Optogenetic Activation of EphB2 receptor in Dendrites Induced Actin Polymerization by Activating Arg Kinase

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Abstract
Erythropoietin-producing hepatocellular (Eph) receptors regulate a wide array of developmental processes by responding to cell-cell contacts. EphB2 is well-expressed in brain and known to be important for dendritic spine development, as well as for the maintenance of the synapses, although the mechanisms of these functions have not been fully understood. Here we studied EphB2’s functions in hippocampal neurons with an optogenetic approach, which allows us to specify spatial regions of signal activation and monitor in real-time the consequences of signal activation. We designed and constructed OptoEphB2, a genetically encoded photoactivatable EphB2. Photoactivation of OptoEphB2 in fibroblast cells induced receptor phosphorylation and resulted in cell rounding -- a well-known cellular response to EphB2 activation. In contrast, local activation of OptoEphb2 in dendrites of hippocampal neurons induces rapid actin polymerization, resulting dynamic dendritic filopodial growth. Inhibition of Rac1 and CDC42 did not abolish OptoEphB2-induced actin polymerization. Instead, we identified Abelson Tyrosine-Protein Kinase 2 (Abl2/Arg) as a necessary effector in OptoEphB2-induced filopodia growth in dendrites. These findings provided new mechanistic insight into EphB2’s role in neural development and demonstrated the advantage of OptoEphB as a new tool for studying EphB signaling.
Introduction

Eph receptors comprise the largest receptor tyrosine kinase (RTK) family in mammals (Boyd et al., 2014). The 14 known members of the Eph receptor family are further divided into two subfamilies: the 9 EphA (A1-A8, A10) receptors primarily bind to GPI (glycosyl phosphatidylinositol)-linked ephrin-A ligands, and the 5 EphB (B1-B4, B6) receptors primarily bind to transmembrane ephrin-B ligands (Janes et al., 2012; Sloniowski and Ethell, 2012). Despite these ligand preferences, multiple studies have shown that cross-subfamily binding is also possible and the Eph receptors’ ligand specificities are not absolute (Kullander and Klein, 2002; Himanen et al., 2004; Noberini et al., 2012; Dai et al., 2014). Nevertheless, in most physiologic cases, both the receptors and their ligands are membrane-bound. Thus Eph receptors’ signaling initiation in vivo typically requires cell-cell contact (Janes et al., 2012; Lisabeth et al., 2013; Boyd et al., 2014). Furthermore, both ephrins and Ephs are capable of transmitting downstream signals into the respective cells presenting them, resulting in the so-called “forward” signaling downstream of the Eph receptors as well as the “reverse” signaling downstream of the ephrins (Janes et al., 2012; Lisabeth et al., 2013). By sensing cell-cell contacts within complex tissue structures, Eph-ephrin interactions regulate a large array of developmental processes such as cell positioning, tissue patterning, axon guidance and synaptogenesis (Sloniowski and Ethell, 2012; Boyd et al., 2014). Dysfunction in Eph/ephrin signaling has also been linked to various pathological processes, such as cancer and Alzheimer’s Disease (Chen et al., 2012; Boyd et al., 2014).

EphB signaling is important for multiple aspects of neural development. One function is to regulate axon pathfinding during embryonic stage. It is believed that EphB mediate this function by causing growth cone collapse (Pabbisetty et al., 2007; Lin et al., 2008; Schaupp et al., 2014). Meanwhile in dendrite (Bouvier et al., 2008), EphB is believed to regulate spine formation in hippocampal and cortical neurons (Sloniowski and Ethell, 2012). Previous studies have shown that deletion or inhibition of EphBs resulted in reduced spine density and dysmorphic spines in hippocampal neurons (Henkemeyer et al., 2003). Consistently, in vitro activation of EphBs by ligands rapidly increased dendritic spine density (Penzes et al., 2003). While these studies established an important role for EphBs in dendritic spine morphogenesis, the molecular mechanisms of these functions are still not fully understood. Current hypothesis is that EphB signaling is initiates at either the dendrite or dendritic filopodia due to contact with innervating axons, which expresses ephrin ligands. However, the exact effects of local EphB activation on dendritic morphologies have not been defined.
To facilitate further studies of Eph receptors’ signaling mechanisms, we sought to develop and characterize better tools to manipulate Eph receptor utilizing optogenetics. The current experimental method for activating Eph receptors relies on the bath application of solubilized ligands, which lacks spatial control and therefore cannot faithfully reproduce endogenous signaling processes that are initiated at subcellular regions of cell-cell contact. In addition, we also seek to overcome the complexity in decoupling consequences of the forward signaling and the backward signaling in the Eph-ephrin interaction, which could be difficult in many systems because the same cells could often express endogenously both the ephrin ligands as well as the Eph receptors.

**Results**

*Optically induced optoEphB2 clustering resulted in receptor activation*

We report the development of OptoEphB2, a genetically-encoded, photoactivatable EphB2 based on the blue light-induced clustering of the *Arabidopsis thaliana* photoreceptor Cryptochrome 2 (Cry2) (Kennedy *et al.*, 2010; Bugaj *et al.*, 2013). The blue light-induced clustering promotes receptor cross-phosphorylation leading to receptor activation (Fig 1a). The strategy have been previously used to achieve optical activation of FGFR and Trk (Chang *et al.*, 2014; Kim *et al.*, 2014), two other members of the RTK family. However, we found that OptoEphB2 designed using wild-type Cry2 did not yield consistent receptor phosphorylation. We suspected that this is because, unlike most RTKs, which only need receptor dimerization for activation, Eph receptors are known to require high-order cluster formation (Davis *et al.*, 1994; Stein *et al.*, 1998), and wild-type Cry2 did not generate clusters that are big enough. Thus a recently-identified mutant, Cry2olig (Cry2 E490G), which has a higher tendency to form high-order clusters (Taslimi *et al.*, 2014) was used in our final design. In addition, we replaced the extracellular domain (ECD) and the transmembrane sequence of the EphB2 with an N-terminal myristoylation signal peptide (derived from c-Src) (Fig. 1a&b, Fig. S1). This was done to ensure that only the forward signaling, and not a combination of both the forward and the reverse signaling, is being activated. Conversely, expressing the ECD domains could cause inadvertent receptor activation due to interactions with endogenous ephrins, as well as ECD-mediated receptor-receptor interactions (Himanen *et al.*, 2010). Indeed, constructs that employed full-length Eph receptor sequences failed to localize to the plasma membrane (Fig. S2) in HEK296 cells and were found mainly on intracellular vesicles, suggesting receptor activation and internalization.
The design of OptoEphB2 shown in Fig. 1 resulted in plasma membrane localization and robust blue light-induced clustering (Fig. 1b). OptoEphB2-expressing mouse embryonic fibroblasts (MEFs) subjected to blue LED light illumination exhibited significantly higher overall tyrosine phosphorylation compared to cells left in the dark (Fig. 1c). In contrast, blue light produced no increase in tyrosine phosphorylation in cells expressing kinase-dead optoEphB2 (KD-optoEphB2), which contained a mutation (K99M in OptoEphB2) in the kinase domain (Fig. 1c). The most significant increase in phosphorylation was observed near 135 kDa, consistent with the size of OptoEphB2. Anti-phosphotyrosine blot analysis of immune-precipitated OptoEphB2 indeed verified this and showed an approximately 29-fold increase of tyrosine phosphorylation (Fig. 1c) in blue light-illuminated samples.

To further test whether OptoEphB2 phosphorylation serves a functional role, we examined if photoactivation of OptoEphB2 produced the same cellular phenotypes as those caused by ligand-mediated activation. One of the most widely reported cell phenotype upon Eph activation is retraction of cellular protrusions and cell rounding (Zou et al., 1999; Elowe et al., 2001; Zimmer et al., 2003; Lin et al., 2008; Lisabeth et al., 2013). We first verified that the MEF cell line indeed has such a response when stimulated with pre-clustered ephrinB1-Fc ligands (Fig. S3). Consistently, we found that photoactivation of OptoEphB2 in MEFs also quickly induced cell rounding (Fig. 1d, Video S1). As expected, the observed cell rounding phenotypes were kinase-dependent, as blue light illumination of KD-optoEphB2 produced receptor clusters but did not result in significant reduction in cell area (Fig. 1d). Furthermore, when photoactivation is restricted to sub-cellular regions using digital light patterning (Guo et al., 2009), both receptor clustering and cell retraction were spatially restricted to the photoactivated regions, while non-illuminated regions were unaffected (Fig. 1e), demonstrating the ability of spatially controlling EphB signaling with OptoEphB2.

**Kinetics of OptoEphB activation**

To characterize the kinetics of OptoEphB2 activation (Fig. 2), we analyzed the rate of tyrosine phosphorylation (Fig. 2a) as well as cluster formation in cells with live cell time-lapse imaging (Fig. 2b). We found that the OptoEphB2 cluster density increases quickly with a time constant of ~15 sec, when cells were illuminated with 440-nm blue LED (~10 mW/cm², 0.1 Hz pulsed). In comparison, Western analysis of receptor phosphorylation (Fig. 2a) in cell lysates collected at various time delays after blue light illumination (~0.5 mW/cm²
blue LED) showed a similarly rapid increase, with a time constant of ~50 sec. Saturation of phosphorylation was reached at about ~3 min and the phosphorylation level remained constant until the end of the experiment (10 min). The kinetics of the cell rounding after OptoEphB2 activation was also quantified by measuring cell area in time-lapses microscope data (Fig. 2c). As expected, the kinetics of cell rounding is significantly slower than the receptor phosphorylation and cluster formation, as the signal propagate from the receptor to the downstream effectors that ultimately remodel actin cytoskeleton and cell morphology. Collapse of membrane cell protrusions was observed with only a delay of 1-2 min, and cell rounding typically takes ~10 min to finish.

Previous report has shown that Cry2olig cluster formation is reversible (Taslimi et al., 2014). Thus, we tested if the OptoEphB2-induced cell phenotype was also reversible. By leaving a pre-stimulated cell in the dark, we observed that the receptor clusters dissipated within 5 min (Fig. 2d, Video S2). This is somewhat surprising because the dissipation rate is faster than previously reported for cytoplasmic Cry2olig-mCherry (Taslimi et al., 2014). This may be due to differences in clustering on the plasma membrane vs cytosol or the effects of EphB2 ICD signaling. Additionally, the cell started to re-expand by generating highly dynamic membrane protrusions (Fig. 2d, Video S2), indicating that the whole process was reversible. In addition, after recovery of the cell area, we could reinitiate the cell rounding process again by a new round of blue light illumination, indicating that the OptoEphB2 receptor activation together with its downstream processes are indeed reversible.

**OptoEphB2 activation in dendrites of hippocampal neurons induced actin nucleation**

We first tested whether OptoEphB2 expression alters endogenous EphB2 localization in hippocampal neurons (Fig. S6). EphB2 was detected primary in soma-dendritic compartments and appears to be slightly clustered along the dendritic shaft (Fig. S6). We detected no significant changes in this localization pattern in cells expressing OptoEphB2-mCherry (Fig. S6). Next, we performed OptoEphB2 activation in primary hippocampal neurons of DIV9-11. We found that localized photoactivation of OptoEphB2 induced formation of dynamic filopodia-like protrusions within the region of activation (Fig. 3a-b, Video S3). The results were compared with cells expressing KD-optoEphB2 or LI-optoEphB2, an OptoEphB2 mutant carries a D387A change in the Cry2 sequence, making the molecule light-insensitive (LI). Clusters were produced from OptoEphB2 or KD-optoEphB2, but not LI-optoEphB2 (Fig. 3a). However, of OptoEphB2 cells produced filopodia
protrusions. Thus, the observed effect requires both clustering and the kinase activity. Filopodia appeared and disappeared transient during the experiment. To quantify these dynamic morphological changes in dendrites, we generated maximum intensity projection images by always taking the brightest pixels over 5-minute time-lapse segments (Fig 3b). Thus, the cell area in the final image represented total area filopodia explored during their dynamic protrusion/retraction process. We measured cell areas of the maximum intensity projection images and performed multivariate linear regression analyses against stimulation time. We found that that cells expressing OptoEphB2, but not KD-optoEphB2, exhibit significant increased cell area after activation (p = 0.008). Furthermore, after 5-minute photoactivation, t-test showed significant differences in cell areas between OptoEphB2 cells and KD-optoEphb2 cells (Fig. 3c).

Dendritic filopodia are membrane protrusions supported by actin cytoskeleton (Korobova and Svitkina, 2010). To see if OptoEphB2-induced filopodia are indeed a result of actin polymerization, we labeled F-actin by transflect cells with mCherry-Lifeact construct (Addgene plasmid #54491). We found that after photo-activation of OptoEphB2, Lifeact accumulated in a punctate distribution on the periphery of the dendrite in the activation ROI (Fig. 4a), indicating increased actin polymerization at the base of newly formed filopodia. To further examine this phenomenon, we designed a double-activation protocol to test whether the effects were sensitive to CK666, an Arp2/3-dependent actin nucleation inhibitor. The protocol was based on earlier finding that OptoEphB2 signal is reversible (Fig. 2d). Similarly, OptoEphB2 induced filopodia retracts after sample sit in the dark for 20 min, allowing a second round of photoactivation at the same dendritic region, which produce the dynamic filopodia again (Fig. 4b). To test effect of CK666, we treated cell with CK666 right before the 2nd stimulation and compare the results with the 1st stimulation (Fig 4c). Indeed we found (Fig. 4d) that the induction of filopodia can be blocked by treatment of CK-666 (Fig. 4c,d), suggesting that EphB activation induces branched actin nucleation, which in turn give rise to increased F-actin and formation of new filopodia.

It has been suggested that axon-dendritic contact during neural development lead to EphB activation directly in dendritic filopodia (Sloniowski and Ethell, 2012). Therefore, we further tested whether OptoEphB2 activation can induced actin polymerization specifically in dendritic filopodia. The experiments were carried out in primary hippocampal neurons of DIV9-11 co-expressing OptoEphB2-Venus and mCherry-Lifeact. Filopodia were stimulated
by targeting blue light to a region of illumination (ROI) oriented lengthwise along, but offset from, the dendritic shaft. We found that indeed OptoEphB2 activation resulted in significantly increased Lifeact signal (~57% increase) in dendritic filopodia (Fig. 4e,f), consistent with the idea that the stimulation promote F-actin nucleation just like in dendritic shafts.

*Arg kinase act downstream of EphB2 and is required for OptoEphB2-induced dendritic actin polymerization*

To elucidate the molecular mechanism of OptoEphB2 induced actin polymerization in dendrites, we performed a high-throughput phosphotyrosine profiling assay previously described (Machida *et al.*, 2007) and dubbed as the “Rosette” assay. The Rosette assay is a reverse-phase protein-binding assay using a library of purified Src-homology 2 (SH2) proteins as probes to detect interaction with small amount of cell lysate spotted on membrane. Because SH2 domains are the phosphotyrosine-binding modules of many important RTK effectors, the assay allows identification of potential SH2-containing effectors by examining changes in their binding to cell lysates. Using a library of 48 probes, we performed the screening on lysates from cells stimulated with OptoEphB2 or KD-optoEphB2 (Fig. 5a,b and Fig. S4). For comparison, a similar assay is also performed on cells expressing wt-EphB2 stimulated with ephrinB1 ligand (Fig. 5c,d and Fig. S5). In both cases, we found that the SH2 probe from Arg, a non-receptor tyrosine kinase, exhibited highest induced binding to activated lysates. To further validate the interaction between EphB2 and Arg, we performed co-clustering experiment in cells co-expressing Arg and OptoEphB2 (Fig. 5e). Using total internal reflection fluorescence microscopy (TIRFM), we found that Arg molecules cluster spontaneously on cell membrane. However, after blue-light photoactivation, the number of Arg clusters increased significantly and co-localized with the light-induced OptoEphB2 clusters (Fig. 5e), consistent with the hypothesis that OptoEphB2 interacts with Arg. As expected, the co-clustering was not observed in KD-optoEphB2 cells (Fig. 5e).

Arg is a regulator of actin cytoskeleton. It has been shown to play critical role in processes such as cell protrusion (Lapetina *et al.*, 2009) and dorsal-wave formation (Boyle *et al.*, 2007). In neurons, Arg has been shown to be important for the maintenance of cytoskeleton stability in the dendritic spines (Lin *et al.*, 2013). Thus, we suspect Arg acted downstream of OptoEphB2 to promote dendritic actin polymerization. To test this hypothesis, we co-expressed Arg and OptoEphB2 in hippocampal neuron and found that photoactivation of
OptoEphB2 resulted in its co-clustering with Arg (Fig. 6a) in dendrites, suggesting interactions between the two in dendrites. When kinase-dead Arg (KD-Arg) mutant was expressed, OptoEphB2 still cluster normally in response to light (Fig. 6b). However, the KD-Arg inhibited the filopodia-inducing effects of OptoEphB2 (Fig 6b, Video S4). Importantly, such inhibitory effect is specific to Arg, as expressing of kinase-dead Src (KD-Src), which is also a non-receptor tyrosine kinase with an SH2 domain, did not exhibit such effects (Fig. 6c). Similarly, we also found that the filopodia-inducing effects of OptoEphB2 is also abolished in the presence GNF2, a chemical inhibitor specific to the Abl/Arg family kinases (Fig. 6c,d). Combined, these results indicated that Arg is a required effector in the EphB2 pathway to promote actin polymerization.

**OptoEphB2 induced dendritic filopodia without activating Rac1 and CDC42**

Previous studies of EphB signaling have identified several RhoGEFs, including that of Rac1 and CDC42, that can bind to EphB (Penzes et al., 2003, Irie and Yamaguchi, 2002). Both Rac1 and CDC42 are central regulators of actin cytoskeleton, and therefore could play important roles in OptoEphB2-induced dendritic actin polymerization. On the other hand, existing literature on Arg suggested that Arg regulates actin cytoskeleton directly by activating actin nucleation promoters, such as cortactin (Boyle et al., 2007), or Nck (Antoku et al., 2008). Thus, its action may be independent of the RhoGTPases activation in dendrites. To see if this is true, we further performed OptoEphB2 photoactivation experiment while inhibiting either Rac1 or CDC42 by overexpressing dominant-negative (T17N) Rac1 (DN-Rac1) or dominant-negative (T17N) CDC42 (DN-CDC42). We found that neither condition resulted in strong inhibition of OptoEphB2-induced filopodial growth (Fig. 7). Subtle effects on filopodia morphology can be observed (Fig. 7a). For example, expressing DN-Rac1 seems to result in formation of longer filopodia after OptoEphB2 activation. Nevertheless, induction of filopodia were observed in all cells tested after OptoEphB2 activation. The results suggested that Arg-dependent mechanism for inducing filopodia is independent of the RhoGTPases pathway downstream of EphB.

**Discussion**

Despite extensive research into the mechanisms of Eph-ephrin signaling, the system has often defied efforts of establishing a unified signaling pathway to explain Eph receptor functions. The complexity of the system is manifested, in part, from the multitude of cellular responses observed from the signal activation, many of which seem to be apparently contradictory of
each other. For example, Eph-ephrin signaling was found to be capable of both suppressing (Miao et al., 2001) and activating (Genander et al., 2009) proliferation, and both strengthening (Carter et al., 2002) and weakening (Miao et al., 2000) focal adhesions. While Eph genes are consistently implicated in various forms of cancers (Pasquale, 2010), they can serve both as cancer suppressor (Batlle et al., 2005) and as promoter (Fang et al., 2005). In our study, this complexity was again demonstrated in that stimulation of OptoEphB2 have vastly different morphological consequences in fibroblasts (cell rounding) and in neurons (filopodial protrusion).

The complexity of EphB pathway demands more versatile research tools. Here we demonstrated a new optogenetic construct for activating Eph receptor signaling. It is worthwhile to discuss the key advantages, as well as potential problems, of the optogenetic method in comparison to ligand-mediated activation: (i) The main advantage of the optogenetic method is accurate spatial and temporal control over Eph receptor signaling. This specific feature carries particular importance for Ephs, whose in vivo activation is, by definition, spatially and temporally confined due to the nature of cell-cell contact. (ii) We also demonstrated that OptoEphB activation is reversible, whereas ligand activation is essentially irreversible. This reversibility is useful for experiments that require repeated cycles of activation in a controlled experimental setting, as we have demonstrated in this paper. (iii) Finally, although not directly utilized in the current study, OptoEphB presumably allows for testing the biological functions of specific members among the Eph family or, by extension, the effect of co-clustering two or more Eph receptors in a precise manner. Ligand-mediated stimulation does not allow control over cluster composition, since ligand binding may be promiscuous (Kullander and Klein, 2002; Himanen et al., 2004; Noberini et al., 2012; Dai et al., 2014) and receptor-receptor binding interfaces allow interactions between Ephs of different types (Himanen et al., 2010; Janes et al., 2011, 2012), thus any ligand may result in activation of multiple receptor subtypes. On the other hand, a potential disadvantage is that OptoEphB2 requires ectopic expression, which may perturb cell physiology. Eph receptors are also known to have ligand-independent and kinase-independent signaling activities (Genander et al., 2009; Himanen et al., 2010; Seiradake et al., 2010; Boyd et al., 2014). Whether these activities are perturbed by OptoEphB expression has not been fully investigated yet.
Our results also provided new insights into EphB signaling in synaptogenesis and dendritic spine development. While it is not entirely surprising that EphB2 play a role in regulating actin cytoskeleton, previous studies have been largely focusing on EphB2 signaling onto the RhoGTPase pathway, e.g., by activating Rac1 or CDC42 (Penzes et al., 2003, Irie and Yamaguchi, 2002); the importance of the Arg activation in this signaling pathway has not been previously identified. Furthermore, our data indicated that Arg could activate dendritic actin polymerization independent of the RhoGTPase activation. While this is consistent with the current literatures on Arg’s biochemical functions, it is interesting to note that previous experiments have found that inhibiting RhoGTPases Rac1 could block EphrinB-induced synaptic spine formation (Penzes et al., 2003). A possible explanation is that Rac1 is needed for the reorganization of the presynaptic structure, which is a part of the synaptogenesis process; while our experiments focused entirely on the dendritic region.

The importance for EphB2 in dendritic spine morphogenesis had been well accepted, but the exact mechanism has been under debate. Comparison between EphB1/B3 double-knockout and EphB TKO neurons suggested that EphB2 enhances the motility of dendritic filopodia, thereby increasing the probability of axo-dendritic contact formation (Kayser et al., 2008). However, one study of ligand-mediated stimulation of neurons suggested that EphB signaling shortens and stabilizes existing dendritic filopodia (Moeller et al., 2006), a dynamic change thought to initiate a transition from dendritic filopodia to dendritic spines (Ziv and Smith, 1996). Our experiments provided a real-time view of the cellular response from EphB2 activation, and provided unambiguous evidences that increased F-actin accumulation, as opposed to the stabilization of the F-actin, is the key consequence here. It also seems that EphB2 signaling may serves as a positive feedback mechanism in spine formation by promoting a high density of dendritic filopodia near sites of axo-dendritic contact, which would lead to more local axo-dendritic contacts.

**Materials and Methods**

*Reagents and cell culture:* All primary antibodies used in the study are purchased from respective commercial sources, including anti-phosphotyrosine (Cell Signaling, Danvers, MA), anti-tubulin (Thermo Scientific, Waltham, MA), anti-mCherry (Thermo Scientific, Waltham, MA), and anti-EphB2 (Santa Cruz Biotechnology, Dallas, TX). IRDye 680- and IRDye 800-labeled secondary antibodies were purchased from LI-COR (Lincoln, NE).
other chemicals used for the experiments were purchased from Sigma-Aldrich (St. Louis, MO).

OptoEphB2 as well as all variants and control constructs were derived from CRY2olig-mCherry or CRY2oligPHR-mCherry plasmids (Kennedy et al., 2010; Taslimi et al., 2014) obtained from Addgene (Cambridge, MA). A gateway cassette (ThermoFisher, Watham, MA) was cloned into the multiple cloning regions of these vectors to convert them into Gateway vectors. The intracellular domain (aa 565-986) of EphB2, together with an N-term myristoylation signal peptide (MGSNKSKPK) was amplified and cloned into gateway entry vector pDONR223 (ThermoFisher). The final optoEphB2 construct was obtained via LR recombination of the entry clone with CRY2oligPHR-mCherry-derived gateway vector. For two-color microscopy experiments, an mVenus labeled variant (OptoEphB2-Venus) was often used in place of the mCherry labeled one (OptoEphB2-mCherry), because it was detected that long exposure of green light could potentially activate OptoEphB2 to some degree. Using OptoEphB2-Venus can help reducing the total exposure of cells to green excitation in some experiments. OptoEphB2-Venus was constructed by replacing mCherry in OptoEphB2-mCherry via standard PCR cloning method. All other related variants were constructed in a similar manner. The kinase-dead KD-optoEphB2 construct was made by site-directed mutagenesis to introduce a K98M mutation. The light-insensitive Cry2 mutant, containing a D387A mutation, was previously described (Bugaj et al., 2013), and the mutation was introduced by subcloning fragments that contained the mutation. Both Arg and KD-Arg constructs are gifts from the Koleske lab (Yale). The mCherry-Lifeact plasmid was a gift from Michael Davidson (Addgene plasmid #54491).

To obtain cell lines expressing OptoEphB2, the complete OptoEphB2-mCherry coding sequence was subcloned into a lentiviral vector pLIX401, a gift from David Root (Addgene plasmid #41390). Viral particles produced from HEK293FT cells (ThermoFisher) were used to infect MEF-TetOff cells (Clontech, Mountain View, CA). All cell lines used were maintained in Dulbecco’s Modified Eagle Medium (Lonza, Switzerland) with fetal bovine serum (BioWest, Kansas City, MO). Cell lines were from commercial sources and had not been independently authenticated in the lab. Primary hippocampal neurons were plated and maintained as previously described (Tatavarty et al., 2012). Cells were isolated from pre-dissected embryonic hippocampi (E17-19) of Sprague-Dawley rats (Brainbits Inc, Springfield, IL) and plated onto plasma-cleaned 30-mm coverslips coated with 0.05% poly-
L-lysine at 90,000-100,000 cells/dish. Transient transfections of neurons were carried out using Lipofectamine 2000 (ThermoFisher), following manufacturer’s protocol with some modification 1-2 days before imaging.

Biochemical Assays
For western assays, cells were lysed in modified kinase lysis buffer (Ditlev et al., 2012) (KLB) as previously described, with 0.1% SDS added to aid in solubilizing large OptoEphB2 or optoEphB2-KD clusters. SH2 domain rosette screening was performed as described previously (Machida et al., 2007). Briefly, lysates were diluted with 2x spotting solution (100 mM Tris–HCl, pH6.8, 30% glycerol, 2% SDS) to approximately 4 μg/μL and spotted in duplicate in a rosette pattern on nitrocellulose membranes. Membranes were blocked with 5% milk in TBST (25 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05 % (v/v) Tween-20) and incubated with 200 nM GST-SH2 domains labeled with GSH-HRP for 2 h in a 96-well chamber plate. Each well was washed with TBST and chemiluminescent detection and quantification was performed using Carestream Image station system and Carestream MI software.

Microscopy and Image Analysis:
Live Cell Imaging and Optogeneic Control of OptoEphB2: Most live cell imaging experiments were carried out on a Nikon (Tokyo, Japan) Ti-E inverted fluorescence microscope with a 60x TIRF objective (NA = 1.49, Nikon). Images were acquired with an iXon Ultra EM-CCD (Andor, Oxford Instruments, Abingdo, Oxfordshire, UK). The microscope was placed within a temperature-regulated imaging chamber and cells were maintained at 37°C during imaging. For imaging mammalian cell lines, cells were kept in DMEM/F12 containing 2% FBS and 20 mM HEPES. For imaging neurons, the cells were kept in imaging medium containing 117 mM NaCl, 5 mM KCl, 1.25 mM NaH₂PO₄, 20 mM HEPES, 50 mM dextrose, 1 mM MgCl₂, 2 mM CaCl₂, and 100 mg/L BSA. Co-clustering assay was performed on an Olympus (Tokyo, Japan) IX81 TIRF microscope equipped with a 60x TIRF objective (NA = 1.49, Olympus) and a TE-cooled EM-CCD (PhotonMax, Princeton Instruments, Trenton, NJ). The 488-nm line of an argon ion laser was used to excite GFP and to photoactivate OptoEphB2, a 562-nm DPSS laser was used to excite mCherry, and a 442-nm DPSS laser was alternatively used for photo-activation of optoEphB2. Spatial control of OptoEphB2 was achieved using a Mosaic illumination system (Andor) coupled to a 440-nm LED (CoolLED, Andover, Hampshire, UK) on the Nikon Ti-E
microscope, unless otherwise noted. For focal illumination of dendrites, the mosaic was used to deliver blue light to a 40-pixel-diameter circular region. The ROI was expanded to cover the whole mosaic for global illumination. All image quantifications and analyses were performed in ImageJ.
References


Figure 1. Optogenetic activation of EphB2. (a) Schematic illustrations of OptoEphB2 domain structure and the photoactivation process. Blue light illumination induces Cry2 clustering, which results in receptor autophosphorylation (Y, tyrosine and pY, phosphotyrosine) and downstream signaling. ECD: extracellular domain. TM: transmembrane domain. ICD: intracellular domain. Myr: myristoylation signal peptide. FP: fluorescent protein. (b) TIRFM images of optoEphB2-expressing HEK293 cells before and after photo-activation (440 nm, three 250-ms pulses delivered 4.5 s apart), showing
optoEphB2 clustering. (C) Left: western blot analysis of whole cell lysates collected from MEFs expressing OptoEphB2-mCherry or KDoptoEphB2-mCherry that were illuminated by blue LED light (~10^{-2} W/cm^2), or left in the dark, for 1 minute. Right: quantification of OptoEphB2 phosphorylation. Relative tyrosine phosphorylation was assayed in OptoEphB2 immuno-precipitates and quantified by dividing the phosphotyrosine signal by the mCherry signal. Error bars show SEM (n=3). (d) Time-lapse TIRF images of MEFs expressing OptoEphB2-mCherry or KD-optoEphB2-mCherry showing kinase dependent cell-rounding after blue light illumination (10 mW/cm^2, 50-ms pulses at 3 pulses/min). Black dotted lines trace initial cell area and solid line traces the final cell area. (e) Time-lapse fluorescence images of a MEF cell activated by blue light illumination (100-ms pulses, 6 pulses/min.) within the specified region of illumination (ROI, black circle). OptoEphB2 clustering and cell process retraction were spatially restricted to the ROI. Time stamps are relative to the start of blue light illumination.
Figure 2. Kinetics of OptoEphB2 activation. (a) (Left) Western blot (pTyr) of MEF cell lysates after specified time of blue light illumination. (Right) Quantification of normalized total pTyr immunoactivity in cell lysates (n=3). Solid line denotes exponential fit with a time constant of 49.7 sec. (b) OptoEphB2 cluster density (# of cluster / cell area, n = 10) in MEFs under blue light activation. The dotted line denotes exponential fit with a time constant of
~15 seconds. (c) Quantification of MEF cell rounding kinetics. Cell area was normalized to the mean value prior to photoactivation. Both cells expressing OptoEphB2 (black line, n = 10) and the control cells expressing Kinase-dead mutant (gray line, n= 10) were show for comparison. (d) Testing of the reversibility of OptoEphB2-induced cell rounding. MEFs expressing OptoEphB2 were stimulated with multiple trains of blue light (6 x 100-ms pulses, 0.1 Hz), while cell morphology was monitored with TIRFM. Top panel shows selected frames in time-lapse data, showing MEF contraction and recovery after the first round of stimulations, as well as the re-contraction after the second round of stimulations. Bottom panel shows the quantification cell area over time, normalized to the average cell area prior to the first stimulation (2 minutes). Blue bars denote time for optical stimulation. All error bars denote standard error.
Figure 3. OptoEphB2 activation in dendrites induces dynamic filopodial protrusions. (a) Time-lapse images of neurons expressing OptoEphB2-Venus, or its mutant variants, and mCherry (as a volume marker). Cells were photo-activated via blue light illumination (50-ms pulses, 3 pulses/min.) over the indicated ROI (white circles) in dendritic segments. Images were mCherry fluorescence. Time labels are relative to the start of photoactivation. (b) Images of maximum-intensity projection over time (5-min durations) from the dendritic segments shown in (a). (c) Quantification of increased filopodial protrusions. Dendritic areas within the ROI were measured from maximum intensity projections and normalized to measurements before photoactivation. Error bars are SEM (n = 32 for OptoEphB2, n = 16 for KD-optoEphB2). *p < 0.05; t-test, comparing OptoEphB2 to KD-optoEphB2.
Figure 4. OptoEphB2 activation in dendrites induces actin polymerization. (a) Time-lapse images of mCherry-Lifeact in the ROI during photoactivation of OptoEphB2. (b) Maximum-intensity projection images of a dendrite undergoing two rounds of OptoEphB2 photoactivation. Neural cells (DIV11) expressing OptoEphB2-Venus and mCherry (shown) were photoactivated over the indicated ROI (dash line). Two rounds of photoactivation were spaced with 20 minutes of incubation in the dark. (c) Same as in (b) except the second round of photo-activation were carried out with the presence of CK666 (200 μM). (d) The Kolmogorov-Smirnov plot showing the cumulative probability of the cell area increase (based on maximum intensity projection, n = 16 each) from the two-round activation protocol shown in (b). (e) Images of mCherry-Lifeact in a dendritic filopodium before (-1:00) and after (8:00) OptoEphB2 photoactivation. (f) Quantification of Lifeact intensity in filopodia.
Normalized intensity was calculated cell-by-cell by averaging the intensity in all filopodia (>5 per cell) along the ROIs before and after illumination, normalizing to the pre-illumination value, and averaging between cells. Error bars, SEM (n=11 cells for optoEphB2, n=9 cells for KD-optoEphB2). *p < 0.05; t-test.
Figure 5. OptoEphB2 interacts with Arg. (a) Rosetta assay of whole cell lysates (MEFs expressing OptoEphB2-mCherry or KD-optoEphB2-mCherry) probed with a panel of purified SH2 probes (only Arg-SH2 is shown) as well as anti-mCherry and anti-pTyr. The top graph illustrates the lysates spotting pattern. Pervanadate- and PTP1B-treated samples were spotted at half volume and serves as positive and negative controls respectively. (b) Quantification of Arg-SH2 and anti-mCherry (represent total OptoEphB2-mCherry expression) bindings to cell lysates. KD: KD-optoEphb2 lysate (with light stimulation). (c) Same as in (a) except lysates are from cells stimulated with ephrinB1-Fc ligand. Lysates were probed with Arg-SH2, anti-EphB2 and anti-pTyr. (d) Quantification of Arg-SH2 and anti-EphB2 bindings to cell lysates shown in (c). (e) Fluorescence images of COS cells co-
expressing Arg-YFP and OptoEphB2-mCherry showing light-induced co-clustering of Arg and OptoEphb2, but not with KD-optoEphB2.
Figure 6. OptoEphB2-induced dendritic filopodia requires Arg kinase. (a) Blue light induced co-clustering of OptoEphB2 and Arg in dendrites in neuron cells (DV11) expressing OptoEphB2-mCherry and Arg-YFP. (b) Left: Images of maximum-intensity projection from neural cells (DIV11) co-expressing OptoEphB2-mCherry and KD-Arg (top) or KD-Src (bottom). Blue-light induced clustering of OptoEphB2 but no filopodial growth in KD-Arg cells. The inhibitory effect was not observed with KD-Src expression. Right: Quantification of cell area increase (n = 11, based on maximum-intensity projection) in the presence of KD-Arg or KD-Src. (c) Images of maximum-intensity projection from neural cells (DIV 11) co-expressing OptoEphB2-Venus and mCherry. Cells were either treated with GNF2, a specific Arg inhibitor, or DMSO, as controls. The images showed that GNF2 treatment, but not DMSO treatment, abolished OptoEphB2 induced dendritic filopodia growth. (d) Quantification of the effects of GNF2 treatment (n = 16) in comparison to DMSO (n=27). Light-induced dendritic area increases were quantified with maximum-intensity projection images (as shown in c). The left panel showed the average values and the right panels showed the cumulative distributions. *p < 0.05; t-test.
**Figure 7.** OptoEphB2 can induce dendritic filopodia growth without activating Rac1 and CDC42. (a) Time-lapse fluorescence images of neural cell (DIV 10-11) expressing OptoEphB2-mCherry and dominant negative CDC42 (DN-CDC42-YFP, top) or dominant negative Rac1 (DN-Rac1-YFP, bottom). Fluorescence signals were from OptoEphB2. (b) Images of maximum-intensity projection before and after photoactivation (10-15 min) from cells shown in (a). (c) Quantifications of cell area increases (based on maximum-intensity projection) for cells expressing DN-Rac1-YFP (n=15), DN-CDC42-YFP (n = 13) and YFP only (n=11). Results were compared to control dataset of cells treated with actin polymerization inhibitor Cytochalasin D (n=4). * p < 0.05; t-test.
Supplementary Figures


Fig S1. OptoEphB2 sequence.
Figure S2. Confocal images of HEK293 cells expressing various Eph receptor fusion with Cry2PHR: (a) EphB1-Cry2PHR-mCherry, (b) EphB2-Cry2PHR-mCherry, (c) Myr-EphB2ICD-Cry2PHR-mCherry and (d) Myr-EphB2ICD-Cry2OligPHR-mCherry (optoEphB2). Expressing full-length EphB fusion with Cry2 resulted in internalization of the receptor. In comparison, optoEphB design resulted membrane localization. Scale bars, 10 µm.
**Figure S3.** Ligand activation of EphB2 activation causes MEF cell rounding. Cells were stimulated with either human Fc (left column) or EphrinB1-Fc (right column), both preclustered with anti-Human-Fc. Dotted lines denote cell area before stimulation and solid line denote that after stimulation.
Figure S4. Summary of the Rosetta assay results on OptoEphB2 activation. Cell lysates were collected from MEF cells expressing OptoEphB2 either activated with blue light (1 min) or left in the dark. Only the probes showing induced binding were plotted.
Figure S5. Summary of the Rosetta assay results on EphB2 activation using ephrinB1 ligand. Cell lysates were collected from EphB2-expressing MEF cells either treated with preclustered EphrinB1-Fc or Fc only. Only the probes showing induced binding were plotted.
Figure S6. OptoEphB2 expression does not affect endogenous EphB2 localization. Cultured hippocampal neurons were transfected with either vector control or optoEphB2-mcherry on DIV10 and imaged on DIV11. Cells were fixed and endogenous EphB2 was immune-labeled (R&D systems AF467) with Cy5. Cells were imaged at either 20x (a) or 100x (b) magnification. EphB2 signal was found to be highest in the somadendritic compartment of neurons, but also in axons and glial cells to certain degree (a). At high magnification, membrane localization of EphB2 is evident in dendrites and EphB2 appears to be slightly clustered (b). No overt changes in EphB2 localization was detects in cells expressing optoEphB2 and cells activated with blue light.
Movies

Video S1: Blue light illumination induce OptoEphB2 clustering in COS cells expressing either OptoEphB2-mCherry (left) or KD-optoEphB2-mCherry (right). Cell rounding was only observed in OptoEphB2-expressing cells. The blue dot denotes the time of blue light illumination.
Video S2: Reversible OptoEphB2 clustering. MEF cells expressing OptoEphB2-mCherry exposed to periodical blue light illumination and followed via mCherry fluorescence. The blue dot denotes the time of blue light illumination.
Video S3: OptoEphB2 activation induce filopodial protrusions in the dendrite. OptoEphB2 was locally photo-activated in the dendrite of cultured hippocampal neurons. The cell co-expressed OptoEphB2 and mCherry. Fluorescence signal is from mCherry. The blue circle denotes the area of blue light illumination. The blue dot denotes the time of blue light illumination.
Video S4: KD-Arg inhibited OptoEphB2 induced dendritic filopodia. The video shows the OptoEphB2 fluorescence signal from a hippocampal neuron co-expressing KD-Arg and OptoEphB2. Blue light activation triggered OptoEphB2 clustering, but did not produce filopodial growth. Blue light was turned on at time 0:00 and remained on throughout the video. The blue circle denotes the area of blue light illumination.