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Rho GTPases regulate PTP μ -mediated nasal neurite outgrowth and temporal repulsion of retinal ganglion cell neurons

Denice L. Major and Susann M. Brady-Kalnay *

Department of Molecular Biology and Microbiology, Case Western Reserve University, School of Medicine, Cleveland, OH

Abstract

Members of the Receptor Protein Tyrosine Phosphatase (RPTP) subfamily of cell adhesion molecules (CAMs) mediate neurite outgrowth and growth cone repulsion. PTP μ is a growth permissive substrate for nasal retinal ganglion cell (RGC) neurites and a growth inhibitory substrate for temporal RGCs. In this manuscript, we demonstrate that the distinct PTP μ -dependent phenotypes of nasal outgrowth and temporal repulsion are regulated by Rho GTPases. The role of Rho GTPases in the regulation of nasal outgrowth and temporal repulsion was tested by utilizing dominant negative and constitutively active forms of Rac1, RhoA and Cdc42 in Bonhoeffer stripe assays. Nasal neurite outgrowth on PTP μ was blocked by Cdc42-DN. Temporal repulsion to a PTP μ substrate was substantially reduced by addition of Cdc42-DN. The molecule that regulates the switch between permissive versus repulsive responses to PTP μ is Rac1 for temporal neurons. Inhibition of Rac1 is required for repulsion of temporal neurons. Interestingly, adding Rac1-CA to temporal RGC neurons converted PTP μ -dependent repulsion to a permissive response. In addition, adding exogenous Rac1-DN to nasal neurons induced a phenotype switch from a permissive to repulsive response to PTP μ . Together these data suggest that Cdc42 activity is required for both permissive and repulsive responses to PTP μ . However, the key to PTP μ -dependent repulsion is inhibition of Rac1 activity in temporal RGC neurons.

Keywords

receptor protein tyrosine phosphatase; cell adhesion molecule; retinal ganglion cell; tyrosine phosphorylation; axon guidance; Rho GTPase; actin cytoskeleton

Introduction

Spatiotemporal patterning of the visual system is a structured event in which retinal ganglion cell (RGC) axons from a particular region of the retina migrate and innervate a specific region of the optic tectum (McLaughlin et al., 2003; O'Leary and McLaughlin, 2005; Thanos and Mey, 2001; van Horck et al., 2004). For example, nasal axons innervate the posterior tectum, while temporal neurons innervate the anterior tectum. Studies have suggested that RGC projection to the tectum is partially due to the graded expression of Eph receptor tyrosine kinases and their ephrin ligands (McLaughlin et al., 2003; O'Leary and McLaughlin, 2005).

*Corresponding author: Susann M. Brady-Kalnay, Department of Molecular Biology and Microbiology, School of Medicine, Case Western Reserve University, Cleveland, OH, 44106-4960, Phone: (216) 368-0330, Fax: (216) 368-3055, Email: susann.brady-kalnay@case.edu.

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Therefore, tyrosine phosphorylation is key to the development of the visual system but little is known about the function of tyrosine phosphatases in this system. Receptor tyrosine phosphatases (RPTPs), which catalyze the dephosphorylation of tyrosine residues, are expressed in the nervous system and recent evidence suggests that they may be involved in guiding retinal axons to their targets during development (Brady-Kalnay, 2001; Ensslen-Craig and Brady-Kalnay, 2004). A subfamily of RPTPs, including PTP μ , has cell adhesion molecule-like extracellular segments and intracellular domains with tyrosine phosphatase activity (Brady-Kalnay, 2001; Ensslen-Craig and Brady-Kalnay, 2004; Johnson and Van Vactor, 2003).

The PTP μ -like subfamily of RPTPs includes four members: PTP μ , PTP κ , PTP ρ and PCP-2 (Brady-Kalnay, 2001). The PTP μ -like subfamily members contain motifs found in CAMs including a MAM domain, an immunoglobulin (Ig) domain and four FNIII repeats in their extracellular segment. The motifs present in PTP μ suggested that it might function in cell-cell adhesion. We demonstrated that expression of PTP μ induced the aggregation of nonadhesive Sf9 insect cells (Brady-Kalnay et al., 1993; Gebbink et al., 1993). PTP κ and PCP-2 have also been shown to mediate aggregation (Cheng et al., 1997; Sap et al., 1994). These studies demonstrated that the binding is homophilic (i.e. the “ligand” for a transmembrane PTP is an identical PTP molecule on an adjacent cell). Both PTP μ -dependent adhesion and neurite outgrowth are mediated by homophilic binding (Brady-Kalnay and Tonks, 1994; Ensslen-Craig and Brady-Kalnay, 2005). The homophilic binding site resides in the immunoglobulin domain (Brady-Kalnay and Tonks, 1994). The MAM domain of PTP μ also plays a role in cis dimerization (Aricescu et al., 2006; Cismasiu et al., 2004) and cell aggregation by sorting cells expressing PTP μ into distinct aggregates from cells expressing related molecules such as PTP κ (Zondag et al., 1995). Together, these studies demonstrate that PTP μ mediates cell-cell adhesion and suggest it may transduce signals in response to adhesion that are required for neurite outgrowth.

The juxtamembrane domain of the PTP μ -like enzymes contains a region of homology to the intracellular domain of the cadherins (Brady-Kalnay, 2001). We demonstrated that PTP μ interacts specifically with N-cadherin, E-cadherin and R-cadherin (Brady-Kalnay et al., 1998). Both N- and R-cadherin are expressed in the retina and control retinal histogenesis (Redies, 1997; Redies and Takeichi, 1996). Importantly, we have demonstrated a functional role for the PTP μ /N-cadherin interaction because we found that PTP μ regulates N-cadherin-dependent neurite outgrowth of retinal ganglion cells (Burden-Gulley and Brady-Kalnay, 1999).

PTP μ is expressed in a gradient in both the retina and the tectum (Burden-Gulley et al., 2002). Since the axons of RGCs form the optic nerve and are the sole output from the retina to the brain, the expression of PTP μ on these cells was consistent with a putative role for PTP μ in axonal migration. The ability of retinal ganglion cell axons to grow out of a retinal explant onto a purified protein substrate has been used to study axonal (neurite) growth (Lemmon et al., 1992). To test whether PTP μ promoted neurite outgrowth, we used purified PTP μ as a substrate and demonstrated that it promoted neurite outgrowth from E8 retinal explants (Burden-Gulley and Brady-Kalnay, 1999). More importantly, PTP μ is a permissive substrate for neurite outgrowth from nasal RGC neurons, while it is inhibitory to temporal RGC neurons (Burden-Gulley et al., 2002). The molecular details of the intracellular signals that regulate axonal growth and guidance are not well defined.

It is intriguing that PTP μ is able to mediate both permissive and repulsive responses in RGC neurons. PTP μ is expressed at low levels in nasal neurons and is permissive for nasal RGC neurite outgrowth (Burden-Gulley et al., 2002). PTP μ is expressed at high levels in temporal RGCs and is repulsive to those neurons (Burden-Gulley et al., 2002). Our recent data suggest

that the gradient of PTP μ expression in the retina regulates whether PTP μ is a repulsive or permissive guidance cue (Ensslen-Craig and Brady-Kalnay, 2005). We found that expression of exogenous PTP μ in nasal neurites, which increases PTP μ levels to those found in temporal neurons, resulted in a phenotypic switch from a permissive to a repulsive response to a PTP μ substrate (Ensslen-Craig and Brady-Kalnay, 2005). Catalytic activity of PTP μ is necessary for both permissive and repulsive guidance events downstream of PTP μ binding. Since both expression and catalytic activity of PTP μ are required, this implies that PTP μ homophilic binding results in a tyrosine phosphatase-dependent signal that is necessary for PTP μ -mediated nasal outgrowth and temporal repulsion, adding support to the growing body of evidence that RPTP catalytic activity is important for axon guidance (Garritty et al., 1999; Johnson et al., 2001). Most importantly, these results demonstrate a functional significance for the observed gradients in retinotectal expression of PTP μ . In this manuscript, we investigate the molecular mechanisms by which PTP μ regulates both nasal outgrowth and temporal repulsion.

The Rho subfamily of small G-proteins plays a central role in regulating the actin cytoskeleton (Raftopoulou and Hall, 2004; Ridley, 2004). Activation of Cdc42 results in the formation of filopodia while Rac activation induces lamellipodia formation, and Rho activation results in stress fibers in fibroblasts (Nobes and Hall, 1995). Rho GTPases are molecular switches, cycling between GTP-bound “on” and GDP-bound “off” states. This cycle is controlled by GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and GDP-dissociation inhibitors (Bishop and Hall, 2000; Kaibuchi et al., 1999).

The role of Rho GTPase activation in the regulation of nasal outgrowth and temporal repulsion was tested by utilizing dominant negative and constitutively active TAT-tagged forms of Rac1, RhoA and Cdc42, which were directly taken up by cells. We prepared alternating stripes of PTP μ and laminin as substrates for single stripe assays and a combination of PTP μ mixed with laminin versus laminin only lanes for the mixed stripe assays. Single stripes measure permissive responses, i.e. the ability to grow or not on one adhesion molecule. Mixed stripes measure repulsive responses because a repulsive substrate-like PTP μ is mixed with a normally permissive cue, such as laminin. The TAT-Rho GTPase fusion proteins were added to the stripe assays and changes in nasal outgrowth or temporal crossing were observed. Nasal neurite outgrowth and temporal repulsion to a PTP μ substrate were substantially reduced by the addition of Cdc42-DN, while inhibition of Rac1 is required for temporal repulsion to PTP μ response. These data suggest that the key to PTP μ -dependent repulsion is inhibition of Rac1 activity in temporal RGC neurons.

Results

We cultured retinal explants from embryonic day 8 chicks in Bonhoeffer stripe assays (Vielmetter et al., 1990; Walter et al., 1987a; Walter et al., 1987b). The Bonhoeffer stripe assay examines the ability of neurons to grow on two protein substrates presented in alternating lanes. A permissive substrate must be present in a lane to observe any neurite outgrowth on that lane (Lemmon et al., 1992). Very few adhesion molecules promote neurite outgrowth of chick retinal ganglion cell (RGC) neurons (Lemmon et al., 1992). The Receptor Protein Tyrosine Phosphatase PTP μ is able to mediate both permissive and repulsive guidance events (Burden-Gulley et al., 2002). PTP μ is permissive only to ventral nasal RGC neurons (Burden-Gulley and Brady-Kalnay, 1999; Burden-Gulley et al., 2002; Ensslen and Brady-Kalnay, 2004). In Bonhoeffer stripe assay, if a single substrate is plated on a lane and no neurite outgrowth is observed on that lane then it is unclear if that substrate is repulsive (and the neurons are actively avoiding it) or is just not permissive (i.e. no neurite outgrowth is observed in that lane). To distinguish between these two possibilities, a mixed substrate lane assay must be used. Repulsion is measured when neurites avoid a permissive substrate (laminin) that has been mixed with a repulsive substrate (PTP μ) in a single lane. Temporal neurites do not grow on a

PTP μ only substrate lane (Burden-Gulley et al., 2002) nor do they cross a mixed laminin/PTP μ substrate, demonstrating that the PTP μ repulsive signal is dominant over the laminin permissive signal (Ensslen and Brady-Kalnay, 2004). The mixed stripe assay allows one to detect a pharmacological or molecular blockade of the PTP μ repulsive signal since the temporal neurites are then able to grow on the laminin component of the mixed lane. Since both nasal and temporal neurites originate from the same retinal explant, the Bonhoeffer stripe assay provides a powerful means to assess the effects of reagents that alter signaling cascades.

PTP μ is a permissive substrate for ventral nasal RGC neurites, they cross between the PTP μ only and laminin only lanes (Fig. 1). Addition of dominant negative Rac1 (Rac1-DN) and dominant negative Cdc42 (Cdc42-DN) does not affect laminin outgrowth but blocks crossing onto the PTP μ lanes (Fig. 1). Dominant negative RhoA (RhoA-DN) had no effect on RGC crossing onto the PTP μ substrate. Quantitation of the stripe assays was performed using a rating scale as previously described (Ensslen and Brady-Kalnay, 2004; Walter et al., 1987a). Neurites that show no preference for either substrate are assessed at 0, and neurites that grow exclusively on laminin are assessed at 3. An assessment of 2 indicates that most of the neurites grow on the laminin lanes with an occasional neurite crossing over PTP μ lanes, while an assessment of 1 is when there is a significant amount of neurite crossing but a tendency to fasciculate on laminin. Quantitation of the data presented indicates that Rac1 and Cdc42 activity are required for PTP μ to be a permissive growth substrate. However, subsequent experiments demonstrate that the effect of exogenous Rac1-DN addition was to induce nasal repulsion, instead of blocking the permissive response (see Fig. 5). Therefore, we conclude that Cdc42 activation is required for the nasal permissive response to a PTP μ substrate.

We used a mixed stripe assay to test the effect of dominant negative Rho GTPases on PTP μ -dependent temporal repulsion. Only addition of Cdc42-DN substantially reduced temporal repulsion on a PTP μ substrate (Fig. 2). Neither Rac1-DN nor RhoA-DN altered temporal repulsion to PTP μ (Fig. 2). These data indicate that Cdc42 activity is required for PTP μ -dependent repulsion. We then tested whether the constitutively active (CA) mutants of the Rho GTPases altered temporal repulsion to a PTP μ substrate. Interestingly, Rac1-CA blocks temporal repulsion to PTP μ in the mixed stripe assay (Fig. 3). Neither Cdc42-CA or RhoA-CA alter temporal repulsion to PTP μ (Fig. 3). These data suggest that Rac1 inhibition is required for PTP μ -dependent temporal repulsion.

To test whether Rac1-CA was simply blocking temporal repulsion in a mixed stripe assay or altering the response of temporal neurons to the PTP μ substrate, we performed single stripe assays. The single stripe assay measures the ability of temporal neurons to respond to the presence of PTP μ alone with either a permissive or repulsive response. Rac1-CA caused the temporal neurons to behave as if PTP μ were a permissive substrate (Fig. 4). Neither Cdc42-CA nor RhoA-CA was able to induce this phenotypic switch from repulsion to a permissive response (Fig. 4). Together, our data suggest that Rac1 activation converts PTP μ to a permissive substrate and that Rac1 inhibition is required for PTP μ -mediated temporal repulsion.

PTP μ is expressed in the retina in a gradient at low levels in nasal neurons and is permissive. In contrast, PTP μ is expressed at high levels in temporal RGCs and is repulsive to those neurons (Burden-Gulley et al., 2002; Ensslen-Craig and Brady-Kalnay, 2005). We found that expression of exogenous PTP μ in nasal neurites, which increases PTP μ levels to those found in temporal neurons, results in a phenotypic switch from a permissive to repulsive response to a PTP μ substrate (Ensslen-Craig and Brady-Kalnay, 2005).

The data presented thus far in this study indicate that Rac1 inhibition is required for PTP μ to be a repulsive substrate. If that were the case, artificially inhibiting Rac1 activation (with exogenous Rac1-DN) in nasal neurons might convert a PTP μ -dependent permissive response

to repulsion in a mixed stripe assay. Figure 5 shows that addition of Rac1-DN did, in fact, cause a phenotypic switch in nasal neurons from a PTP μ -mediated permissive response to repulsion in mixed stripe assays. Neither Cdc42-DN nor RhoA-DN was able to induce this phenotypic switch in nasal neurons (Fig. 5). Therefore, the data in Figure 1 indicate that exogenous Rac1-DN induced repulsion, instead of blocking the permissive response to PTP μ . These data suggest that inhibition of Rac1 activity is required for PTP μ to be a repulsive substrate.

To determine if Rac1 and Cdc42 are in distinct or related pathways, we performed epistasis experiments. Rho GTPase mutants were added singly or in combination. Explants from E8 nasal retina were grown on alternating stripes of PTP μ or laminin only (single stripe assay) and either Rac1-CA, Cdc42-DN or the combination or Rac1-CA + Cdc42-DN were added using protein transduction via their TAT-tag. Control and Rac1-CA transduced nasal neurites cross onto a PTP μ substrate (Fig. 6). Following addition of Cdc42-DN or the combination of Rac1-CA + Cdc42-DN, however, nasal neurites are no longer able to cross onto PTP μ lanes. These results suggest that activation of Cdc42 is required for a permissive response to a PTP μ substrate and that Cdc42 is dominant over Rac1.

To confirm that Cdc42 is required for PTP μ to be a permissive substrate, E8 temporal retina explants were grown on alternating stripes of PTP μ or laminin only (single stripe assay), and either Rac1-CA, Cdc42-DN or the combination of Rac1-CA + Cdc42-DN were added by protein transduction. Control and Cdc42-DN transduced temporal neurites avoided PTP μ (Fig. 7). Following addition of Rac1-CA, temporal neurites crossed over onto the PTP μ lanes. Addition of Cdc42-DN to Rac1-CA blocked the conversion of PTP μ to a permissive signal for temporal neurons. These experiments confirm that Cdc42 activity is required for the permissive response to PTP μ , and altering Rac1 is not enough to elicit a permissive response. Rac1-CA is sufficient to reduce repulsion to PTP μ , indicating that it is capable of effectively converting the high PTP μ in temporal neurons to a low PTP μ signal found in nasal neurons.

Figure 5 demonstrates that exogenous Rac1 inhibition converts the permissive to a repulsive response to PTP μ in nasal neurites. On alternating stripes of PTP μ mixed with laminin (PTP μ /LN) versus laminin only lanes, explants from E8 nasal retina were grown with the addition of either Rac1-DN, Cdc42-CA or the combination of Rac1-DN + Cdc42-CA. Control and Cdc42-CA transduced nasal neurites crossed onto the PTP μ mixed substrate lanes because both PTP μ and laminin are permissive substrates for nasal neurons (Fig. 8). Following addition of exogenous Rac1-DN, nasal neurites now avoid the PTP μ -containing lanes. These results suggest that inhibition of Rac1 activity induces a phenotypic switch in the nasal neurons from a permissive to a repulsive response to a PTP μ substrate. The combination of Rac1-DN + Cdc42-CA produced the same result as Rac1-DN alone. These data suggest inhibition of Rac1 is key to repulsion in response to PTP μ , and that exogenous Cdc42 activation cannot overcome this Rac1 inhibiting signal.

To test whether Cdc42 activity is absolutely required for temporal repulsion, explants from E8 temporal retina were grown on alternating stripes of PTP μ mixed with laminin (PTP μ /LN) versus laminin only lanes and either Rac1-DN, Cdc42-DN or the combination of Rac1-DN + Cdc42-DN dominant negative (DN) mutants were added. Control and Rac1-DN transduced temporal neurites avoided the PTP μ mixed substrate lanes (Fig. 9). Following addition of Cdc42-DN or the combination of Rac1-DN + Cdc42-DN, temporal neurites crossed the PTP μ -containing lanes. These results suggest that inhibition of Cdc42 activity affects temporal repulsion to a PTP μ substrate. Exogenous Rac1 inhibition in temporal neurons is not sufficient to overcome the requirement for Cdc42 activity in temporal neurons. Together, the combination experiments confirm that Cdc42 activity is required for both permissive and repulsive responses, and that Rac1 inhibition is required for PTP μ to be repulsive. Rac1 and Cdc42 do

not appear to influence one another in the combination experiments. These results may suggest that Rac1 and Cdc42 are in distinct pathways. Our combination experiments also do not suggest that one molecule is either upstream or downstream of one another.

Discussion

In this manuscript, we tested whether the Rho GTPases regulate PTP μ -dependent nasal outgrowth and temporal repulsion. The data presented here indicate that Cdc42 activity is required for both nasal outgrowth and temporal repulsion (Fig. 10), which is consistent with our previous study that demonstrated a requirement of Cdc42 activity for PTP μ -dependent growth cone rearrangement (Rosdahl et al., 2003). In the previous study, the stimulation of E6 retinal cultures with purified PTP μ resulted in a morphological change in the growth cone, which became dominated by filopodia within five minutes of PTP μ addition. We perturbed GTPase signaling, and found that PTP μ -induced growth cone rearrangement required Cdc42 but not Rac1 or Rho. Dominant-negative forms of Rac1 had no effect on PTP μ -dependent growth cone rearrangement (Rosdahl et al., 2003) whereas a dominant-negative mutant of Cdc42 blocked PTP μ -induced rearrangement (Rosdahl et al., 2003). Importantly, we observed that PTP μ signaling in growth cones resulted in both lamellipodial loss and filopodial extension, which is a phenotype very similar to that observed in static images of nasal outgrowth on a PTP μ substrate (Burden-Gulley and Brady-Kalnay, 1999). It is interesting to note that both lamellipodial loss and filopodial extension are hallmarks of Rac inhibition and Cdc42 activation, respectively (Rosdahl et al., 2003).

In order to examine the differential behavior of nasal or temporal RGCs in response to a PTP μ substrate, we utilized the Bonhoeffer stripe assay. The stripe assay allows the assessment of neurite outgrowth on alternating substrate lanes. In this manuscript, Bonhoeffer stripe assays with nasal and temporal neurons, where distinct signals are perturbed, has yielded information about which signals PTP μ utilizes to induce nasal permissive responses and temporal repulsive responses. The data presented in this manuscript indicate that Cdc42 activation is required for both nasal outgrowth and temporal repulsion on a PTP μ substrate. In addition, we demonstrated that PTP μ -dependent temporal repulsion requires inhibition of Rac1 activity.

We hypothesize that the activation of Cdc42 by PTP μ likely involves IQGAP1 (Fig. 11). PTP μ binds IQGAP1 (Phillips-Mason et al., 2006), which interacts with the Rho GTPases Rac1 and Cdc42 in their GTP-bound state (Briggs and Sacks, 2003). PTP μ binds IQGAP1, which was originally identified as a putative RasGAP due to the fact that it contains a region with significant homology to the catalytic domain of RasGAPs (Weissbach et al., 1994). IQGAP1 neither exhibits RasGAP activity nor binds to Ras, but specifically interacts with the Rho GTPases Rac1 and Cdc42 in their GTP-bound state (Briggs and Sacks, 2003). IQGAP1 has no GAP activity towards Cdc42 or Rac1 (Erickson et al., 1997). Rather, IQGAP1 stabilizes Cdc42 in its GTP-bound state (Hart et al., 1996; Swart-Mataraza et al., 2002; Zhang et al., 1997). Both *in vitro* and *in vivo* IQGAP1 appears to have a higher affinity for Cdc42 over Rac1 (Erickson et al., 1997; Hart et al., 1996; Kuroda et al., 1996). Interestingly, IQGAP1 binds directly to PTP μ and their binding is increased by activated Cdc42 (Phillips-Mason et al., 2006). We hypothesize that the requirement for Cdc42 in both nasal outgrowth and temporal repulsion is dependent upon IQGAP1–PTP μ interaction.

IQGAP1 was recently shown to promote neurite outgrowth although the molecular mechanisms are unclear (Li et al., 2005). It is widely known that the Rho GTPases including Cdc42 and Rac1 regulate the cytoskeleton and neurite outgrowth (Govek et al., 2005; Nikolic, 2002). IQGAP1 is an intriguing molecule because it can link several proteins that have the potential to regulate both the actin cytoskeleton and microtubules, hence neurite outgrowth. IQGAP1 functions as a scaffolding protein and interacts with E-Cadherin (Kuroda et al.,

1998; Li et al., 1999), β -catenin (Briggs et al., 2002; Kuroda et al., 1998), CLIP-170 (Fukata et al., 2002), actin (Erickson et al., 1997; Mateer et al., 2002) and the ERK2 mitogen-activated protein kinase (MAPK) (Roy et al., 2004). We have recently demonstrated that PTP μ associates with an IQGAP1 complex that also contains ERK2 (Phillips-Mason et al., 2006) (Fig. 11). Furthermore, ERK2 is known to regulate neurite outgrowth in response to neurotrophins and CAMs (Dimitropoulou and Bixby, 2000; Doherty et al., 2000; Yamada et al., 1997). Interestingly, MAPK activation is required for PTP κ , a PTP μ -subfamily member, to promote neurite outgrowth (Drosopoulos et al., 1999).

Our results also suggest that a key difference between the two phenotypic responses to a PTP μ substrate, nasal crossing and temporal repulsion, is the activity of the Rac1 GTPase (Fig. 10). We have found that Rac1 inhibition is required for temporal repulsion to a PTP μ substrate. If constitutively active Rac1 (Rac1-CA) is added to temporal neurons, a phenotypic switch occurs which converts PTP μ to a permissive substrate. Neither Cdc42-CA nor Rho-CA is able to induce this phenotypic switch. Consistent with this hypothesis, addition of dominant-negative Rac1 to nasal neurons in a single stripe assay and a mixed substrate assay (used to measure repulsion) induces repulsion of nasal neurons on a PTP μ substrate. It is interesting that inhibition of Rac1 includes repulsion. Most studies link RhoA activation to growth cone collapse and repulsion. Our study found no effect of RhoA on PTP μ -dependent neurite outgrowth or repulsion. The molecular mechanism by which PTP μ inhibits Rac1 is currently unknown and is the subject of future studies.

PTP μ also interacts with RACK1, a WD-40 repeat protein, that was originally identified as a protein that binds to activated protein kinase C (PKC) (Ron et al., 1994) (Fig. 11). Interestingly PKC δ , a RACK1 binding protein, also interacts with PTP μ in a signaling complex in the retina (Rosdahl et al., 2002). We recently found that PKC δ activity is required for both permissive and inhibitory responses of RGCs to PTP μ , with higher levels of PKC δ activation associated with temporal growth cone collapse and repulsion (Ensslen and Brady-Kalnay, 2004). A potential mechanism for differential PKC δ activation is due to the gradient of PTP μ expression in the retina. A lower level of PKC δ activity is required for nasal outgrowth on a PTP μ substrate (Rosdahl et al., 2002).

The same signaling molecule can regulate both permissive and repulsive responses. For example, Protein Kinase C mediates signals downstream of both growth permissive (Bixby and Jhabvala, 1990; Kabir et al., 2001; Kolkova et al., 2000) and inhibitory cues (Mikule et al., 2003; Powell et al., 2001; Theodore et al., 1995; Xiang et al., 2002). The Rho family of GTPases (Giniger, 2002), cyclic nucleotides (Song et al., 1998; Song et al., 1997), MAPK kinases (Dimitropoulou and Bixby, 2000; Perron and Bixby, 1999; Tong et al., 2003) and calcium (Nishiyama et al., 2003; Zheng, 2000), mediate both repulsive and attractive behaviors in neurons (Skaper et al., 2001). The magnitude of second messenger signal in the growth cone controls the permissive versus repulsive response. It is intriguing that PTP μ signaling involves regulation of some of these enzymes that mediate both permissive responses and repulsion (Fig. 11). Our combined studies suggest that the magnitude of PTP μ -dependent signaling controls the switch from a permissive response to repulsion (Ensslen-Craig and Brady-Kalnay, 2005; Ensslen and Brady-Kalnay, 2004), and that Cdc42 is a likely candidate molecule in the PTP μ signaling cascade.

Tyrosine phosphatases have been implicated in the control of neurite outgrowth (Bixby and Jhabvala, 1992, 1993; Burden-Gulley and Brady-Kalnay, 1999). Some CAM-like RPTPs have been shown to promote neurite outgrowth and act as attractive or repulsive cues. PTP κ has been shown to promote neurite outgrowth of cerebellar neurons (Drosopoulos et al., 1999). PTP δ promotes neurite outgrowth of forebrain and cerebellar neurons (Wang and Bixby, 1999) and a local gradient of soluble PTP δ acts as a chemoattractant, which depends upon PTP

activity in forebrain neurons (Sun et al., 2000). The RPTP CRYP α regulates interactions between neurons and glial cells in the retina as well as retinal axon growth (Haj et al., 1999; Ledig et al., 1999). Phosphacan, the soluble splice variant of RPTP ζ/β , can promote or inhibit neurite outgrowth depending upon the context (Maeda and Noda, 1996). The *Drosophila* RPTPs, DLAR, DPTP69D and DPTP99A were shown to be crucial for certain neurons to recognize guidance cues that help them innervate appropriate target muscles (Desai et al., 1996; Desai et al., 1997; Krueger et al., 1996). Perturbation of the leech LAR homolog (HmLAR2) results in abnormal neuronal projections (Gershon et al., 1998). Recent studies suggest that HmLAR2 may regulate mutual repulsion via a homophilic binding mechanism (Baker and Macagno, 2000a, b; Baker et al., 2000). The regulation of tyrosine phosphorylation by RPTPs may affect neurite outgrowth by “steering” growth cones along the appropriate pathway to establish a spatiotemporal pattern of neuronal connections, via both permissive and repulsive responses.

Experimental Methods

Culturing of Chick Retinal Explants

Chick embryonic day 8 embryos were staged according to Hamburger and Hamilton (Hamburger, 1951). Retinal explants were prepared as described (Drazba and Lemmon, 1990; Halfter et al., 1983). E8 (stage 32) neural retinas were dissected, flattened on concanavalin-coated nitrocellulose filters, and cut into 350 μ m wide explants. Explants were placed retinal ganglion side down on substrate-coated dishes and grown in 10% fetal bovine serum (Hyclone, Logan, UT), 2% chick serum (Sigma, St. Louis, MO), 2mM L-glutamine (Invitrogen, Carlsbad, CA), and 2 units/ml penicillin, 2 μ g/ml streptomycin, 5ng/ml amphotericin (Sigma) in RPMI-1640 (Invitrogen) for 48 hours for stripe assays.

Bonhoeffer Stripe Assay

The substrate lane assay used was a slightly modified version of the Bonhoeffer method (Vielmetter et al., 1990), as described (Burden-Gulley et al., 2002). Tissue culture dishes were coated with nitrocellulose (Lagenaur and Lemmon, 1987) and dried before applying the silicon lane matrix to the dish surface. The PTP μ Fc chimera has been described (Rosdahl et al., 2003). Laminin was purchased from BTI (Stoughton, MA). 80ng of PTP μ -Fc chimera containing a small amount of Texas-Red conjugated BSA (for visualization of the lanes, Molecular Probes, Eugene, OR) was injected into the channels of the matrix, incubated for 10 minutes, aspirated then replaced with a fresh aliquot of the same substrate. All remaining binding sites within the lanes were blocked with bovine serum albumin (BSA; fraction V; Sigma) and rinsed with calcium-magnesium free phosphate buffer (CMF). The matrix was removed and 1.75 μ g of laminin was spread across the lane area and incubated for 20 minutes. The mixed substrate assays were performed as described (Ensslen and Brady-Kalnay, 2004). For mixed substrate lanes, 160ng of PTP μ -Fc chimera was mixed with 0.9 μ g laminin containing Texas-Red BSA and was used as the first substrate as described above. For the second substrate 1.75 μ g of laminin was spread over the lane area. The entire dish was blocked with BSA and rinsed with RPMI. Explants were cultured for 48 hours prior to imaging. For Rho GTPase perturbations, 6.5 μ g of TAT-tagged mutant protein was added at the time of plating explants. Representative images from a minimum of three separate experiments are shown. Quantitation of the stripe assays was performed using a rating scale as described (Ensslen and Brady-Kalnay, 2004; Walter et al., 1987a). Neurites that show no preference for either substrate are assessed at 0, and neurites that grow exclusively on one substrate are assessed at 3. An assessment of 2 indicates that most of the neurites grow on the laminin lanes with an occasional neurite crossing over PTP μ lanes, while an assessment of 1 is when there is a significant amount of neurite crossing but a tendency to fasciculate on laminin. Data from a minimum of three experiments were combined (with a minimum sample size of 9 explants

per condition) to determine the average degree of avoidance for each condition, then plotted using Microsoft Excel.

TAT Protein Expression and Purification

Expression plasmids for constitutively active (CA) and dominant negative (DN) Rac1, RhoA and Cdc42 were kindly provided by Dr. Steven Dowdy (UCSD, CA) (Soga et al., 2001). The expression plasmids were transformed into BL21 (DE3) pLysS competent cells (Novagen). Protein expression was induced with 200 μ M IPTG (final concentration).

Cells were resuspended in 1X PBS containing 2 μ L/mL PIC (Sigma) and 1 μ L/mL 1M benzamidine, sonicated on ice three times for 30 seconds, and centrifuged at 4200rpm for 25min. at 4°C. The supernatant was applied to 1mL packed Talon Metal Affinity Resin (BD Biosciences, Clontech) to bind to the His-tag of the Rho GTPase fusion proteins and rocked overnight at 4°C. Beads were pelleted (1500rpm, 3min, 4°C) and washed three times with 10mL 1XPBS/PIC/benzamidine followed by 3 additional washes with 10mL 1X PBS. Protein was eluted from beads with 1XPBS containing 200mM imidazole.

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References

- Aricescu AR, Hon WC, Siebold C, Lu W, van der Merwe PA, Jones EY. Molecular analysis of receptor protein tyrosine phosphatase mu-mediated cell adhesion. *Embo J* 2006;25:701–712. [PubMed: 16456543]
- Baker MW, Macagno ER. RNAi of the receptor tyrosine phosphatase HmLAR2 in a single cell of an intact leech embryo leads to growth-cone collapse. *Curr Biol* 2000a;10:1071–1074. [PubMed: 10996077]
- Baker MW, Macagno ER. The role of a LAR-like receptor tyrosine phosphatase in growth cone collapse and mutual-avoidance by sibling processes. *J Neurobiol* 2000b;44:194–203. [PubMed: 10934322]
- Baker MW, Rauth SJ, Macagno ER. Possible role of the receptor protein tyrosine phosphatase HmLAR2 in interbranch repulsion in a leech embryonic cell. *J Neurobiol* 2000;45:47–60. [PubMed: 10992256]
- Bishop AL, Hall A. Rho GTPases and their effector proteins. *Biochem J* 348 Pt 2000;2:241–255. [PubMed: 10816416]
- Bixby JL, Jhabvala P. Extracellular matrix molecules and cell adhesion molecules induce neurites through different mechanisms. *J Cell Biol* 1990;111:2725–2732. [PubMed: 2277083]
- Bixby JL, Jhabvala P. Inhibition of tyrosine phosphorylation potentiates substrate-induced neurite growth. *Journal of Neurobiology* 1992;23:468–480. [PubMed: 1279111]
- Bixby JL, Jhabvala P. Tyrosine phosphorylation in early embryonic growth cones. *The Journal of Neuroscience* 1993;13:3421–3432. [PubMed: 7688039]
- Brady-Kalnay, SM. Protein tyrosine phosphatases. Oxford University Press; Oxford, UK: 2001.
- Brady-Kalnay SM, Boghaert ER, Zimmer S, Brackenbury R. Increasing N-CAM-mediated cell-cell adhesion does not reduce invasion of RSV-transformed WC5 rat cerebellar cells. *Clin Exp Metastasis* 1993;11:313–324. [PubMed: 8391406]
- Brady-Kalnay SM, Mourton T, Nixon JP, Kinch M, Chen H, Brackenbury R, Rimm DL, Del Vecchio RL, Tonks NK. Dynamic interaction of PTP μ with multiple cadherins *in vivo*. *J Cell Biol* 1998;141:287–296. [PubMed: 9531566]
- Brady-Kalnay SM, Tonks NK. Identification of the homophilic binding site of the receptor protein tyrosine phosphatase PTP mu. *J Biol Chem* 1994;269:28472–28477. [PubMed: 7961788]

- Briggs MW, Li Z, Sacks DB. IQGAP1-mediated stimulation of transcriptional co-activation by beta-catenin is modulated by calmodulin. *J Biol Chem* 2002;277:7453–7465. [PubMed: 11734550]
- Briggs MW, Sacks DB. IQGAP proteins are integral components of cytoskeletal regulation. *EMBO Rep* 2003;4:571–574. [PubMed: 12776176]
- Burden-Gulley SM, Brady-Kalnay SM. PTP μ Regulates N-Cadherin-dependent Neurite Outgrowth. *J Cell Biol* 1999;144:1323–1336. [PubMed: 10087273]
- Burden-Gulley SM, Ensslen SE, Brady-Kalnay SM. Protein tyrosine phosphatase- μ differentially regulates neurite outgrowth of nasal and temporal neurons in the retina. *J Neurosci* 2002;22:3615–3627. [PubMed: 11978837]
- Cheng J, Wu K, Armanini M, O'Rourke N, Dowbenko D, Lasky LA. A novel protein-tyrosine phosphatase related to the homotypically adhering κ and μ receptors. *J Biol Chem* 1997;272:7264–7277. [PubMed: 9054423]
- Cismasiu VB, Denes SA, Reilander H, Michel H, Szedlacsek SE. The MAM (meprin/A5-protein/PTP μ) domain is a homophilic binding site promoting the lateral dimerization of receptor-like protein-tyrosine phosphatase μ . *J Biol Chem* 2004;279:26922–26931. [PubMed: 15084579]
- Desai C, Gindhart JG, Goldstein LSB, Zinn K. Receptor tyrosine phosphatases are required for motor axon guidance in the *Drosophila* embryo. *Cell* 1996;84:599–609. [PubMed: 8598046]
- Desai CJ, Sun Q, Zinn K. Tyrosine phosphorylation and axon guidance: of mice and flies. *Curr Opin Neuro* 1997;7:70–74.
- Dimitropoulou A, Bixby JL. Regulation of retinal neurite growth by alterations in MAPK/ERK kinase (MEK) activity. *Brain Res* 2000;858:205–214. [PubMed: 10700616]
- Doherty P, Williams G, Williams EJ. CAMs and axonal growth: a critical evaluation of the role of calcium and the MAPK cascade. *Mol Cell Neurosci* 2000;16:283–295. [PubMed: 11085868]
- Drazba J, Lemmon V. The role of cell adhesion molecules in neurite outgrowth on Müller cells. *Dev Biol* 1990;138:82–93. [PubMed: 2407579]
- Drosopoulos NE, Walsh FS, Doherty P. A soluble version of the receptor-like protein tyrosine phosphatase κ stimulates neurite outgrowth via a Grb2/MEK1-dependent signaling cascade. *Mol Cell Neurosci* 1999;13:441–449. [PubMed: 10383829]
- Ensslen-Craig SE, Brady-Kalnay SM. Receptor protein tyrosine phosphatases regulate neural development and axon guidance. *Dev Biol* 2004;275:12–22. [PubMed: 15464569]
- Ensslen-Craig SE, Brady-Kalnay SM. PTP μ expression and catalytic activity are required for PTP μ -mediated neurite outgrowth and repulsion. *Mol Cell Neurosci* 2005;28:177–188. [PubMed: 15607952]
- Ensslen SE, Brady-Kalnay SM. PTP μ signaling via PKC δ is instructive for retinal ganglion cell guidance. *Mol Cell Neurosci* 2004;25:558–571. [PubMed: 15080886]
- Erickson JW, Cerione RA, Hart MJ. Identification of an actin cytoskeletal complex that includes IQGAP and the Cdc42 GTPase. *J Biol Chem* 1997;272:24443–24447. [PubMed: 9305904]
- Fukata M, Watanabe T, Noritake J, Nakagawa M, Yamaga M, Kuroda S, Matsuura Y, Iwamatsu A, Perez F, Kaibuchi K. Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. *Cell* 2002;109:873–885. [PubMed: 12110184]
- Garrity PA, Lee CH, Salecker I, Robertson HC, Desai CJ, Zinn K, Zipursky SL. Retinal axon target selection in *Drosophila* is regulated by a receptor protein tyrosine phosphatase. *Neuron* 1999;22:707–717. [PubMed: 10230791]
- Gebbink MFBG, Zondag GCM, Wubbolts RW, Beijersbergen RL, van Etten I, Moolenaar WH. Cell-cell adhesion mediated by a receptor-like protein tyrosine phosphatase. *J Biol Chem* 1993;268:16101–16104. [PubMed: 8393854]
- Gershon TR, Baker MW, Nitabach M, Macagno ER. The leech receptor protein tyrosine phosphatase HmLAR2 is concentrated in growth cones and is involved in process outgrowth. *Dev* 1998;125:1183–1190.
- Giniger E. How do Rho family GTPases direct axon growth and guidance? A proposal relating signaling pathways to growth cone mechanics. *Differentiation* 2002;70:385–396. [PubMed: 12366376]
- Govek EE, Newey SE, Van Aelst L. The role of the Rho GTPases in neuronal development. *Genes Dev* 2005;19:1–49. [PubMed: 15630019]

- Haj F, McKinnell I, Stoker A. Retinotectal ligands for the receptor tyrosine phosphatase CRYPalpha. *Mol Cell Neurosci* 1999;14:225–240. [PubMed: 10493824]
- Halfter W, Newgreen DF, Sauter J, Schwarz U. Oriented axon outgrowth from avian embryonic retinae in culture. *Dev Biol* 1983;95:56–64. [PubMed: 6825932]
- Hamburger H, Hamilton H. A series of normal stages in the development of the chick embryo. *Journal of Morphology* 1951;88:49–92.
- Hart MJ, Callow MG, Souza B, Polakis P. IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. *Embo J* 1996;15:2997–3005. [PubMed: 8670801]
- Johnson KG, McKinnell IW, Stoker AW, Holt CE. Receptor protein tyrosine phosphatases regulate retinal ganglion cell axon outgrowth in the developing *Xenopus* visual system. *J Neurobiol* 2001;49:99–117. [PubMed: 11598918]
- Johnson KG, Van Vactor D. Receptor protein tyrosine phosphatases in nervous system development. *Physiol Rev* 2003;83:1–24. [PubMed: 12506125]
- Kabir N, Schaefer AW, Nakhost A, Sossin WS, Forscher P. Protein kinase C activation promotes microtubule advance in neuronal growth cones by increasing average microtubule growth lifetimes. *J Cell Biol* 2001;152:1033–1044. [PubMed: 11238458]
- Kaibuchi K, Kuroda S, Fukata M, Nakagawa M. Regulation of cadherin-mediated cell-cell adhesion by the Rho family GTPases. *Curr Opin Cell Biol* 1999;11:591–596. [PubMed: 10508646]
- Kolkova K, Novitskaya V, Pedersen N, Berezin V, Bock E. Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway. *J Neurosci* 2000;20:2238–2246. [PubMed: 10704499]
- Krueger NX, Van Vactor D, Wan HI, Gelbart WM, Goodman CS, Saito H. The transmembrane tyrosine phosphatase DLAR controls motor axon guidance in *Drosophila*. *Cell* 1996;84:611–622. [PubMed: 8598047]
- Kuroda S, Fukata M, Kobayashi K, Nakafuku M, Nomura N, Iwamatsu A, Kaibuchi K. Identification of IQGAP as a putative target for the small GTPases, Cdc42 and Rac1. *J Biol Chem* 1996;271:23363–23367. [PubMed: 8798539]
- Kuroda S, Fukata M, Nakagawa M, Fujii K, Nakamura T, Ookubo T, Izawa I, Nagase T, Nomura N, Tani H, Shoji I, Matsuura Y, Yonehara S, Kaibuchi K. Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin-mediated cell-cell adhesion. *Science* 1998;281:832–835. [PubMed: 9694656]
- Lagenaur C, Lemmon V. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. *Proc Natl Acad Sci U S A* 1987;84:7753–7757. [PubMed: 3478724]
- Ledig MM, Haj F, Bixby JL, Stoker AW, Mueller BK. The receptor tyrosine phosphatase CRYPalpha promotes intraretinal axon growth. *J Cell Biol* 1999;147:375–388. [PubMed: 10525542]
- Lemmon V, Burden SM, Payne HR, Elmslie GJ, Hlavin ML. Neurite growth on different substrates: Permissive versus instructive influences and the role of adhesive strength. *The Journal of Neuroscience* 1992;12:818–826. [PubMed: 1545241]
- Li Z, Kim SH, Higgins JM, Brenner MB, Sacks DB. IQGAP1 and calmodulin modulate E-cadherin function. *J Biol Chem* 1999;274:37885–37892. [PubMed: 10608854]
- Li Z, McNulty DE, Marler KJ, Lim L, Hall C, Annan RS, Sacks DB. IQGAP1 promotes neurite outgrowth in a phosphorylation-dependent manner. *J Biol Chem*. 2005
- Maeda N, Noda M. 6B4 proteoglycan/phosphacan is a repulsive substratum but promotes morphological differentiation of cortical neurons. *Development* 1996;122:647–658. [PubMed: 8625816]
- Mateer SC, McDaniel AE, Nicolas V, Habermacher GM, Lin MJ, Cromer DA, King ME, Bloom GS. The mechanism for regulation of the F-actin binding activity of IQGAP1 by calcium/calmodulin. *J Biol Chem* 2002;277:12324–12333. [PubMed: 11809768]
- McLaughlin T, Hindges R, O'Leary DD. Regulation of axial patterning of the retina and its topographic mapping in the brain. *Curr Opin Neurobiol* 2003;13:57–69. [PubMed: 12593983]
- Mikule K, Sunpaweravong S, Gatlin JC, Pfenninger KH. Eicosanoid activation of protein kinase C epsilon: involvement in growth cone repellent signaling. *J Biol Chem* 2003;278:21168–21177. [PubMed: 12665507]
- Nikolic M. The role of Rho GTPases and associated kinases in regulating neurite outgrowth. *Int J Biochem Cell Biol* 2002;34:731–745. [PubMed: 11950591]

- Nishiyama M, Hoshino A, Tsai L, Henley JR, Goshima Y, Tessier-Lavigne M, Poo MM, Hong K. Cyclic AMP/GMP-dependent modulation of Ca²⁺ channels sets the polarity of nerve growth-cone turning. *Nature* 2003;424:990–995. [PubMed: 12944936]
- Nobes CD, Hall A. Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem Soc Trans* 1995;23:456–459. [PubMed: 8566347]
- O'Leary DD, McLaughlin T. Mechanisms of retinotopic map development: Ephs, ephrins, and spontaneous correlated retinal activity. *Prog Brain Res* 2005;147:43–65. [PubMed: 15581697]
- Perron JC, Bixby JL. Distinct neurite outgrowth signaling pathways converge on ERK activation. *Mol Cell Neurosci* 1999;13:362–378. [PubMed: 10356298]
- Phillips-Mason PJ, Gates TJ, Sacks DB, Brady-Kalnay SM. The receptor protein tyrosine phosphatase PTP μ interacts with IQGAP1. *J Biol Chem* 2006;281:4903–4910. [PubMed: 16380380]
- Powell EM, Mercado ML, Calle-Patino Y, Geller HM. Protein kinase C mediates neurite guidance at an astrocyte boundary. *Glia* 2001;33:288–297. [PubMed: 11246227]
- Raftopoulou M, Hall A. Cell migration: Rho GTPases lead the way. *Dev Biol* 2004;265:23–32. [PubMed: 14697350]
- Redies C. Cadherins and the formation of neural circuitry in the vertebrate CNS. *Cell Tissue Res* 1997;290:405–413. [PubMed: 9321704]
- Redies C, Takeichi M. Cadherins in the developing central nervous system: an adhesive code for segmental and functional subdivisions. *Dev Biol* 1996;180:413–423. [PubMed: 8954714]
- Ridley AJ. Rho proteins and cancer. *Breast Cancer Res Treat* 2004;84:13–19. [PubMed: 14999150]
- Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D. Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins [published erratum appears in *Proc Natl Acad Sci U S A* 1995 Feb 28;92(5):2016]. *Proc Natl Acad Sci U S A* 1994;91:839–843. [PubMed: 8302854]
- Rosdahl JA, Ensslen SE, Niedenthal JA, Brady-Kalnay SM. PTP μ -dependent growth cone rearrangement is regulated by Cdc42. *J Neurobiol* 2003;56:199–208. [PubMed: 12884260]
- Rosdahl JA, Mourton TL, Brady-Kalnay SM. Protein kinase C delta (PKCdelta) is required for protein tyrosine phosphatase μ (PTP μ)-dependent neurite outgrowth. *Mol Cell Neurosci* 2002;19:292–306. [PubMed: 11860281]
- Roy M, Li Z, Sacks DB. IQGAP1 binds ERK2 and modulates its activity. *J Biol Chem* 2004;279:17329–17337. [PubMed: 14970219]
- Sap J, Jiang YP, Friedlander D, Grumet M, Schlessinger J. Receptor tyrosine phosphatase R-PTP- κ mediates homophilic binding. *Mol Cell Biol* 1994;14:1–9. [PubMed: 8264577]
- Skaper SD, Moore SE, Walsh FS. Cell signalling cascades regulating neuronal growth-promoting and inhibitory cues. *Prog Neurobiol* 2001;65:593–608. [PubMed: 11728645]
- Soga N, Namba N, McAllister S, Cornelius L, Teitelbaum SL, Dowdy SF, Kawamura J, Hruska KA. Rho family GTPases regulate VEGF-stimulated endothelial cell motility. *Exp Cell Res* 2001;269:73–87. [PubMed: 11525641]
- Song HJ, Ming GL, He Z, Lehman M, McKerracher L, Tessier-Lavigne M, Poo M. Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* 1998;281:1515–1518. [PubMed: 9727979]
- Song HJ, Ming GL, Poo MM. cAMP-induced switching in turning direction of nerve growth cones. *Nature* 1997;388:275–279. [PubMed: 9230436]
- Sun QL, Wang J, Bookman RJ, Bixby JL. Growth cone steering by receptor tyrosine phosphatase delta defines a distinct class of guidance cue. *Mol Cell Neurosci* 2000;16:686–695. [PubMed: 11083928]
- Swart-Mataraza JM, Li Z, Sacks DB. IQGAP1 is a component of Cdc42 signaling to the cytoskeleton. *J Biol Chem* 2002;277:24753–24763. [PubMed: 11948177]
- Thanos S, Mey J. Development of the visual system of the chick. II. Mechanisms of axonal guidance. *Brain Res Brain Res Rev* 2001;35:205–245. [PubMed: 11423155]
- Theodore L, Derossi D, Chassaing G, Llirbat B, Kubes M, Jordan P, Chneiweiss H, Godement P, Prochiantz A. Intraneuronal delivery of protein kinase C pseudosubstrate leads to growth cone collapse. *J Neurosci* 1995;15:7158–7167. [PubMed: 7472470]

- Tong J, Elowe S, Nash P, Pawson T. Manipulation of EphB2 regulatory motifs and SH2 binding sites switches MAPK signaling and biological activity. *J Biol Chem* 2003;278:6111–6119. [PubMed: 12486127]
- van Horck FP, Weini C, Holt CE. Retinal axon guidance: novel mechanisms for steering. *Curr Opin Neurobiol* 2004;14:61–66. [PubMed: 15018939]
- Vielmetter J, Stolze B, Bonhoeffer F, Stuermer CA. In vitro assay to test differential substrate affinities of growing axons and migratory cells. *Exp Brain Res* 1990;81:283–287. [PubMed: 2397757]
- Walter J, Henke-Fahle S, Bonhoeffer F. Avoidance of posterior tectal membranes by temporal retinal axons. *Development* 1987a;101:909–913. [PubMed: 3503703]
- Walter J, Kern-Veits B, Huf J, Stolze B, Bonhoeffer F. Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. *Development* 1987b;101:685–696. [PubMed: 3503693]
- Wang J, Bixby JL. Receptor Tyrosine Phosphatase-delta Is a Homophilic, Neurite-Promoting Cell Adhesion Molecule for CNS Neurons. *Mol Cell Neurosci* 1999;14:370–384. [PubMed: 10588391]
- Weissbach L, Settleman J, Kalady MF, Snijders AJ, Murthy AE, Yan YX, Bernards A. Identification of a human rasGAP-related protein containing calmodulin-binding motifs. *J Biol Chem* 1994;269:20517–20521. [PubMed: 8051149]
- Xiang Y, Li Y, Zhang Z, Cui K, Wang S, Yuan XB, Wu CP, Poo MM, Duan S. Nerve growth cone guidance mediated by G protein-coupled receptors. *Nat Neurosci* 2002;5:843–848. [PubMed: 12161754]
- Yamada M, Ikeuchi T, Hatanaka H. The neurotrophic action and signalling of epidermal growth factor. *Prog Neurobiol* 1997;51:19–37. [PubMed: 9044427]
- Zhang B, Wang ZX, Zheng Y. Characterization of the interactions between the small GTPase Cdc42 and its GTPase-activating proteins and putative effectors. Comparison of kinetic properties of Cdc42 binding to the Cdc42-interactive domains. *J Biol Chem* 1997;272:21999–22007. [PubMed: 9268338]
- Zheng JQ. Turning of nerve growth cones induced by localized increases in intracellular calcium ions. *Nature* 2000;403:89–93. [PubMed: 10638759]
- Zondag G, Koningstein G, Jiang YP, Sap J, Moolenaar WH, Gebbink M. Homophilic interactions mediated by receptor tyrosine phosphatases μ and κ . *J Biol Chem* 1995;270:14247–14250. [PubMed: 7782276]

Nasal (Single Stripe)

Figure 1. Cdc42 activity is required for PTPμ to be a permissive growth substrate for nasal neurons
 Explants from E8 nasal retina were grown on alternating stripes of PTPμ or laminin only lanes (single stripe assay) and either Rac1, Cdc42 or RhoA dominant negative (DN) mutants were added using protein transduction via their TAT-tag. Control and RhoA-DN transduced nasal neurites cross onto a PTPμ substrate. Following addition of Rac1-DN or Cdc42-DN, however, nasal neurites are no longer able to cross onto PTPμ lanes. These results suggest that activation of Cdc42 is required for a permissive response to a PTPμ substrate. Quantitation of the degree of nasal avoidance of PTPμ lanes is shown following addition of dominant-negative mutants of the Rho GTPases to single stripe assays. The average degree of avoidance (mean $\pm$ STDEV) was plotted for each condition as described in Experimental Methods.

Temporal (Mixed Stripe)

Figure 2. Cdc42 activity is required for PTPμ-dependent temporal repulsion

Explants from E8 temporal retina were grown on alternating stripes of PTPμ mixed with laminin (PTPμ/LN) versus laminin only lanes and either Rac1, Cdc42 or RhoA dominant negative (DN) mutants were added. Control, RhoA-DN and Rac1-DN transduced temporal neurites avoided the PTPμ mixed substrate lanes. Following addition of Cdc42-DN, temporal neurites crossed the PTPμ-containing lanes. These results suggest that inhibition of Cdc42 activity affects temporal repulsion to a PTPμ substrate. Quantitation of the degree of temporal avoidance of PTPμ lanes is shown following addition of dominant-negative mutants of the Rho GTPases to mixed stripe assays. The average degree of avoidance (mean $\pm$ STDEV) was plotted for each condition.

Temporal (Mixed Stripe)

Figure 3. Rac1 inhibition is required for PTPμ-dependent temporal repulsion

Explants from E8 temporal retina were grown on alternating stripes of PTPμ mixed with laminin (PTPμ/LN) versus laminin only lanes and either Rac1, Cdc42 or RhoA constitutively active mutants were added. Control, Cdc42-CA and RhoA-CA transduced temporal neurites avoided the PTPμ mixed substrate lanes. Following addition of Rac1-CA, however, temporal neurites crossed onto the PTPμ-containing lanes. These results suggest that inhibition of Rac1 activity is required for temporal repulsion by a PTPμ substrate. Quantitation of the degree of temporal neurite avoidance of PTPμ lanes is shown following addition of constitutively active mutants of the Rho GTPases to mixed stripe assays. The average degree of avoidance (mean ± STDEV) was plotted for each condition.

Temporal (Single Stripe)

Figure 4. Rac1-CA converts PTPμ to a permissive signal in single stripe, PTPμ only lanes
Explants from E8 temporal retina were grown on alternating stripes of PTPμ or laminin only lanes and either Rac1, Cdc42 or RhoA constitutively active mutants were added. Control, Cdc42-CA and RhoA-CA transduced temporal neurites avoided PTPμ. Following addition of Rac1-CA, temporal neurites crossed over onto the PTPμ only lanes. These results suggest that activation of Rac1 converts PTPμ to a permissive substrate for temporal neurons. Quantitation of the degree of temporal neurite avoidance of PTPμ lanes is shown following addition of constitutively active mutants of the Rho GTPases to single stripe assays. The average degree of avoidance (mean $\pm$ STDEV) was plotted for each condition.

Figure 5. Inhibition of Rac1 converts the PTPμ permissive signal to repulsion in nasal neurites
Explants from E8 nasal retina were grown on alternating stripes of PTPμ mixed with laminin (PTPμ/LN) versus laminin only lanes and either Rac1, Cdc42 or RhoA dominant negative mutants were added. Control, RhoA-DN and Cdc42-DN transduced nasal neurites crossed onto the PTPμ mixed substrate lanes because both PTPμ and laminin were permissive substrates for nasal neurons. Following addition of Rac1-DN, nasal neurites avoided the PTPμ-containing lanes. These results suggest that inhibition of Rac1 activity induces a phenotypic switch in the nasal neurons from a permissive to a repulsive response to a PTPμ substrate. Quantitation of the degree of nasal avoidance of PTPμ lanes is shown following addition of dominant-negative mutants of the Rho GTPases to mixed stripe assays. The average degree of avoidance (mean $\pm$ STDEV) was plotted for each condition.

Nasal (Single Stripe)

Figure 6. Cdc42 is dominant over Rac1 in the permissive response of nasal neurites to PTPμ
Explants from E8 nasal retina were grown on alternating stripes of PTPμ or laminin only lanes (single stripe assay) and either Rac1-CA, Cdc42-DN or the combination of Rac1-CA + Cdc42-DN mutants were added. Control and Rac1-CA transduced nasal neurites cross onto a PTPμ substrate. Following addition of Cdc42-DN or Rac1-CA + Cdc42-DN, however, nasal neurites are no longer able to cross onto PTPμ lanes. These results suggest that activation of Cdc42 is required for a permissive response to a PTPμ substrate. Quantitation of the degree of nasal avoidance of PTPμ lanes is shown following addition of dominant-negative mutants of the Rho GTPases to single stripe assays. The average degree of avoidance (mean ± STDEV) was plotted for each condition.

Temporal (Single Stripe)

Figure 7. Cdc42 is required for PTPμ to be a permissive substrate

Explants from E8 temporal retina were grown on alternating single stripes of PTPμ or laminin only lanes and either Rac1-CA, Cdc42-DN or the combination of Rac1-CA + Cdc42-DN were added. Control and Cdc42-DN transduced temporal neurites avoided PTPμ. Following addition of Rac1-CA, temporal neurites crossed over onto the PTPμ only lanes. These results suggest that activation of Rac1 converts PTPμ to a permissive substrate for temporal neurons. Quantitation of the average degree of avoidance (mean ± STDEV) was plotted for each condition. The addition of Cdc42-DN to Rac1-CA blocked the permissive response. These results confirm that activation of Cdc42 is required for a permissive response to PTPμ.

Nasal (Mixed Stripe)

Figure 8. Exogenous Rac1 inhibition converts the permissive to a repulsive response to PTPμ by nasal neurites, and Cdc42 activation cannot overcome this response

Explants from E8 nasal retina were grown on alternating stripes of PTPμ mixed with laminin (PTPμ/LN) versus laminin only lanes and either Rac1-DN, Cdc42-CA or the combination of Rac1-DN + Cdc42-CA mutants were added. Control and Cdc42-CA transduced nasal neurites crossed onto the PTPμ mixed substrate lanes because both PTPμ and laminin were permissive substrates for nasal neurons. Following addition of Rac1-DN or Rac1-DN + Cdc42-CA, nasal neurites avoided the PTPμ-containing lanes. These results suggest that inhibition of Rac1 activity induces a phenotypic switch in the nasal neurons from a permissive to a repulsive response to a PTPμ substrate. In addition, Rac1-DN is dominant in the induced nasal repulsion. Quantitation of the average degree of nasal avoidance (mean ± STDEV) was plotted for each condition.

Temporal (Mixed Stripe)

Figure 9. Exogenous Rac1 inhibition in temporal neurons is not sufficient to overcome the requirement for Cdc42 activity in temporal neurons

Explants from E8 temporal retina were grown on alternating stripes of PTPμ mixed with laminin (PTPμ/LN) versus laminin only lanes and either Rac1-DN, Cdc42-DN or the combination of Rac1-DN + Cdc42-DN mutants were added. Control and Rac1-DN transduced temporal neurites avoided the PTPμ mixed substrate lanes. Following addition of Cdc42-DN or Rac1-DN + Cdc42-DN, temporal neurites crossed the PTPμ-containing lanes. These results suggest that Cdc42 activity is required for temporal repulsion to a PTPμ substrate. Quantitation of the average degree of temporal avoidance (mean ± STDEV) was plotted for each condition.

Cdc42 Activation and Rac1 Inhibition Distinguish Permissive from Repulsive Responses to PTP μ

Figure 10. Summary diagram of the results from perturbation of RhoGTPase activity on PTP μ -dependent nasal crossing and temporal repulsion

Cdc42 activation and Rac1 inhibition distinguish permissive from repulsive responses to PTP μ .

Figure 11.
Diagram of the possible molecular mechanisms involved in PTPμ-dependent regulation of Cdc42 and Rac1 activity.