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Ephrin-B2 elicits differential growth cone collapse and axon retraction in retinal ganglion cells from distinct retinal regions

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Abstract

The circuit for binocular vision and stereopsis is established at the optic chiasm, where retinal ganglion cell (RGC) axons diverge into the ipsilateral and contralateral optic tracts. In the mouse retina, ventrotemporal (VT) RGCs express the guidance receptor EphB1, which interacts with the repulsive guidance cue ephrin-B2 on radial glia at the optic chiasm to direct VT RGC axons ipsilaterally. RGCs in the ventral retina also express EphB2, which interacts with ephrin-B2, whereas dorsal RGCs express low levels of EphB receptors. To investigate how growth cones of RGCs from different retinal regions respond upon initial contact with ephrin-B2, we utilized time-lapse imaging to characterize the effects of ephrin-B2 on growth cone collapse and axon retraction in real time. We demonstrate that bath application of ephrin-B2 induces rapid and sustained growth cone collapse and axon retraction in VT RGC axons, whereas contralaterally-projecting dorsotemporal RGCs display moderate growth cone collapse and little axon retraction. Dose response curves reveal that contralaterally-projecting ventronasal axons are less sensitive to ephrin-B2 treatment compared to VT axons. Additionally, we uncovered a specific role for Rho kinase signaling in the retraction of VT RGC axons but not in growth cone collapse. The detailed characterization of growth cone behavior in this study comprises an assay for the study of Eph signaling in RGCs, and provides insight into the phenomena of growth cone collapse and axon retraction in general.

Keywords

Retinal ganglion cells; ipsilateral projection; growth cones; Eph receptors and ephrins; Rho kinase

INTRODUCTION

Ensuring that neuronal projections form appropriate connections with their intended target is an essential feature of brain development. Neuronal growth cones often travel great distances and encounter numerous decision points where they integrate an array of attractive and repulsive guidance cues that steer axons towards the proper route. Repellent guidance cues that divert axons away from undesirable destinations can be broadly divided into soluble/diffusible factors, which establish an inhibitory concentration gradient, and contact-

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mediated cues, which normally cause the growth cone to collapse and recover its forward growth in an alternate direction. Since the discovery of the first repulsive guidance cue nearly 20 years ago (Luo et al., 1993), the effect of repellent cues on growth cone behavior and axon guidance have been studied intensively in many different model systems, most notably in the developing visual pathways.

After exiting the eye, RGC axons from both retinæ travel through the optic nerve and converge at the ventral diencephalon to form the optic chiasm. The decision to project ipsilaterally or contralaterally at the optic chiasm is the first step in establishing the binocular pathway and is essential for stereopsis. In mice, which have a relatively small binocular field, the vast majority of RGC axons traverse the chiasm midline with only a small percentage of RGCs projecting ipsilaterally (~3–5%). This ipsilateral projection arises from the peripheral ventrotemporal (VT) retina.

Differential growth cone behavior associated with this chiasm-crossing decision was first examined in DiI-labeled RGC growth cones as they encounter radial glia processes at the optic chiasm midline (Godement et al., 1990; Guillery et al., 1995; Marcus et al., 1995; Mason and Sretavan, 1997). As RGC growth cones encounter the optic chiasm, their normally smooth extension and streamlined morphology within the optic nerve transforms into cycles of saltatory extension, pausing and retraction, accompanied by adoption of complex morphologies (Godement et al., 1990; Godement et al., 1994; Marcus et al., 1995; Mason and Wang, 1997; Mason and Erskine, 2000). Growth cones of VT RGCs usually pause longer than their contralaterally-projecting counterparts and eventually project into the ipsilateral optic tract by consolidation of a backwards-oriented filopodium. These observations led to the idea that VT RGCs are prevented from crossing the chiasm by contact-mediated cues expressed on radial glia cells.

Work in both *Xenopus* and mice (Nakagawa et al., 2000; Williams et al., 2003) identified members of the EphB family of receptor tyrosine kinases and their ligands, the ephrin-Bs, as playing critical roles in formation of the ipsilateral retinal pathway. During formation of the ipsilateral projection (E13–E16.5 in mice), EphB1 expression is restricted to RGC axons originating from the peripheral VT crescent, whereas ephrin-B2 is expressed by radial glia at the optic chiasm. *EphB1*^{−/−} mice have a strongly reduced ipsilateral projection (Williams et al., 2003), and ectopic expression of EphB1 into non-VT RGCs can reroute these fibers into the ipsilateral optic tract (Petros et al., 2009).

However, this apparently simple mechanism is complicated by the fact that RGCs express other EphB receptors that have similar binding affinities for ephrin-B2 (Flanagan and Vanderhaeghen, 1998): EphB2 is expressed in a high-ventral to low-dorsal gradient in RGCs (Williams et al., 2003; McLaughlin and O'Leary, 2005) and EphB3 appears to be expressed homogeneously throughout the retina at E14.5 (Birgbauer et al., 2000; Williams et al., 2003). Therefore RGC's exhibit distinct EphB expression profiles dependent on their location within the retina: VT RGCs are EphB1+/EphB2+/EphB3+; ventronasal (VN) RGCs are EphB1−/EphB2+/EphB3+; and dorso-temporal (DT) RGCs are EphB1−/EphB2−/EphB3+. Although combinatorial EphB receptor signaling has not been ruled out, the mild repulsion of VN axons by ephrin-B2 substrates *in vitro* (Williams et al., 2003) and weak rerouting effect of ectopic EphB2 expression *in vivo* (Petros et al., 2009) suggests that EphB1-ephrin-B2 interaction is the primary mechanism for directing the ipsilateral retinal projection. This is in contrast to the retinotectal (McLaughlin and O'Leary, 2005) and thalamocortical (Vanderhaeghen et al., 2000; Cang et al., 2005) projections where gradients of multiple Eph receptors and ephrins direct growth cones to their appropriate destination, making the optic chiasm a relatively simple system in which to study the mechanisms underlying ephrin-induced growth cone behavior.

In this study, we have attempted to broaden our understanding of this mechanism by characterizing the initial effect of ephrin-B2 on growth cone collapse and axon retraction of RGCs from different retinal regions. To this end, we combined time-lapse imaging with the established *in vitro* collapse assay (Raper and Kapfhammer, 1990; Kapfhammer et al., 2007), allowing one to study the real-time effects of a compound on growth cone behavior. We found that ephrin-B2 induces rapid growth cone collapse and sustained axon retraction of VT RGCs, whereas ephrin-B2 has little effect on DT RGCs. VN RGCs behave similar to VT RGCs at high ephrin-B2 concentrations but are less sensitive than VT RGCs at more moderate ephrin-B2 concentrations. Additionally, we demonstrate that blockade of Rho-kinase (ROCK), a likely downstream component of the EphB1 signaling cascade, abrogates ephrin-B2-induced axon retraction of VT RGCs but not growth cone collapse. In summary, these findings characterize the differential ephrin-B2-induced responses of RGC growth cones arising from distinct retinal regions, and represent an assay for examining additional downstream signaling components of EphB receptors and their role in RGC axon guidance.

METHODS

Retinal explant cultures, bath application of ephrin-B2 or human-Fc, and immunostaining for post-hoc analysis

Ventrotemporal (VT), dorsotemporal (DT) and ventronasal (VN) retinal explants were dissected from E14.5 murine retina and plated in 250 μ l serum free medium (SFM) + 0.2% methylcellulose on glass bottom culture dishes (MatTek, Ashland MA) that had been coated with poly-L-ornithine (2 hrs at 37°C) and laminin (20 μ g/ml, O/N at 4°C), as described previously (Williams et al., 2003). Ephrin-B2 (gift of N. Gale, Regeneron) and human-Fc (Jackson Immunologicals) were clustered for 2 hours prior to application with 10-fold excess of goat anti-human Fc antibody (ICN Biomedicals, Aurora OH). 18–20 hours after plating, culture medium was completely removed and replaced with SFM containing 0.5 μ g/ml clustered ephrin-B2 or human-Fc for 15 or 30 minutes. Explants were then fixed with 4% paraformaldehyde in PBS. After blocking with 10% NDS + 0.2% Tween-20 (Sigma) in PBS for 1 hour, mouse IgG anti-neurofilament (2H3, gift of T. Jessell, diluted at 1:5 in 50% blocking solution) was incubated at 4°C O/N. After several PBS washes, secondary antibodies were diluted in 50% blocking solution and incubated at RT for 2–4 hours. Cy3-conjugated phalloidin (1:200, Molecular Probes) was added during secondary antibody incubations. Coverslips were removed from culture dishes, mounted onto slides with Gelmount (Sigma) and imaged on a Zeiss Axioplan-2 epifluorescent microscope equipped with AxioCam cameras (Zeiss) and Openlab deconvolution software (Improvision).

Real-time DIC microscopy of live growth cones

After incubation for 18–20 hours, dishes containing retinal explants were transferred to a Zeiss Axiovert 200T inverted microscope surrounded by a 37°C incubation chamber. Growth cones were captured using differential interference contrast (DIC) imaging at 20 $\times$, 40 $\times$ or 63 $\times$ magnification, with a motorized stage allowing for serial image acquisition from 4–6 fields of view per culture dish, with each field encompassing 2–6 growth cones. Metamorph[®] software was used to collect images every 30 seconds for 15 minutes prior to ephrin-B2 or Fc treatment, and for 15–60 minutes following treatment. For treatment, the imaging session was briefly paused, and the SFM + methylcellulose was aspirated off and replaced with 250 μ l SFM containing pre-clustered ephrin-B2-Fc or human-Fc protein (0.005–5.0 μ g/ml per well). For ROCK inhibition experiments, explants were pre-treated for 1–2 hours with 10 μ M Y-27632 (Sigma), which was also present in the ephrin-B2 or Fc treatment media.

Quantification of growth cone behavior

Metamorph[®] software was used to reconstruct a time-lapse series for each field of view and for analyzing the extension and retraction of RGC growth cones. The position of individual growth cones was marked on the first image of each series ($t = 0$), and the distal tip of the growth cone was tracked relative to this point for each subsequent image. Axonal elongation was measured as a positive value, whereas retraction was assigned a negative value. RGC's exhibiting negative growth or collapsed growth cones during the initial 15-minute observation period were excluded from the study to prevent biasing of results due to factors other than ligand treatment.

Growth cone collapse was scored after treatment of ephrin-B2 or human-Fc. Growth cones were considered collapsed if they were devoid of lamellipodia and filopodia protrusions that were present in prior imaging frames. For all conditions, growth cones from several different retinal explants from a minimum of three separate culture dishes were used for analysis.

Fisher's exact test was used to compare the number of collapsed growth cones for each condition (Table 1). ANOVA followed by modified t-tests was used to compare differences in the delay time of growth cone collapse between each condition (Table 1), and to compare the total distance of axon retraction after ephrin-B2 or Fc treatment between each condition (i.e., $t_{45}-t_{15}$ values for Figure 3, $t_{30}-t_{15}$ values for Figure 4A). All values in text are mean $\pm$ s.e.m.

RESULTS

Ephrin-B2 induces rapid growth cone collapse and axon retraction from VT but not DT retinal explants

Previous experiments plated retinal explants on ephrin-B2 substrates and borders to demonstrate that ephrin-B2 preferentially inhibits neurites from VT explants compared to neurites from other retinal regions (Williams et al., 2003; Petros et al., 2009). Although ephrin ligands are membrane-bound contact dependent cues *in vivo*, bath application of soluble ephrins has been utilized in other studies to produce repellent effects (Knoll and Drescher, 2004; Evans et al., 2007; Zimmer et al., 2007) because it allows precise control of the timing and concentration of ephrin-B2, which is limited in other substrate assays. Therefore, we first verified that bath application of ephrin-B2 to RGC explants mimicked the behavior of ephrin-B2 substrates.

After 18 hours in culture, retinal explants from VT and DT retina were treated with 0.5 $\mu\text{g}/\text{ml}$ ephrin-B2 (or 0.5 $\mu\text{g}/\text{ml}$ human-Fc as control), as this concentration consistently produces differential effects between VT and DT explants in our substrate assays (Lee et al., 2008; Petros et al., 2009). Cultures were fixed 30 minutes after treatment and immunostained for neurofilament and actin to visualize axons and growth cones, respectively. The majority of neurites from VT and DT explants had prominent growth cones 30 minutes after treatment with Fc protein (Figure 1). Addition of ephrin-B2 had little effect on DT axons, as most had visible growth cones at their tips 30 minutes after treatment. In contrast, the vast majority of RGC growth cones from VT explants were collapsed 30 minutes following 0.5 $\mu\text{g}/\text{ml}$ ephrin-B2 treatment (Figure 1). Similarly collapsed growth cone profiles were observed when explants were fixed 15 minutes after ephrin-B2 exposure (data not shown). These findings are consistent with previous analysis of VT and DT explants on ephrin-B2 substrate assays (Lee et al., 2008; Petros et al., 2009) and confirm that this assay is an effective means for investigation of ephrin-B2-mediated chemorepulsion.

However, these results are based on static images; growth cone behaviors upon initial response to ephrin-B2 cannot be analyzed with this method. In order to characterize the initial effects of ephrin-B2 on RGC axon and growth cone behavior, we combined bath application of pre-clustered ephrin-B2 to retinal explants with time-lapse imaging. RGC axons/growth cones were imaged for 15 minutes before and 30 minutes after treatment with either ephrin-B2 or human-Fc protein (Figure 2; Supplementary Movies 1–4). Any growth cones that were collapsed and/or retracting prior to ephrin-B2 or Fc exposure were excluded from analyses. Two types of analyses were performed. First, we characterized the time course of growth cone collapse after ephrin-B2 or Fc treatment (Table 1). Second, we tracked the extension and retraction of RGC axons before and after ephrin-B2 or Fc treatment. For each frame, we determined the distance between the original growth cone position ($t_0=0$) and the current growth cone position, with extension indicated by a positive value and retraction indicated by a negative value (Figures 2 & 3). Pre-clustered ephrin-B2 or Fc was added at t_{15} .

Application of Fc protein induced very little growth cone collapse from either DT (29% collapsed, $n=28$ GCs) or VT (4% collapsed, $n=26$ GCs) RGC axons (Figures 2A, Table 1, & Supplementary Movie 1). Both VT and DT retinal axons continued to advance at rates similar to pre-treatment (~ 1.6 $\mu\text{m}/\text{minute}$), with VT RGC axons extending 49.0 μm and DT RGC axons extending 56.7 μm on average 30 minutes after Fc treatment (Figures 2A, 3A & 3D).

In contrast, ephrin-B2 initiated rapid collapse of nearly all VT RGC growth cones (98% collapsed, $n=48$), with most growth cones collapsing within 90 seconds of treatment (1.54 ± 0.12 minutes; Figure 2B, Table 1 & Supplementary Movie 2). Concomitant with growth cone collapse, ephrin-B2 induced rapid and extensive retraction of VT RGC axons (250 ± 36 μm VT axon retraction 30 minute after ephrin-B2 treatment, $n=40$ GCs Figures 3B & 3D). Some VT axons displayed such extensive retraction that they retracted out of the field of view, and thus our analyses actually underestimate the severity of retraction. Very few VT RGC growth cones had recovered 30 minutes after ephrin-B2 treatment, in agreement with our results described above (Figure 1). Thus, bath application of 0.5 $\mu\text{g}/\text{ml}$ ephrin-B2 induces rapid and sustained effect on growth cone collapse and axon retraction of VT RGC axons.

Surprisingly, the majority of DT RGC growth cones also collapsed upon 0.5 $\mu\text{g}/\text{ml}$ ephrin-B2 treatment (59% collapse, $n=22$; Supplementary Movie 3), which was a moderate but significant increase from the DT + Fc condition (59% vs. 29%, $p < .05$). However, this ephrin-B2-induced collapse of DT RGC growth cones was significantly less than the percentage of collapsed VT RGC growth cones (59% vs. 98%, $p < .0001$) and significantly delayed compared to VT growth cones (1.54 ± 0.12 minutes for VT vs. 7.54 ± 1.38 minutes for DT, $p < .0001$, Figure 2C & Table 1). Importantly, most collapsed DT growth cones had recovered by 30 minutes (data not shown), which would be consistent with the lack of DT growth cone collapse observed in Figure 1. The effect of ephrin-B2 treatment on RGC axon outgrowth from DT explants was also variable, with some DT axons continuing to advance while other DT axons displayed moderate levels of retraction (Supplementary Movie 3). On average, DT axons retracted 50 ± 34 μm 30 minute after ephrin-B2 treatment ($n=22$ GCs), significantly less than the 250 ± 36 μm retraction from VT axons over the same time period ($p < .0001$, Figures 3C & 3D).

In summary, ephrin-B2 causes rapid growth cone collapse of nearly all VT RGCs, with little recovery after 30 minutes (Table 1). The majority of DT growth cones collapse in response to ephrin-B2, but this collapse is significantly delayed compared to the behavior of VT growth cones. The differential effects of ephrin-B2 on RGC axons are even more prominent

for axon retraction; VT retinal axons rapidly retract with little recovery, whereas some DT axon advance and others undergo moderate retraction, culminating in a significantly delayed and milder axon retraction compared to VT RGC axons (Figure 3D).

Ephrin-B2 has differential, concentration-dependent effects on RGC axon retraction

Although EphB1 is the only Eph receptor whose retinal expression is restricted to RGCs in the peripheral VT crescent, EphB2 displays a high ventral-to-low dorsal gradient in RGCs at E14.5 (Birgbauer et al., 2000; Williams et al., 2003). EphB1 and EphB2 have similar binding affinities to ephrin-B2 (Flanagan and Vanderhaeghen, 1998), and we have shown that ectopic expression of EphB2 can redirect a small percentage of non-VT axons into the ipsilateral optic tract (Petros et al., 2009). Additionally, VN RGC axons are moderately repelled by ephrin-B2 substrates (Williams et al., 2003). Since EphB1+/EphB2+ VT RGC axons project ipsilaterally *in vivo* whereas EphB1-/EphB2+ VN RGC axons project contralaterally, we investigated whether bath application of ephrin-B2 could reproduce the differential behaviors of VT and VN RGC axons observed *in vivo*.

To address this issue, we exposed VT, VN and DT retinal explants to varying concentrations of ephrin-B2 ranging from 5.0 ng/ml to 5.0 μ g/ml (Figure 4A). Consistent with our previous data, DT axons displayed little retraction at low ephrin-B2 concentrations and very slight retraction at the highest ephrin-B2 levels ($n \geq 12$ GCs for each ephrin-B2 concentration). In contrast, the speed and severity of VT axon retraction varied dramatically with ephrin-B2 concentration. VT axons rapidly retracted upon application of 5.0 μ g/ml ($n=23$ GCs) and 0.5 μ g/ml ($n=12$ GCs) ephrin-B2 (Figure 4A). 50 ng/ml ($n=30$ GCs) ephrin-B2 caused a slightly delayed and less severe retraction of VT axons compared to the higher concentrations, whereas 5.0 ng/ml ($n=11$ GCs) had almost no effect.

Similar to VT retinal axons, VN RGCs displayed rapid growth cone collapse and axon retraction at the two highest ephrin-B2 concentrations examined, 5.0 μ g/ml ($n=17$ GCs) and 0.5 μ g/ml ($n=19$ GCs). However, at 50 ng/ml, VN RGC axons ($n=19$ GCs) were less sensitive to ephrin-B2 compared to VT axons; VT axons retracted 90.8 ± 10.4 μ m compared to 46.3 ± 12.6 μ m for VN axons ($p < .01$, Figure 4A, arrowheads). Little effect was observed at 0.5 ng/ml ephrin-B2 ($n=18$ GCs). Thus, EphB1+/EphB2+ VT RGC axons and EphB1-/EphB2+ VN RGC axons have similar responses to high or low ephrin-B2 concentrations. Only at more moderate ephrin-B2 levels (50 ng/ml) does the differential behavior of VT and VN axons become apparent.

In order to further quantify differential ephrin-B2 sensitivity of VT, VN and DT RGC axons, dose-response curves were prepared to examine the maximum rate of axon retraction at each ephrin-B2 concentration. Axon retraction rates were calculated for the first four minutes immediately after addition of ephrin-B2, which is when maximal axon retraction occurs (Figure 4A). Plotting the axon retraction rate against the log ephrin-B2 concentrations revealed that VT axons generally retract faster at 5–500 ng/ml ephrin-B2 concentrations compared to VN axons (Figure 4B). Although not statistically significant, there is an apparent leftward shift in the VT dose-response curve compared to VN, in agreement with the above findings and previous studies (Williams et al., 2003) that demonstrate VT axons display increased sensitivity to ephrin-B2. If one estimates that the axon retraction rate is peaking around 20 μ m/min at 5000 ng/ml ephrin-B2, then the ephrin-B2-induced retraction rate of VT axons is approximately 1.7-fold more sensitive than VN axons ($EC_{50} \approx 115$ ng/ml for VT axons, $EC_{50} \approx 190$ ng/ml for VN axons) (Figure 4B). Additionally, both VT and VN are significantly more sensitive to ephrin-B2 compared to DT axons ($p \leq .005$ at 50, 500 and 5000 ng/ml ephrin-B2). Thus, both in terms of the rate of axon retraction and total axon retraction, VT axons display increased sensitivity to ephrin-B2 compared to VN axons, while both VT and VN axons are significantly more sensitive than DT axons.

Rho-kinase (ROCK) signaling is required for ephrin-B2-induced axon retraction, but not growth cone collapse

Eph receptors can regulate growth cone cytoskeleton via interactions with the large family of Rho GTPases, several of which are closely associated with guidance cue-mediated alterations in growth cone behavior (Dickson, 2001; Schmandke and Strittmatter, 2007). Numerous studies have shown that Rac and Rho activity is required for ephrin-induced growth cone collapse (Wahl et al., 2000; Shamah et al., 2001; Gallo et al., 2002; Journey et al., 2002; Cowan et al., 2005; Sahin et al., 2005; Beg et al., 2007). Most signaling originated by Rho family members converges downstream on Rho kinase (ROCK), which can be inhibited with the drug Y-27632.

To investigate if ROCK signaling is required for ephrin-B2-induced growth cone collapse and axon retraction of VT RGCs, retinal explants were incubated with 10 μ M Y-27632 prior to bath application of 0.5 μ g/ml ephrin-B2. Pre-treatment with Y-27632 alone had no effect on retinal neurite outgrowth (data not shown). Nearly all VT growth cones pre-incubated with Y-27632 still underwent ephrin-B2-induced collapse (96% collapsed, n=23 GCs; Figure 5A, Table 1, & Supplementary Movie 4), similar to control ephrin-B2-treated VT explants. However, inhibiting ROCK activity caused a significant reduction in ephrin-B2-induced retraction of VT axons (30 ± 19 μ m retraction at t_{45} with Y-27632 vs. 250 ± 36 μ m retraction at t_{45} in the absence of Y-27632, $p < .0001$; Figure 5B,C & Supplementary Movie 4). Additionally, Y-27632 caused a slight but significant delay in ephrin-B2-induced growth cone collapse from VT axons (2.80 ± 0.31 minutes with Y-27632 vs. 1.54 ± 0.12 minutes without Y-27632, $p < .05$). In fact, the axon retraction profile of VT axons treated with Y-27632 and ephrin-B2 is similar to DT axons treated with ephrin-B2 (Figure 5B,C). Thus, ROCK activity is not required for ephrin-B2-induced growth cone collapse of VT RGCs, but ROCK activity is required for rapid and sustained ephrin-B2-induced axon retraction of VT axons.

DISCUSSION

Over the last decade, significant progress has been made in identifying molecular mechanisms that guide retinal axons throughout their pathway and in their primary target areas, the superior colliculus/optic tectum and the lateral geniculate nucleus (Erskine and Herrera, 2007; Petros and Mason, 2008; Clandinin and Feldheim, 2009). At the optic chiasm, the receptor-ligand pair that induces repulsion at the optic chiasm and drives the ipsilateral retinal projection is EphB1 and ephrin-B2, which are expressed by uncrossed VT RGC axons and radial glia cells at the optic chiasm midline, respectively (Williams et al., 2003). In this study, we have extended our knowledge of RGC growth cone response to ephrin-B2 by characterizing growth cone collapse and axon retraction in RGC populations that express different combinations of EphB receptors. Additionally, we found that ROCK function is required for ephrin-B2-induced retraction of VT RGC axons but is dispensable for ephrin-B2-induced growth cone collapse.

Ephrin-B2 has differential and concentration dependent effects on RGC axons from different retinal origins

This study demonstrates that ephrin-B2 induces extremely rapid growth cone collapse and axon retraction from ipsilaterally-projecting EphB1+ VT RGC axons, with very little recovery observed after 30 minutes. This is consistent with previous data of VT RGC axons that contact ephrin-B2 substrates (Williams et al., 2003; Lee et al., 2008; Petros et al., 2009). The striking speed of VT RGC growth cone collapse within minutes of ephrin-B2 application is similar to other reports that carefully examined the temporal component of ephrin-induced growth cone collapse (Knoll and Drescher, 2004; Harbott and Nobes, 2005;

Yue et al., 2008). One study found that clustered ephrin-B1 did not induce significant growth cone collapse of *Xenopus* RGC axons until 30 minutes post-treatment (Mann et al., 2003), but this could be due to differences in species, experimental conditions or the Eph-ephrin receptor-ligand pairs, and for the latter, whether forward or reverse signaling is involved.

Importantly, many of the VT axons retracted out of the field of view during acquisition of the time-lapse series (Figure 2B), and thus our results underestimate the extent and severity of axon retraction in these VT explants. The rapid growth cone collapse and axon retraction of VT growth cones (Figures 2,3 & Table 1) supports the notion that they express EphB1 at the time when this population would encounter ephrin-B2-expressing radial glia cells at the optic chiasm. While application of soluble ephrin-B2 does not replicate the physiological conditions at the optic chiasm, these findings clearly demonstrate the heightened sensitivity of VT axons to ephrin-B2 compared to RGC's from other retinal regions. It would be informative to analyze VT RGC growth cone behavior when ephrin-B2 application is restricted to one side of the growth cone (turning assay), or as RGC growth cones approach and interact with an ephrin-B2-expressing cell in real time.

Surprisingly, the majority of contralaterally-projecting EphB1– DT RGC growth cones also collapsed upon bath application of ephrin-B2. EphB2 is expressed in a high-ventral to low-dorsal gradient, so weak expression of EphB2 in the DT retina cannot be ruled out. Also, EphB3, which appears to be expressed homogeneously throughout the retina at E14.5 (Williams et al., 2003), could play a role in DT growth cone collapse. In support of this hypothesis, *EphB2*^{-/-};*EphB3*^{-/-} double knockout mice display intraretinal guidance defects specifically from the dorsal retina (Birgbauer et al., 2000), implying a functional role for EphB2 and/or EphB3 in dorsal RGC axon projections.

DT growth cones collapse occurred on average 7.5 minutes after ephrin-B2 exposure (Table 1). This long delay compared to VT (and VN) RGC axons could be explained by lower levels of EphB receptor expression on DT growth cones, or an absence or reduction of other downstream signaling proteins, resulting in more delayed signal transduction. This significant difference in lag time makes it difficult to assess how closely linked the growth cone collapse is to ephrin-B2 signaling, as the delayed collapse could be due to an indirect mechanism of ephrin-B2 treatment. Additionally, DT growth cones were more sensitive to control Fc treatment (28% for DT growth cones vs. 4% for VT growth cones, $p < .05$), and thus in general DT growth cones may be more sensitive to environmental changes. Of note, most DT RGC axons recovered by 30 minutes post-ephrin-B2 treatment (Figure 2C). Thus, DT growth cones and axons are significantly less sensitive to ephrin-B2 compared to VT RGC axons, as would be expected for a contralaterally-projecting population of RGCs.

RGC fibers arising from the VN retina present an interesting contrast to VT and DT RGCs as they do not express EphB1, but do express EphB2 (and likely EphB3) (Birgbauer et al., 2000; Williams et al., 2003), both of which can interact with ephrin-B2 (Flanagan and Vanderhaeghen, 1998). Although VN RGC axons project contralaterally, there is evidence that VN axons are sensitive to ephrin-B2 *in vitro* (Williams et al., 2003) and overexpression of EphB2 can redirect axons ipsilaterally *in vivo*, albeit less efficiently compared to EphB1 (Petros et al., 2009). Applying a range of ephrin-B2 concentrations revealed that VT and VN RGC axons respond similarly when exposed to high (5.0-0.5 $\mu\text{g/ml}$) or low (5.0 ng/ml) ephrin-B2 concentrations. However, we found that VT growth cones were significantly more sensitive to an intermediate concentration of 50 ng/ml ephrin-B2 compared to VN growth cones (Figure 4A). Additionally, dose-response curves highlighted a trend for VT axons to retract at higher rates compared to VN axons, thus displaying increased ephrin-B2 sensitivity (Figure 4B).

One implication of this finding is that the level of ephrin-B2 expression at the chiasm must be carefully regulated in order to elicit specific repulsion of VT axons, as too much ephrin-B2 could potentially repel VN axons. Another possibility is that VN axons have cell autonomous mechanisms to repress ephrin-B2-induced repulsion; or conversely, VT axons could have mechanisms to enhance repulsion from lower levels of ephrin-B2. It would be interesting to artificially increase the concentration of ephrin-B2 in radial glia cells at the chiasm midline to see if this would drive VN axons to an ipsilateral fate.

One complication with these results is that the actual levels of EphB receptor proteins on RGC axons from different quadrants are unknown. Whereas EphB1 is restricted to RGCs, EphB2 and EphB3 are expressed in other retinal layers (Williams et al., 2003). Thus, performing qPCR on various quadrants would not accurately capture EphB protein levels on RGC axons and growth cones. Ectopic expression of EphB1 and EphB2 in RGCs revealed that EphB2 is expressed at much higher levels on RGC axons compared to EphB1 (Petros et al., 2009), implicating specific mechanisms to regulate the translation and membrane insertion of different EphB receptor subtypes. Thus, it is difficult to directly correlate the levels of EphB receptor subtype expression with growth cone behavior in response to ephrin-B2. However, our results clearly indicate that EphB1+/EphB2+ VT axons display increased sensitivity to ephrin-B2-induced repulsion compared to EphB1−/EphB2+ VN axons.

ROCK activity in ephrin-B2-induced growth cone collapse

Numerous studies have demonstrated a role for Rho signaling in ephrin-mediated growth cone repulsion (Shamah et al., 2001; Journey et al., 2002; Cowan et al., 2005; Sahin et al., 2005; Beg et al., 2007). In this study, we found that ROCK activity is specifically required for VT RGC axon retraction, but not growth cone collapse. This observation is in agreement with a previous report in which Y-27632 blocked ephrin-A5-induced axon retraction from chick RGCs, but not growth cone collapse (Harbott and Nobes, 2005). However, another study found that Y-27632 did block ephrin-A5-induced growth cone collapse in chick RGCs (Wahl et al., 2000). In this study, growth cone collapse was only examined 30 minutes after ephrin-A5 treatment, which is ample time for the RGC growth cones to have initially collapsed and then recovered.

One possible explanation for the requirement of ROCK in axon retraction but not growth cone collapse comes from close examination of its role in microtubule (MT) and actin reorganization in the growth cone. Rho and ROCK expression appears to be restricted to the central and transition domains of growth cones (Zhang et al., 2003), and thus ROCK inhibition may not directly affect lamellipodia and filopodia collapse in the peripheral domain of the growth cone. Instead, ROCK function may be restricted to the axon shaft and central domain of the growth cone. This is supported by recent evidence indicating that ROCK inhibition perturbs microtubule distribution and consolidation in the central domain of the growth cone (Schaefer et al., 2008), which is likely necessary for proper axon retraction but not growth cone collapse.

The exact role of Rho signaling in growth cone behavior and actomyosin contractility remains unclear. Several studies in chick found that Y-27632 increases lamellipodia and filopodia protrusions from multiple neuronal subtypes (Loudon et al., 2006; Rosner et al., 2007), while Y-27632 significantly decreased filopodial protrusions from *Xenopus* spinal cord neuronal growth cones (Woo and Gomez, 2006). Conflicting results on the role of ephrins and Rho signaling are also present in cell migration assays. Ephrin-A1 stimulation has been shown to both promote (Hjorthaug and Aasheim, 2007) and inhibit (Sharfe et al., 2002) T cell chemotaxis in a Rho-dependent manner. Similarly, ephrin-induced activation of Eph receptors and Rho signaling promotes migration of melanoma cells (Yang et al., 2006) but induces repulsion of carcinoma cells (Parri et al., 2007). Thus, the role of Rho activity

on ephrin-induced growth cone behavior (and cell migration) is likely to be dependent on the cell type, environmental conditions, type of assay, and Eph receptor and ligand involved for each scenario.

Collapse behavior *in vitro* versus *in vivo*

In general, all RGC growth cones display saltatory growth and streamlined morphologies when advancing through each segment of the visual pathway (Godement et al., 1994; Mason and Sretavan, 1997). At the chiasm midline, both contralaterally and ipsilaterally-projecting RGC growth cones undergo cycles of advance and retraction. After a relatively short pause, contralaterally-projecting growth cones traverse the chiasm midline rapidly, whereas uncrossed VT RGC axons stall for longer periods and eventually consolidates a backwards-directed filopodium that becomes the growth cone and leads the axon into the ipsilateral optic tract.

Growth cones of VT RGCs do not appear to undergo such drastic collapse and retraction *in vivo* compared to the behaviors observed in various *in vitro* assays (Godement et al., 1994; Mason and Wang, 1997). This is not surprising since these assays often expose growth cones to higher than normal ligand concentrations and are carried out in the absence of other endogenous cells and environmental cues. However, *in vitro* assays can still shed light onto growth cone behavior *in vivo*. For example, our finding that ephrin-B2 induces growth cone collapse from DT RGCs *in vitro* could correspond to the pausing and exploratory behavior that non-VT growth cones display at the chiasm midline. Additionally, the prominent growth cone collapse and retraction observed by VT RGC axons is consistent with the avoidance and turning away from the chiasm midline *in vivo*. Studies that combine manipulation of gene expression and labeling of RGCs via *in utero* retinal electroporation (Garcia-Frigola et al., 2007; Petros et al., 2009) with live imaging of growth cones at the optic chiasm (Godement et al., 1994; Mason and Wang, 1997) should resolve these issues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Bath application induces growth cone collapse from VT but not DT explants
 Retinal explants were treated with clustered 0.5 $\mu\text{g/ml}$ Fc or ephrin-B2 for 30 minutes, then fixed and immunostained for neurofilament (NF, green), with Cy3-conjugated phalloidin (Phal, red) used to visualize growth cones. Insets depict individual examples of growth cones for each condition. Prominent growth cones with lamellipodia and/or filopodia were visible on VT and DT axons 30 minutes after Fc treatment, as well as DT axons treated with ephrin-B2. Conversely, nearly all VT growth cones were collapsed 30 minutes after ephrin-B2 exposure. Scale bars = 20 μm .

Figure 2. Ephrin-B2 has differential effects on the behavior of VT and DT explants
Still frames taken from DIC time-lapse movies of growth cones from VT or DT retinal explants exposed to 0.5 $\mu\text{g/ml}$ ephrin-B2 or human-Fc at t_{15} minutes (dashed grey arrow). Black and white arrows highlight representative growth cones in each frame, with their corresponding location at t_0 indicated with a black or white dot, respectively. (A) VT growth cones (and DT growth cones, not shown) continue to advance unhindered after application of human-Fc. (B) The majority of VT growth cones collapse and retract within several minutes after exposure to ephrin-B2, with some axons retracting out of the field of view (white arrow). (C) The response of DT RGC growth cones upon ephrin-B2 treatment is

dampened and more variable, with some axons retracting (black arrow) and other axons continuing to advance (white arrow). Scale bars = 20 μ m.

Figure 3. Ephrin-B2 induces axon retraction from VT explants

(A, B) Graphs depicting the extension and retraction of RGC axons from VT explants exposed to either 0.5 μg/ml Fc or ephrin-B2 after 15 minutes (black arrows). Each individual axon is represented by a different colored line. Note that the trace of some VT axons flattens out after ephrin-B2 treatment, indicating that the axon retracted out of the field of view. (C) Traces from individual DT axons reveal significant variability in their response to ephrin-B2, with many axons pausing and then either extending or undergoing moderate levels of retraction. (D) Average trace of VT and DT axon extension and retraction in response to ephrin-B2 or Fc control. Fc treatment has no effect on either VT or DT axon extension. Ephrin-B2 induces strong and rapid retraction of VT axons, whereas DT axons display slower and weaker retraction upon ephrin-B2 exposure. Average extension/retraction $\pm$ standard error for each condition 30 minutes after ephrin-B2 or Fc treatment (t_{45})

– t_{15}), + signifies extension and – signifies retraction: VT + Fc = $+48.97 \pm 20.82 \mu\text{m}$; DT + Fc = $+56.70 \pm 17.10 \mu\text{m}$; VT + ephrin-B2 = $-249.98 \pm 35.63 \mu\text{m}$; DT + ephrin-B2 = $-50.10 \pm 34.10 \mu\text{m}$.

Figure 4. Ephrin-B2 dose response curve reveals differential effects on VT, DT and VN RGCs (A) Traces depict average path of RGC axons from VT, DT and VN retinal explants exposed to varying concentrations (5.0 $\mu\text{g}/\text{ml}$ to 5.0 ng/ml) of ephrin-B2 at t_{15} minutes (black arrow). 5.0 ng/ml ephrin-B2 had little effect on all retinal regions. DT axons (blue traces) undergo very weak axon retraction even at the highest ephrin-B2 doses. Axons from both VT (red traces) and VN (green traces) explants rapidly retract at a similar distance and timescale when treated with 5.0 $\mu\text{g}/\text{ml}$ or 0.5 $\mu\text{g}/\text{ml}$ ephrin-B2, but a significant difference is observed at 50 ng/ml ephrin-B2 (black arrowheads). $n \geq 12$ DT growth cones, $n \geq 17$ VN growth cones, $n \geq 11$ VT growth cones for each condition. (B) Dose-response curves of different ephrin-B2 concentrations (5 ng/ml = 0.70, 50 ng/ml = 1.70, etc.) on the rate of

axon retraction in the first four minutes immediately following ephrin-B2 treatment (t_{16} – t_{20}). Although not statistically significant, VT axons show an increased retraction rate, and thus ephrin-B2 sensitivity, compared to VN axons, as demonstrated by the leftward shift of the VT trace. Both VT and VN are significantly more sensitive compared to DT axons at 50, 500 and 5000 ng/ml ephrin-B2 (*, $p \leq .005$).

Figure 5. Inhibiting Rho-kinase with Y-27632 blocks the rapid and sustained retraction of neurites from VT explants

(A) Still frames taken from DIC time-lapse imaging of VT growth cones incubated in SFM + 10 μM Y-27632 for 1–2 hours (last 15 minutes of Y-27632 pretreatment is t_0 – t_{15}). At t_{15} , medium was replaced with SFM + 10 μM Y-27632 + 0.5 $\mu\text{g/ml}$ ephrin-B2 for 30 minutes. Red and blue arrows highlight representative growth cones that were located at the corresponding red and blue dots at t_0 . (B) Graph displays the extension and retraction of VT axons (individual axons represented by a colored line) pre-exposed to Y-27632, with ephrin-B2 added after t_{15} (black arrow). Note that several axons recovered and extended forward after ephrin-B2 treatment, which was rarely observed in control ephrin-B2-treated VT

explants (Figure 2). $n = 23$ VT axons, average retraction 30 minutes after treatment: VT + ephrin-B2 + Y-27632 = $-29.72 \pm 34.10 \mu\text{m}$ (C) Summary of extension and retraction behaviors of axons from VT and DT explants exposed to Fc, ephrin-B2, and ephrin-B2 + Y-27632. Note that ROCK inhibition causes weaker and delayed axon retraction from VT explants, comparable to DT explants in ephrin-B2. Scale bar = $20 \mu\text{m}$.

Table 1
Percentage and time-course of ephrin-B2-induced growth cone collapse

Table displays the total number of growth cones analyzed for each condition, the number of growth cones that collapsed at any point following ephrin-B2 or human-Fc treatment, and the average time post-treatment that it took for growth cones to collapse.

Treatment	Total # GCs	# GCs collapse (% collapsed)	Delay in GC collapse (min $\pm$ sem)
VT + Fc	26	1 (3.9%)	---
DT + Fc	28	8 (28.6%)	3.13 $\pm$ 0.96
VT + ephrin-B2	48	47 (97.9%)	1.54 $\pm$ 0.12
DT + ephrin-B2	22	13 (59.1%)	7.54 $\pm$ 1.38
VT + Y-27632 + ephrin-B2	23	22 (95.7%)	2.80 $\pm$ 0.31