Elsevier Editorial System(tm) for Biochemical and Biophysical Research Communications
Manuscript Draft

Manuscript Number:

Title: Neurite responses to ephrin-A5 modulated by BDNF: evidence for TrkB-EphA interactions

Article Type: Regular Article

Keywords: ephrin-A; EphA; BDNF; TrkB; topography; retinal ganglion cells

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Dear Professor,

We are pleased to submit to Biochemical and Biophysical Research Communications (BBRC) our manuscript entitled "**Neurite responses to ephrin-A5 modulated by BDNF: evidence for TrkB-EphA interactions**" by Melinda Fitzgerald, Alysia Buckley, Sherralee S. Lukehurst, Sarah A. Dunlop, Lyn D. Beazley and Jennifer Rodger. We believe that this manuscript constitutes a significant and novel contribution that would be facilitated by the rapid publication achieved by BBRC.

In the developing visual system, roles for individual guidance cues in the establishment of topography are well defined. However, during early postnatal life, retinal ganglion cells are exposed simultaneously to multiple guidance cues as rough topography is refined to the mature pattern. In this study we demonstrate that during the establishment of rough topography (P3), growth cones of pure and explanted RGCs treated with combinations of BDNF and ephrin-A5-Fc responded differently than RGCs treated with BDNF or ephrin-A5-Fc alone ($p=0.0083$). The response to the combined treatment mimicked that of RGCs cultured with ephrin-A5-Fc alone once topography refines. We have explored the mechanism of this change and demonstrated that the guidance cue receptors EphA and TrkB co-localise and physically interact. Our results suggest that the conversion of growth cone responses from collapse to stabilisation as topography refines, occurs as a result of interactions between EphA and TrkB receptors.

This manuscript has not been simultaneously submitted elsewhere for publication and we strongly hope that you find it suitable for publication in BBRC. We note that while the manuscript falls well within the 4200 word limit, it has more characters than indicated under the BBRC guidelines for authors. As the manuscript contains only 3 figures, we hope the word count of 3844 is acceptable. Thank you for your time.

Yours sincerely

Melinda Fitzgerald

Neurite responses to ephrin-A5 modulated by BDNF: evidence for TrkB-EphA interactions

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ABSTRACT

In the developing visual system, growing retinal ganglion cell (RGC) axons are exposed to multiple guidance and growth factors. Furthermore, the relative levels of these factors are differentially regulated as topography is roughly established and then refined. We have shown that during the establishment of rough topography (P3), growth cones of pure and explanted RGCs treated with combinations of BDNF and ephrin-A5-Fc responded differently than RGCs treated with BDNF or ephrin-A5-Fc alone ($p=0.0083$). The response to the combined treatment mimicked that of RGCs cultured with ephrin-A5-Fc alone once topography refines. The guidance cue receptors EphA and TrkB were shown to co-localise in RGCs *in vitro*. Furthermore, EphA and TrkB receptors interacted directly in *in vitro* binding assays. Our results suggest that the conversion of growth cone responses from collapse to stabilisation as topography refines, occurs as a result of interactions between EphA and TrkB receptors.

Key words: ephrin-A, EphA, BDNF, TrkB, topography, retinal ganglion cells

INTRODUCTION

Topographic maps are a fundamental organising principle within the nervous system and much attention has been focussed on guidance cues involved in their development [1]. The role of guidance cues in establishing topography has been most widely studied in the visual system [2, 3]. In mammals, retinal ganglion cell (RGC) axons project topographically to a primary visual brain centre, the superior colliculus (SC) and recreate a map of visual space within the brain [1]. Rough topography is established first and requires repulsive signalling by guidance cues, presumably to minimise the formation of connections in inappropriate locations [4]. The rough map is then hypothesized to be refined by neuronal activity which stabilises appropriate connections and removes inappropriate ones [5].

The best characterised of the repulsive cues in the developing visual system are ephrin-A ligands, which bind to EphA receptors [6-9], with studies of knockout mice confirming the key role of these proteins in establishing topography within the SC [10]. In mice, the transition between rough (unstable) to refined (stable) topography occurs between postnatal day (P) 3 to P8, when axon behaviour changes from searching widely to elaborating and stabilising an arborisation in a specific location [11, 12]. Stabilisation is thought to be triggered by the onset of correlated activity from the retina, and is likely to be controlled by the neurotrophin BDNF [13-15]. BDNF is an attractive and growth promoting molecule that is regulated by neuronal activity and encourages branching and terminal arborisation via its receptor TrkB [16-18]. However, repulsive guidance cues such as the Eph/ephrins remain strongly expressed throughout postnatal life, and in some instances in the adult [19], and it is unclear how RGC axons can override their repulsive signalling to establish stable

connections. Here we explore the possibility that growth cone responses to ephrins are modulated by other cues present in the extracellular environment, specifically BDNF.

METHODS

Animals and Anaesthesia

C57Bl/6 mice and PVG rats were bred at the Animal Resources Centre (Murdoch, WA) and were sacrificed at P3 or P8 by overdose with Euthal. Procedures conformed to "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and were approved by The University of Western Australia's Ethics Committee.

Tissue culture

Retinal explants: Explants were prepared from temporal retinae of P3 or P8 mice by partial digestion with 0.12% Trypsin (Sigma) in Hanks Balanced Salt Solution containing 0.004% DNase (Sigma) along with mechanical triturition. Explants were cultured on poly-lysine and or laminin (Gibco, Invitrogen) coated coverslips in Neurobasal A (Gibco) supplemented with 10% FCS and 0.4mg/ml penicillin/streptomycin. After 48 hours, explants were treated similarly to the purified RGCs, although BDNF pre-incubation was for 2 hours. Cells were photographed immediately and at 5 minute intervals following addition of pre-clustered ephrin-A5-Fc or IgG-Fc, for up to 120 minutes. Results were expressed as percent growth cone collapse of total neurites studied. 50-100 neurites were analysed for each treatment. To confirm that neurites from explants were of RGC origin, coverslips were fixed in Acetone/Methanol (1:1) and processed for immunofluorescence with the RGC specific marker (Tuj1, 1:400; Chemicon) and a glial-specific marker (GFAP, 1:400; Sigma). Only Tuj1-positive processes were included in the analysis. Differences in percentage growth cone collapse were detected using ANOVA and differences between individual treatments detected using Bonferroni/Dunn *post hoc* tests.

Purified RGCs: RGCs were isolated from temporal retinae of P3 PVG rats using a two step immunopanning technique as described by Barres *et al*., 1988. Isolated cells were 96.0±0.9% pure RGC's, as indicated by β-III tubulin (Chemicon) expression compared to total cell numbers identified by Hoechst. RGCs were cultured on poly-lysine and laminin, in serum free chemically defined media as detailed [20] for 24 hours. Confocal microscopy (BioRad MRC 1000/1024UV) with motorized stage at 37°C, was used for analysis of growth cone responses of triplicate cultures with >30 neurites/well. Images were obtained in 16 adjacent fields of view, with 4 optical slices (2µm increments) at 10 minute intervals. RGCs of relevant treatment groups were pre-incubated with BDNF (1nM, Chemicon International) for 4 hours, then RGCs imaged for a further 40 minutes, at 10 minute intervals, following addition of either control-IgG-Fc (10µg/mL clustering antibody; Sigma), pre-clustered ephrin-A5-Fc (10µg/mL ephrin-A5-Fc Chimera, R&D Systems), or pre-clustered ephrin-A5-Fc in addition to BDNF [21, 22]. RGC growth cones with outgrowth >10µm during the observation period were defined as advancing [23].

Immunohistochemistry

Fixed retinal explants from P3 mice were incubated overnight at 4ºC in primary antibodies: EphA7 (1:100, Santa Cruz) and TrkB (1:100, BD Transduction Labs). The specificity of the antibody to TrkB has been extensively characterized [24] and characterization of the antibody to EphA7 by western blotting was conducted (not shown). Antibody binding was visualised following 2 hour incubation with anti-rabbit (Alexa Fluor 488, 1:400 Molecular Probes) or anti-mouse (Alexa Fluor 546, 1:400, Molecular Probes) secondary antibodies.

Slides were coverslipped using Fluromount-G (Southern Biotech) and viewed using fluorescence or confocal microscopy. Control explants stained only with secondary antibodies were included in all experiments and showed no fluorescence. Identity of RGCs in double fluorescent labelled explants was determined morphometrically and only growth cones on neurites longer than 100μm were analysed [12].

Surface Plasmon Resonance (SPR)

Analysis of protein interactions by SPR was carried out on a Biacore 3000 biosensor (Biacore). The extracellular domains of ephrin-A2-Fc (RnD systems), TrkB-Fc (RnD systems) and intracellular domains of EphA5-GST (Invitrogen) and TrkB-His (Invitrogen) were immobilised to a CM5 sensorchip (Biacore) by amine coupling according to the manufacturer's instructions. An anti-GST antibody to capture GST tagged EphA5 was included as a positive control. Analytes were diluted in running buffer (extracellular domains: final concentration: 10ng/μl in HBS-EP: 0.01M HEPES, pH 7.4; 0.15M NaCl; 3mM EDTA; 0.005% surfactant P20) or in kinase buffer (intracellular domains: final concentration: 10ng/ μl in 50mM Tris, pH 7.5, 150mM NaCl; 10mM MgCl2; 1mM MnCl2; 3%DMSO) and 60μl injected over the different flow cells of the sensorchip at a rate of 15μl/min. The surface of each flow cell was regenerated after each injection with 25mM NaOH. Sensorgrams were derived by subtraction of the response on the parallel blank channel, which was derivatised under identical conditions in the absence of protein.

RESULTS

Growth cones of RGCs taken during rough or refined topography responded differently to combinations of ephrin-A5-Fc and BDNF *in vitro*

RGC neurite growth cones extending from retinal explants responded differently to ephrin-A5-Fc when explants were taken at P3 or P8. P3 growth cones were more sensitive to pre-clustered ephrin-A5-Fc treatment than P8 growth cones ($p \leq 0.0001$, Fig. 1a). Growth cone collapse typically occurred within 20 minutes of ephrin-A5-Fc treatment. To test whether an increase in BDNF during refinement of topography could be responsible for the altered response to pre-clustered ephrin-A5-Fc, we analysed the response of P3 RGC neurite growth cones in the presence of pre-clustered ephrin-A5-Fc plus BDNF. The combined treatment resulted in less growth cone collapse than treatment with pre-clustered ephrin-A5-Fc alone ($p \leq 0.0001$, Fig. 1a, d-g) and was not significantly different from P8 growth cone response to pre-clustered ephrin-A5-Fc alone.

We confirmed the impact of BDNF on RGC neurite responses to ephrin-A5 using purified P3 RGC growth cone responses (from rough topography), which allowed us to confirm that responses were due to the exogenous cues and not to indirect effects from other cells in mixed cultures. Growth cone collapse was promoted by ephrin-A5 ($p=0.0083$), but not by BDNF alone ($p>0.05$). Addition of BDNF to ephrin-A5-Fc treated RGCs resulted in intermediate growth cone collapse (Fig. 1b). However, growth cone advance was significantly promoted by BDNF compared to ephrin-A5-Fc ($p=0.0083$) and ephrin-A5-Fc + BDNF ($p=0.0083$) treatment of RGCs (Fig. 1c). No significant differences in growth cone

advance were observed between control-IgG-Fc, ephrin-A5-Fc and ephrin-A5-Fc + BDNF treated RGCs ($p>0.05$).

Co-localization of EphA and TrkB *in vitro*

EphA7 and TrkB were co-localised on the growth cones of RGC axons from retinal explants from P3 (Figs. 2a-c) and P8 (Figs. 2d-f) mice. Similar co-localisation was observed for EphA5 and TrkB in the RGC growth cones (Data not shown).

EphA5 and TrkB binding *in silico*

To determine whether EphA and TrkB receptors interact directly, we examined protein interactions between the intracellular domains of EphA5 and the BDNF receptor TrkB by surface plasmon resonance. Immobilised EphA5 was strongly bound by TrkB, and immobilised TrkB by EphA5 (Fig. 3a). The difference in the binding response units for these two interactions was likely to be due to the orientation of the protein after capture. TrkB multimers also formed indicating TrkB-TrkB interactions [25]. An immobilised extracellular domain of TrkB was included as a negative control and was not bound by either the intracellular domains of EphA5 or of TrkB (<100 RU). Anti-GST was added as a positive control that bound exclusively to the GST tag on EphA5 (Fig. 3a). Further evidence for TrkB binding to EphA5 was obtained using EphA5 bound to immobilised anti-GST. The intracellular domain of EphA5 bound to the antibody via its GST tag and TrkB then bound to EphA5 (not shown).

To confirm that ephrin-A binding to TrkB was not responsible for the different responses in our culture assays, we confirmed that ephrin-A2 did not bind to the extracellular domain of TrkB. Furthermore, we performed a series of controls to verify Biacore specificity: as negative controls, ephrin-A2-Fc did not bind to itself, ephrin-B1-Fc, ephrin-A5-Fc, or control IgG-Fc (Fig. 3b). As positive controls, EphB2 extracellular domain bound weakly to the extracellular domain of ephrin-A2 as previously demonstrated [26]. In addition, the extracellular domain of ephrin-A2 bound to EphA5 and EphA3, as expected from previous studies [2, 26] (Fig. 3b).

DISCUSSION

Here we demonstrate that mouse retinal cultures explanted at the time of rough topography were more sensitive to ephrin-A5 than cultures explanted during refined topography. To our knowledge, this is the first demonstration of changes in sensitivity to guidance cues as topography is being established in the visual system. In addition, significant effects of BDNF on growth cone responses to ephrin-A5 and of ephrin-A5 on responses to BDNF, together with co-localisation of and physical interaction between EphA and TrkB receptor suggests that interaction between these receptors and/or their signalling pathways may regulate RGC responses during the transition between rough and refined topography.

Integration of multiple guidance cues

Ephrin-As are a source of repulsive signalling throughout the development of topography, and our results confirm a high proportion of growth cone collapse when RGCs are exposed to the protein. However, in addition to high ephrin-A expression, BDNF mRNA and protein have been shown to increase in the optic tectum during refinement of topography [27-29] Furthermore, RGC growth cones respond to BDNF alone with uniform and marked increases in growth cone size, complexity and branching [18, 30]. Our *in vitro* findings indicate that exposure to BDNF and ephrin-A5 reduces growth cone collapse induced by ephrin-A5, but also prevents extensive branching and terminal arborisation induced by BDNF. The intermediate response suggests a mechanism whereby integration of two opposing signals results in a balance between growth repulsion and promotion, permitting the formation of stable connections.

EphA and TrkB expression in early postnatal development

To investigate the mechanism for the combined impact of ephrin-A5 and BDNF signals on RGC neurite responses, we first confirmed that the switch in growth cone responses was not attributable to a down regulation in EphA receptors as both EphA5 and EphA7 protein expression in the RGC layer increased during this time (data not shown). Furthermore, the similar responses in purified RGC cultures suggest that no other cell types contribute to the response. These results support the possibility that ephrin-A5 and BDNF signals are being integrated at the level of intracellular signalling. Moreover, colocalisation of EphA and TrkB receptors [14, 16, present study] at the growth cones of explanted RGCs suggests a direct interaction between the receptors. EphA and TrkB are therefore at the same place at the same time to allow interaction at a functional level. Similar receptor interactions that have regulated axon pathfinding have been described for focal adhesion kinase and paxillin [31].

EphA and TrkB interactions

EphA and TrkB receptors might interact directly, or indirectly via crosstalk between signalling pathways. We provide evidence for a direct functional interaction through detection of stable binding between the intracellular domains of EphA and TrkB receptors. Upon binding, EphA and TrkB may phosphorylate and activate each other, as has been previously reported between other members of the receptor tyrosine kinase family [32]. Subsequent rapid regulation of EphA and/or TrkB downstream signalling pathways may explain the altered growth cone responses observed following treatment with ephrin-A5 and/or BDNF. Alternatively, EphA and TrkB signalling might converge at a shared

downstream target, resulting in crosstalk between signalling pathways. A possible target is the ankyrin repeat-rich membrane spanning protein (ARMS), which was tyrosine phosphorylated after BDNF or ephrin-B2 treatment [33].

In summary, our data are consistent with previous reports of a switching of growth cone responses from repulsion to attraction via more than one guidance cue. Cyclic nucleotides inhibited the collapse of spinal neuron growth cones induced by Collapsin-1/Semaphorin III/D [34]. Similarly, dorsal root ganglion growth cone responses to semaphorins 3A were altered by either NGF or BDNF [21, 35]. In the visual system, growth of RGC axons was differentially regulated by BDNF and Semaphorin 3E combinations [36]. Similarly, the switching of RGC growth cone responses by treatment with two guidance cues that we report here is likely to result from cross-talk between intracellular signalling pathways activated by EphA/TrkB receptor complexes. Given the sensitivity of BDNF and TrkB expression to neuronal activity, the integration of ephrin and BDNF signals would provide a mechanism that allows the growth cone to modify its growth pattern in response to changes in neuronal activity during development [21, 37].

ACKNOWLEDGEMENTS

This work was funded by the Neurotrauma Research Program, Western Australia. SAD is a Professorial Fellow, National Health & Medical Research Council of Australia (Grant ID: 254670). We are indebted to Professor Ralph Martins for access to the Biacore 3000. We thank Michael Archer for assistance with graphics and Carole Bartlett for technical assistance. The authors acknowledge the facilities, scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterization & Analysis, The University of Western Australia, a facility funded by The University, State and Commonwealth Governments.

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FIGURE CAPTIONS

Fig. 1. Responses of growth cones from retinal explants (a) or purified RGCs (b, c) to treatment with control-IgG-Fc (20nM), BDNF (1nM), pre-clustered ephrin-A5-Fc (10μg/ml) or pre-clustered ephrin-A5-Fc plus BDNF (1nM). Representative images are shown of RGC growth cones from P3 explants before treatment (d, f) and following treatment with ephrin-A5-Fc (e) or ephrin-A5-Fc plus BDNF (g). 50 – 100 neurites were assessed for each treatment group. Statistical analyses were performed using ANOVA and *post hoc* tests (*$p \leq 0.05$ significantly different from controls). Magnification x 25, scale bar = 10μm

Fig. 2. Explants derived from P3 (a-c) or P8 (d-f) animals were stained immunohistochemically with antibodies to TrkB (green, a, d) or EphA7 (red, b, e). Overlayed images are shown in (c) and (f). Magnification x 20, scale bar = 25μm

Fig. 3. Analysis of protein interactions by surface plasmon resonance. Interactions of selected analytes with immobilised intracellular domains of EphA5 (blue), TrkB (pink) and the extracellular domain of TrkB (green) are shown (a). Interactions between immobilised ephrin-A2-Fc and the extracellular domain of TrkB, or selected Ephs and ephrins are shown (b). Protein interactions were indicated by a Response Unit (RU) score greater than 100. R indicates regeneration of the ligand with 25mM NaOH.

Figure 1

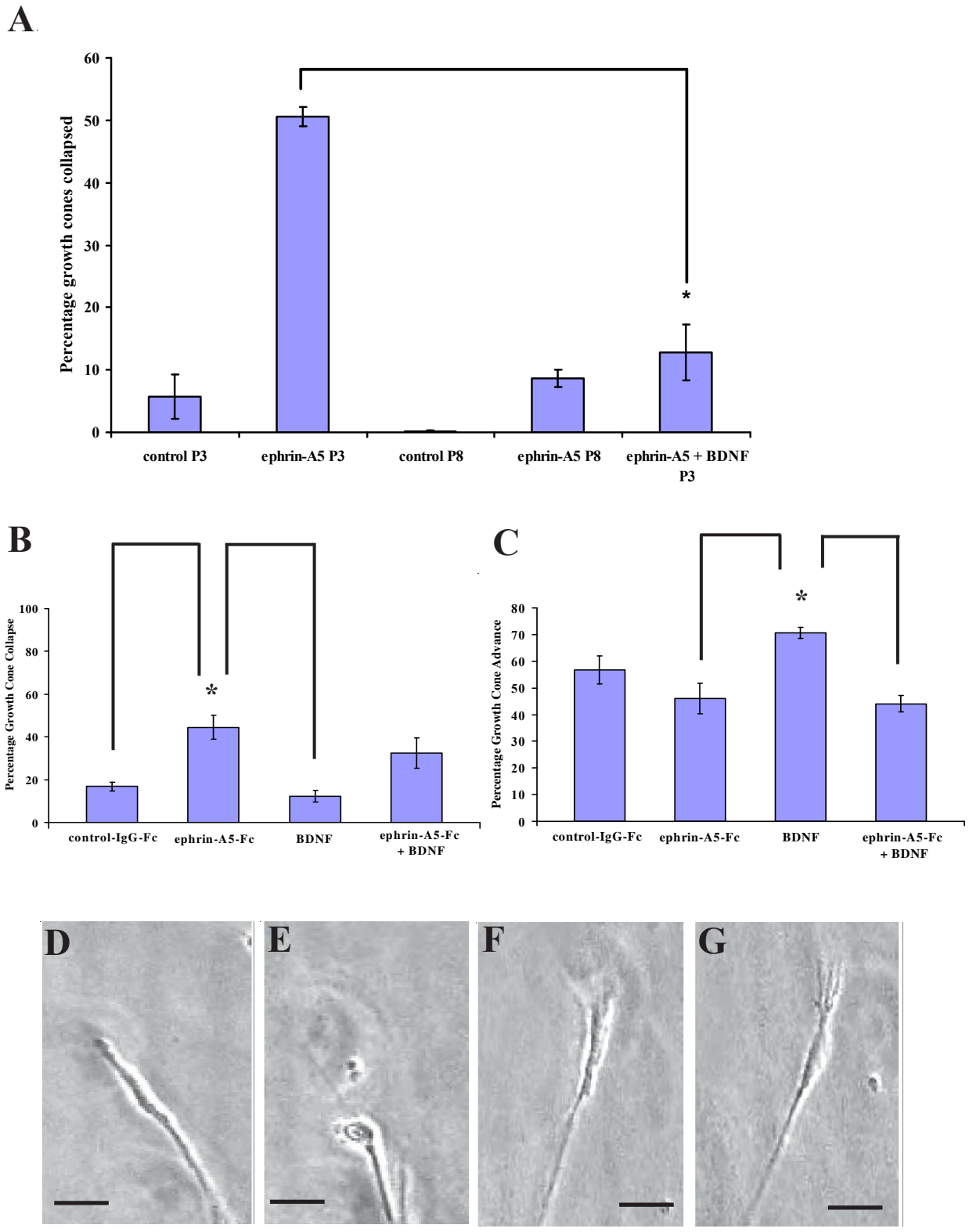
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Figure 2

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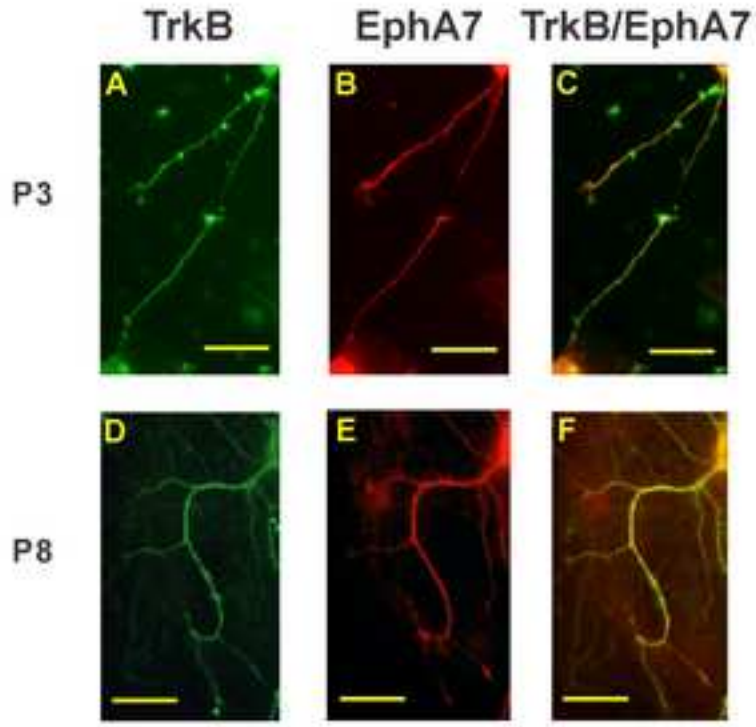
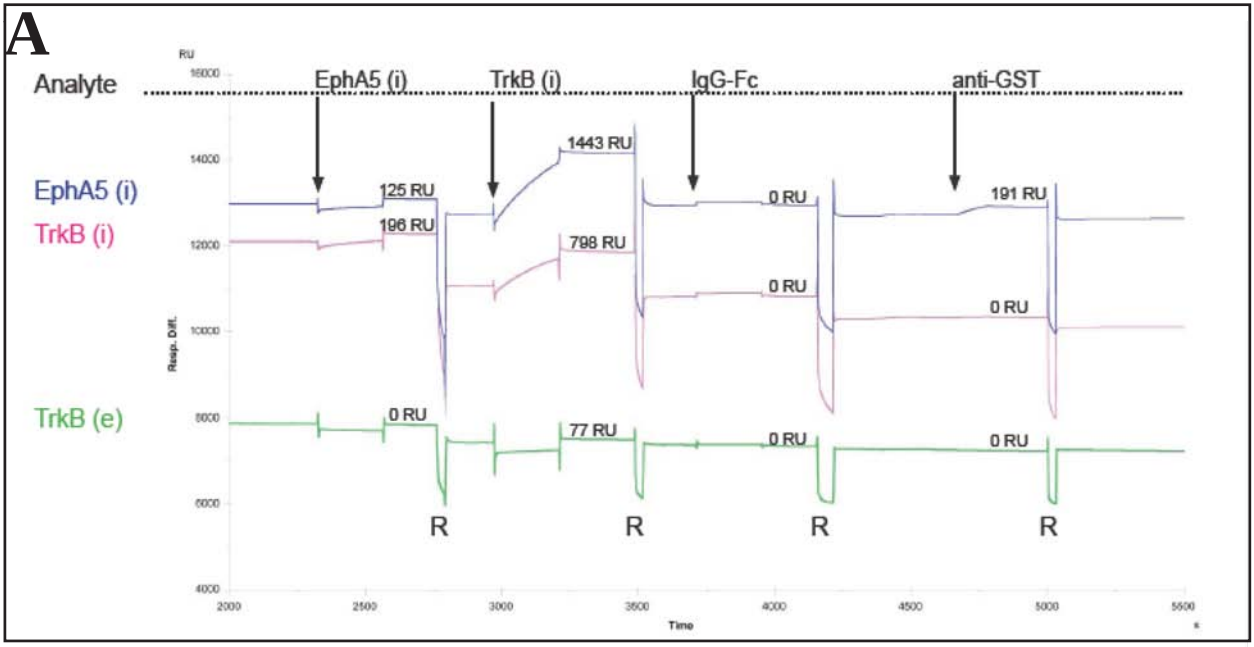
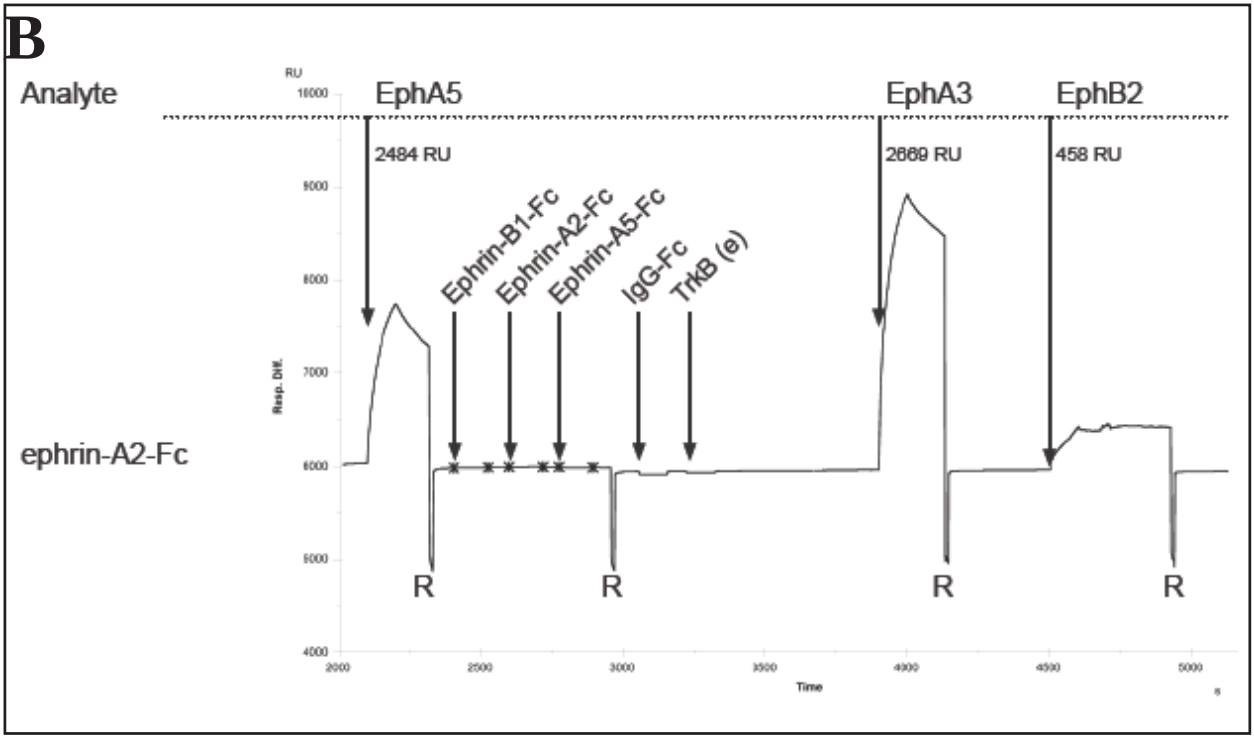



A
Analyte
EphA5 (i)
TrkB (i)
IgG-Fc
anti-GST
EphA5 (i)
TrkB (i)
TrkB (e)
125 RU
196 RU
1443 RU
798 RU
0 RU
0 RU
191 RU
0 RU
0 RU
77 RU
0 RU
0 RU
R
R
R
R
RU
Resp. Diff.
Time


B
Analyte
EphA5
EphA3
EphB2
2484 RU
2869 RU
458 RU
Ephrin-B1-Fc
Ephrin-A2-Fc
Ephrin-A5-Fc
IgG-Fc
TrkB (e)
ephrin-A2-Fc
R
R
R
R
RU
Resp. Diff.
Time