared to DMSO control (C). A reduction in neurite outgrowth

was observed on an R-cadherin substrate in the presence of Rottlerin (H), Gö6976 (L) or Gö6983 (P) when compared to DMSO control (D). Neurite length (Q) and neurite density (R) were quantitated in the presence of Rottlerin (black bars), Gö6976 (hatched bars), Gö6983 (gray bars) or DMSO control (white bars). The asterisk denotes statistically significant changes in neurite length ($p \leq 0.005$) or density ($p \leq 0.001$, $p \leq 0.05$ for R-cadherin) compared to control. Scale bar, 200 μm .

Figure 11.

Model summarizing the potential signaling pathways involved in E-cadherin, N-cadherin and R-cadherin-mediated neurite outgrowth. See discussion for details.