Figure S1:

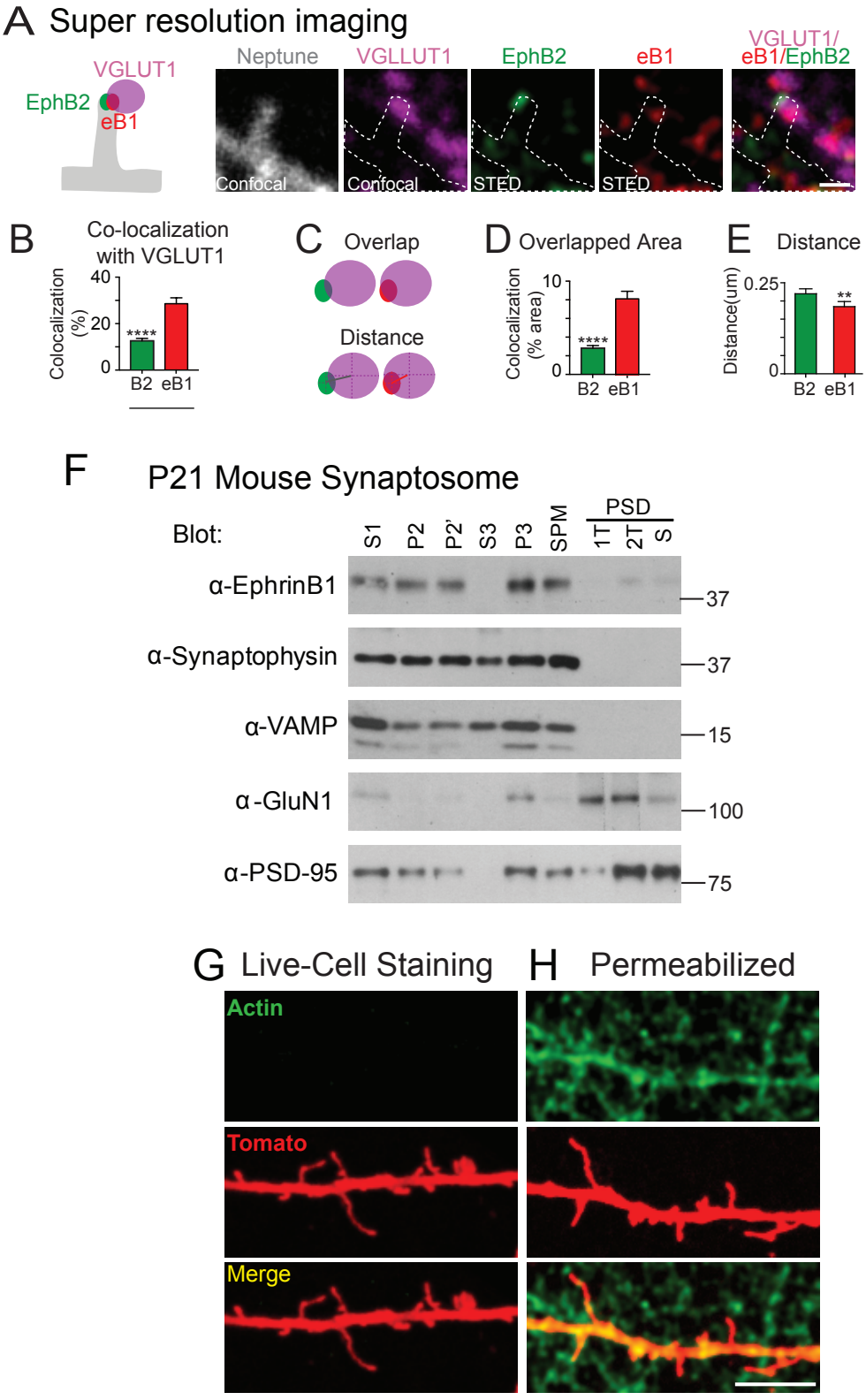
A Super resolution imaging
VGLUT1
EphB2
eB1
Neptune
VGLLUT1
EphB2
eB1
VGLUT1/
eB1/EphB2
Confocal
Confocal
STED
STED
B
Co-localization
with VGLUT1
Colocalization
(%)
40
20
0
****
B2 eB1
C Overlap
Distance
D Overlapped Area
Colocalization
(% area)
10
0
****
B2 eB1
E Distance
Distance(um)
0.25
0
**
B2 eB1
F
P21 Mouse Synaptosome
Blot:
S1
P2
P2'
S3
P3
SPM
PSD
1T
2T
S
α-EphrinB1
37
α-Synaptophysin
37
α-VAMP
15
α-GluN1
100
α-PSD-95
75
G Live-Cell Staining
H Permeabilized
Actin
Tomato
Merge

Figure S1. Controls for STED imaging, ephrin-B localization, and live-cell surface staining. Related to Figure 1.

(A) Localization of EphB2 and ephrin-B1 at nascent synaptic sites in filopodia. The model shows the organization of EphB2 (green, STED), ephrin-B1 (red, STED) and VGLUT1 (purple, confocal). Images show a filopodium with EphB2 (green), ephrin-B1 (red), and VGLUT1 (purple). Scale bar = 0.5 μm. (B) Quantification of the percent colocalization of VGLUT1 and EphB2 (green) or VGLUT1 and ephrin-B1 (red) (n = 17 imaging fields, each field contained >100 puncta, $p < 0.0001$, paired t-test). (C). Illustration of the methods used to determine overlap and distance between EphB2 and VGLUT1 or ephrin-B1 and VGLUT1 puncta. (D) Quantification of the overlap of EphB2 (green) and ephrin-B1 with VGLUT1 (n = 17, $p < 0.0001$, paired t-test). (E) Quantification of the distance between the centers of mass of EphB2 or ephrin-B1 and VGLUT1 puncta. (n = 66, $p = 0.008$, paired t-test). (F) PSD fractionation of P21 wild-type mouse brain. Ephrin-B1 is enriched with presynaptic markers synaptophysin and VAMP but not with postsynaptic markers PSD-95 or GluN1. S1: Homogenates. P2: Crude synaptosomal fraction. P2': Washed crude synaptosomal fraction. S3: Crude synaptic vesicle fraction. P3: Lysed synaptosomal membrane fraction. SPM: Synaptic plasma membranes. PSD: Postsynaptic density fractions. (G) Live cell staining for actin (green, left, no permeabilization) and (H) immunostaining for actin following permeabilization after fixation (green, right) in DIV7-10 neurons transfected with tdTomato (red). Scale bars = 5 μm. **** $p \leq 0.0001$; ** $p \leq 0.01$; Error bars indicate SEM

Figure S2:

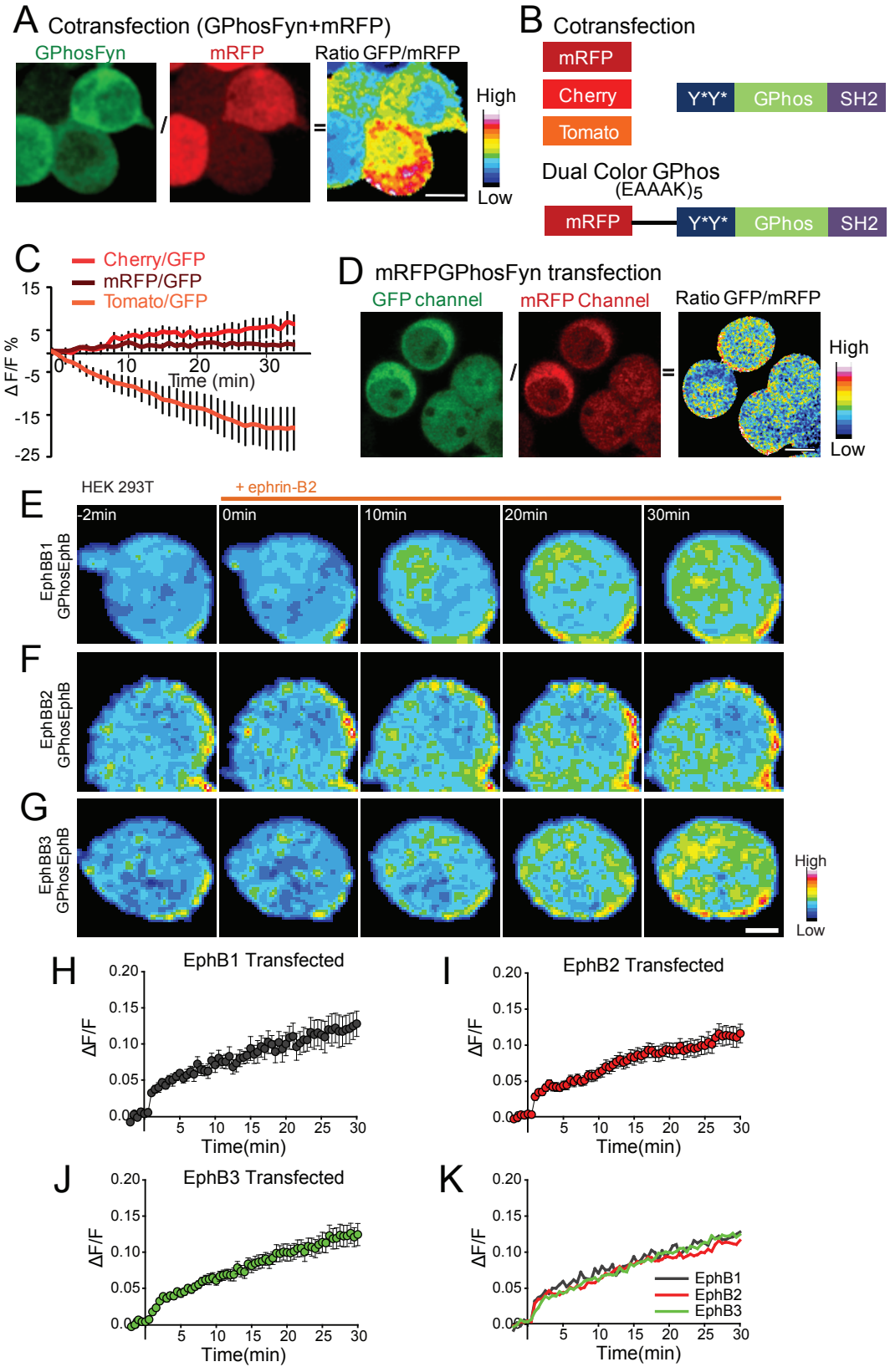
A Cotransfection (GPhosFyn+mRFP)
GPhosFyn
mRFP
Ratio GFP/mRFP
High
Low
B Cotransfection
mRFP
Cherry
Tomato
Y*Y*
GPhos
SH2
Dual Color GPhos
(EAAAK)5
C
Cherry/GFP
mRFP/GFP
Tomato/GFP
Δ F/F %
Time (min)
D mRFPGPhosFyn transfection
GFP channel
mRFP Channel
Ratio GFP/mRFP
E
HEK 293T
+ ephrin-B2
-2min
0min
10min
20min
30min
EphBB1 GPhosEphB
F
EphBB2 GPhosEphB
G
EphBB3 GPhosEphB
H
EphB1 Transfected
ΔF/F
Time(min)
I
EphB2 Transfected
J
EphB3 Transfected
K
EphB1
EphB2
EphB3

Figure S2. The design and testing of the dual color GPhos indicator. Related to Figure 3.

(A) HEK293T cells were co-transfected with both GPhosFyn and mRFP. Variant expression of mRFP was combined with variant expression of GPhos. (B) Introduction of second fluorescent protein. $[EAAAK]_5$ α-helix repeats were added as a linker to prevent FRET. (C) The bleaching rate of mRFP is most similar to that of GPhos. (D) HEK293T cells were transfected with the dual-color mRFP-GPhosFyn indicator. Background mRFP channel is more stable and ratiometric images have less variability. (E-G) Ratiometric pseudocolor images of single optical sections of HEK293T cells transfected with EphB1 (E), B2 (F) or B3 (G) and GPhosEphB at 0, 10, 20, and 30 minutes after ephrin-B2 treatment (Orange bar). Images were collected once every 30 seconds. The pseudocolored lookup table (16 colors) indicates the ratiometric GPhosEphB signal. Scale bars = 2 μm. (H) Quantification of the effects of ephrin-B2 treatment on GPhos signal in HEK 293T cells transfected with EphB1 (n = 10). (I) Quantification of the effects of ephrin-B2 treatment on GPhos signal in HEK 293T cells transfected with EphB2 (n = 25). (J) Quantification of the effects of ephrin-B2 treatment on GPhos signal in HEK 293T cells transfected with EphB3 (n = 25). (K) The comparison of GPhosEphB signal in HEK 293T cells transfected with EphB1, B2 and B3. Error bars indicate SEM in H, I, J.

Figure S3:

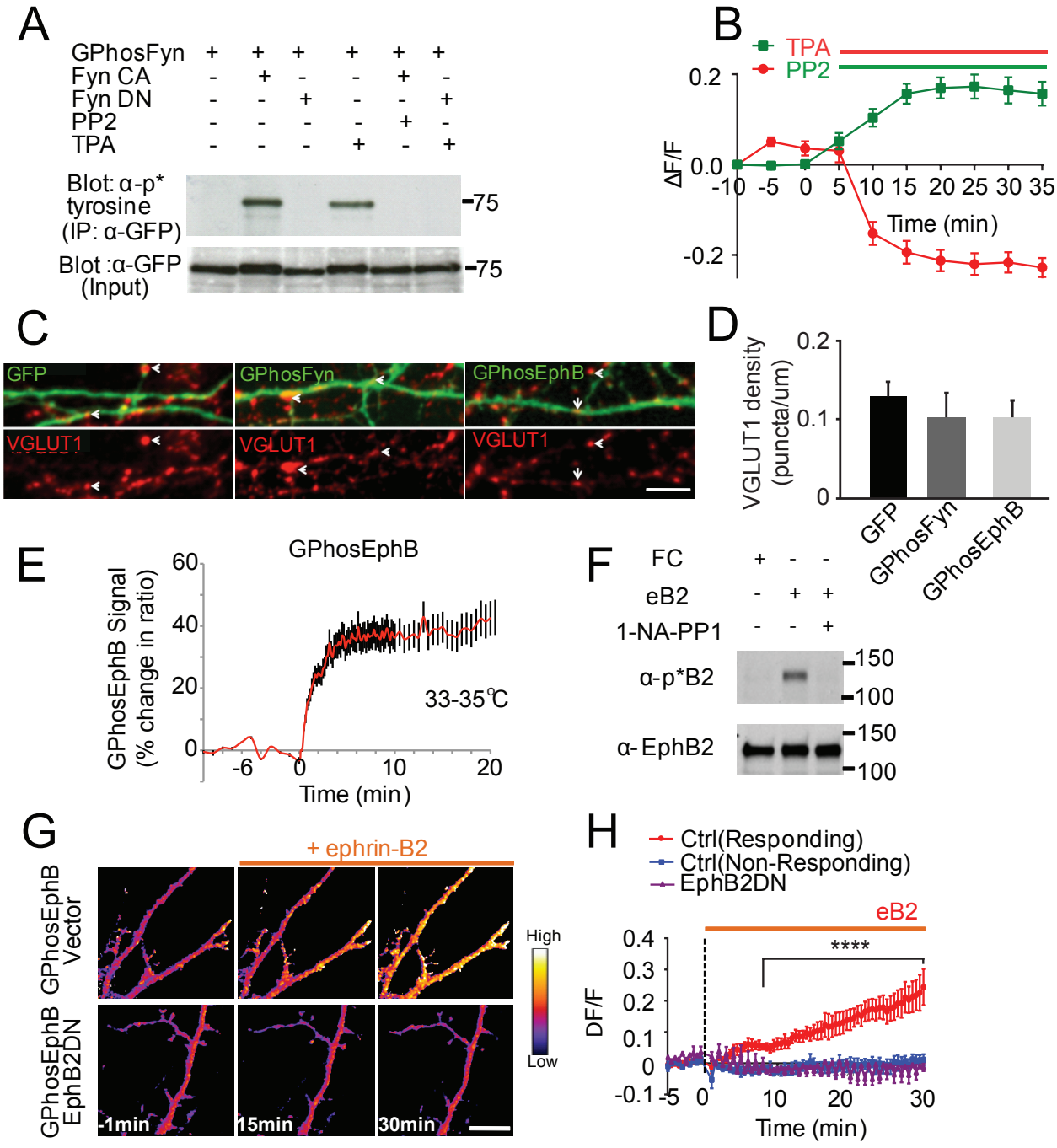
A
GPhosFyn + + + + + +
Fyn CA - + - - + -
Fyn DN - - + - - +
PP2 - - - - + -
TPA - - - + - +
Blot: α-p* tyrosine (IP: α-GFP)
75
Blot :α-GFP (Input)
75
B
TPA
PP2
0.2
0.0
-0.2
ΔF/F
-10 -5 0 5 10 15 20 25 30 35
Time (min)
C
GFP
GPhosFyn
GPhosEphB
VGLUT1
VGLUT1
VGLUT1
D
VGLUT1 density (puncta/um)
0.2
0.1
0
GFP
GPhosFyn
GPhosEphB
E
GPhosEphB
GPhosEphB Signal (% change in ratio)
60
40
20
0
33-35℃
-6 0 10 20
Time (min)
F
FC + - -
eB2 - + +
1-NA-PP1 - - +
α-p*B2
150
100
α-EphB2
150
100
G
+ ephrin-B2
GPhosEphB Vector
GPhosEphB EphB2DN
High
Low
-1min
15min
30min
H
Ctrl(Responding)
Ctrl(Non-Responding)
EphB2DN
eB2
****
DF/F
0.4
0.3
0.2
0.1
0
-0.1
-5 0 10 20 30
Time (min)

Figure S3. GPhos indicator reports phosphorylation of tyrosine kinase in HEK cells and Neurons. Related to Figure 3.

(A) GPhos indicator is phosphorylatable. Immunoprecipitation of HEK 293T cells transfected with GPhosFyn, mRFP, FynCA or FynDN. Co-transfection with FynCA but not Fyn DN revealed a 75 KD band indicative of phosphorylation. (B) Activating tyrosine phosphorylation with a phorbol-ester (100 nM, TPA, n = 10) resulted in an increase in the GPhosFyn signal. Inhibiting tyrosine kinase activity (3 μM, PP2, n = 10) resulted in a decrease in GPhosFyn signal. Bars indicate drug application. Error bars indicate SEM. (C) GPhos indicator does not affect synapse density. Representative images of neurons transfected with GFP, GPhosFyn, or GPhosEphB. Neurons were fixed and stained with anti-VGLUT1 antibodies to mark excitatory presynaptic terminals (red). Arrows indicate VGLUT1 puncta (red) within transfected neurons (green). Scale bars = 5 μm (D) Quantification of VGLUT1 puncta density. There is no significant difference between the three groups. (E) Effects of ephrin-B2 treatment on GPhosEphB signal in HEK293T cells transfected with EphB2 at 33-35°C. Images were collected every 20s (n = 17). (F) Western blot analysis of EphB phosphorylation from cultured EphB TKI mouse. 1-NA-PP1 blocked EphB phosphorylation confirming findings in Soskis et al (2012). (G) GPhosEphB reported no EphB kinase activity when neurons were transfected EphB2DN and treated with activated ephrin-B2. Representative images of dendrites of neurons transfected with either GPhosEphB and vector control (top panels) or GPhosEphB and dominate negative EphB2 containing only its extracellular domain (EphB2DN, bottom panels). (H) Quantification of the effect of ephrin-B2 treatment on transfected neurons. Imaged neurons fell into three groups. GPhosEphB transfected non-responding (Ctrl/Non-responding, n = 5, blue), GPhosEphB responding (Ctrl/Responding, n = 7, red), and GPhosEphB and EphB2DN transfected neurons (EphB2DN, n = 3, purple). Non-responding neurons had less than 5% changes during the recording period. Scale bar = 10 μm. Error bars indicate SEM.

Figure S4:

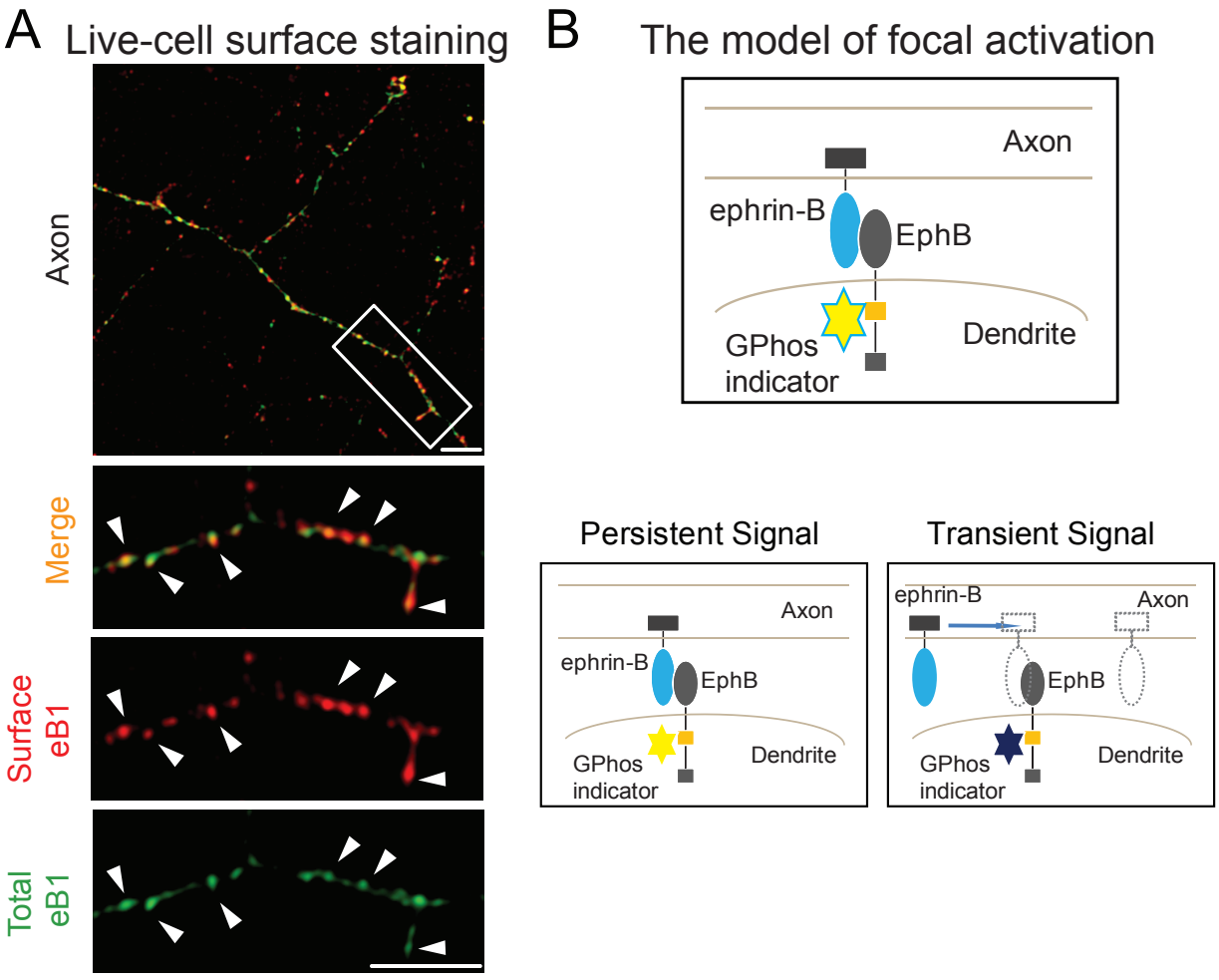

Figure S4. Live-cell surface staining of neurons transfected with mTurquoise2-eB1 and model for modes of GPhosEphB activation upon contact with axonal ephrin-B1. Related to Figure 4.

(A) Representative pseudocolored images of live-cell surface staining of neurons transfected with mTurquoise2-ephrin-B1. Arrowheads indicate colocalization of surface ephrin-B1 (red, stained with anti-HA, Cy3) and total ephrin-B1 staining (green, stained with anti-HA, DyLight 647). Scale bar = 5 μm. (B) Schematic illustration of ephrin-B-EphB binding and activation of GPhosEphB indicator. The model of persistent signal shows that EphB is constantly activated at stable eB1 puncta. The model of transient signal shows that EphB is only activated when contacting eB1 puncta.

Figure S5:

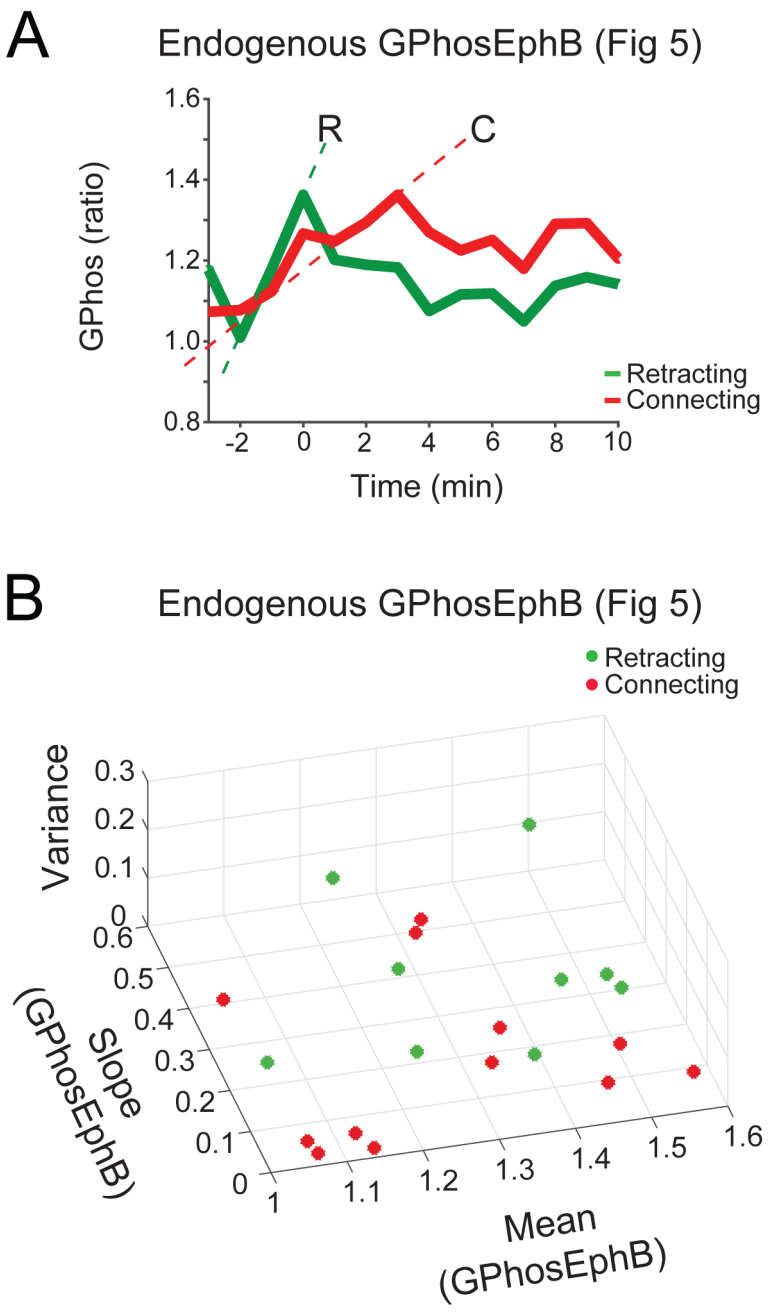

Figure S5. Data related to modeling of EphB kinase activity in filopodia. Related to Figure 5

(A) Graph of the mean GPhos data for Retracting and Connecting filopodia used for classification in (B). Retracting filopodia are shown in green, connecting data are shown in red. Time 0 indicates the time the filopodia began contacting a labeled axon. (B) Graph showing results of classification. The behavior of filopodia was examined using cross-validated classification accuracy of different observations by three classifiers implemented in Matlab.

Two linear classifiers (a linear Support Vector Machine and linear discriminant) and a non-linear classifier (logistic regression) were employed. The classifiers were trained and tested on three features of each observation: the slope, mean and variance of the signal. Similar results were obtained by using only pairs of the three features. The average linear discriminant for these groups was 98%. Green dots represent Retracting filopodia and red dots represent Connecting filopodia.

Figure S6:

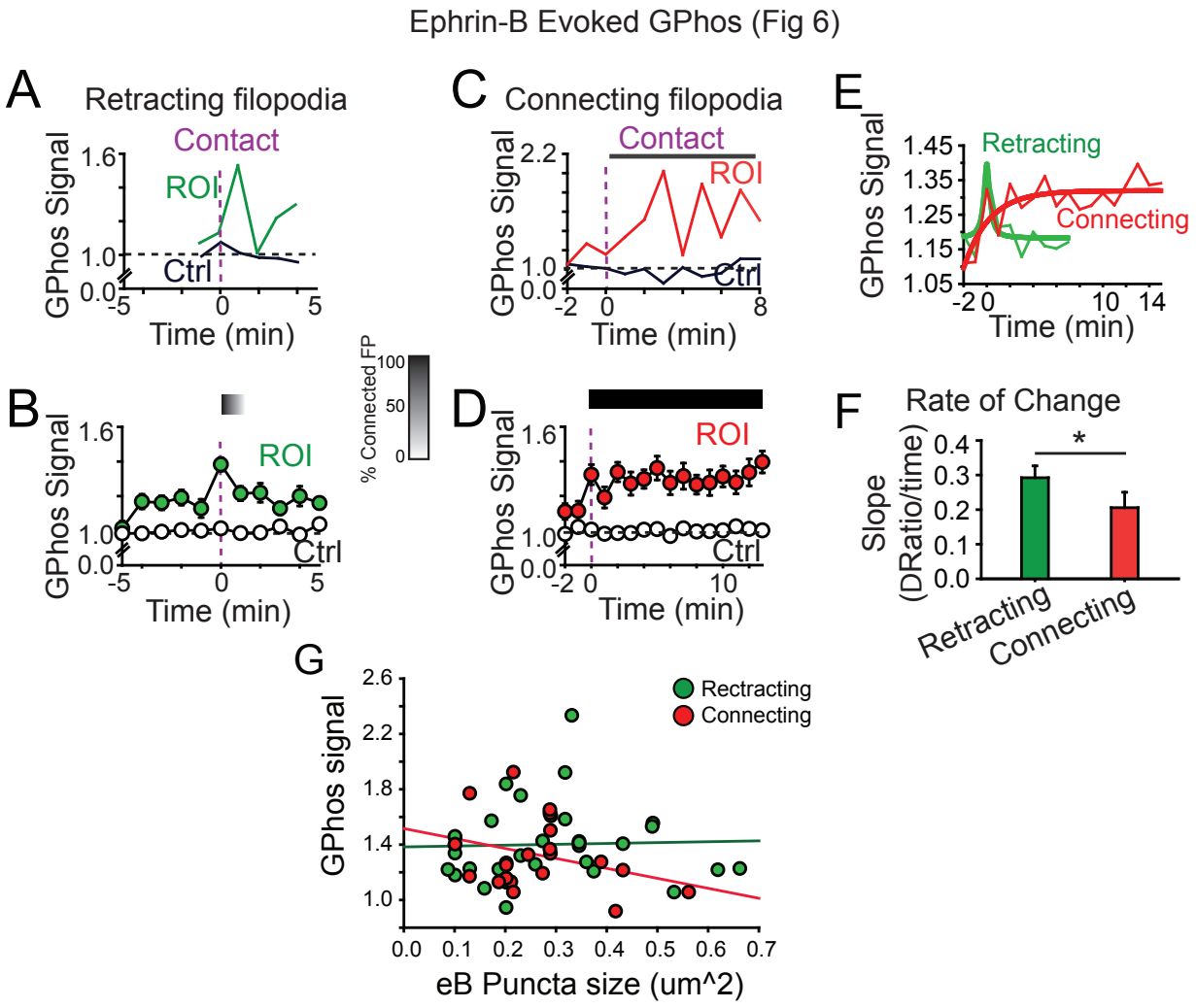

Figure S6. Data related to exogenous application of ephrin-B on filopodial behavior. Related to Figure 6.

(A) Quantification of GPhosEphB signal of the example shown in figure 6J. (B) Quantification of GPhosEphB signal in the pooled data set. (ROI: n = 33, Ctrl: n = 33, $p \leq 0.001$, K-S Test). (C) Quantification of GPhosEphB signal in figure 6K. (D) Quantification of GPhosEphB signal in the pooled data set. (ROI: n = 20, Ctrl: n = 20, $p \leq 0.001$, K-S Test). (E) The kinetics of average GPhosEphB signal. As in Figure 5H, data of Retracting group was best fit with a peak model whereas data of connecting group was best fit with an exponential model. The same exponential could be used to fit the GPhosEphB signal from both endogenous (Figure 5H) and exogenous stimulation conditions suggesting a similar mechanism ($p = 0.16$). (F) The slope of GPhos signal in Retracting filopodia was significantly different from that in Connecting filopodia

(Retracting: 0.29 ± 0.03, n = 31; Connecting: 0.2 ± 0.04, n = 20, p = 0.033, Mann-Whitney U test). * $p < 0.05$. Error bars indicate SEM. (G). The sizes of ephrin-B puncta are not correlated with the strength of GPhos signal. The magnitude of the GPhosEphB signal at each filopodium measured is shown in relation to the size of the ephrin-B puncta. Retracting filopodia are shown in green, Connecting filopodia are shown in red. For comparison, the size of each ephrin-B puncta was determined at time 0 in both Retracting and Connecting filopodia. Retracting: R = 0.0347. R^2 = 0.0012, n = 33 (green line); Connecting: R = 0.315. R^2 = 0.0991, n = 20 (red line).

Figure S7:

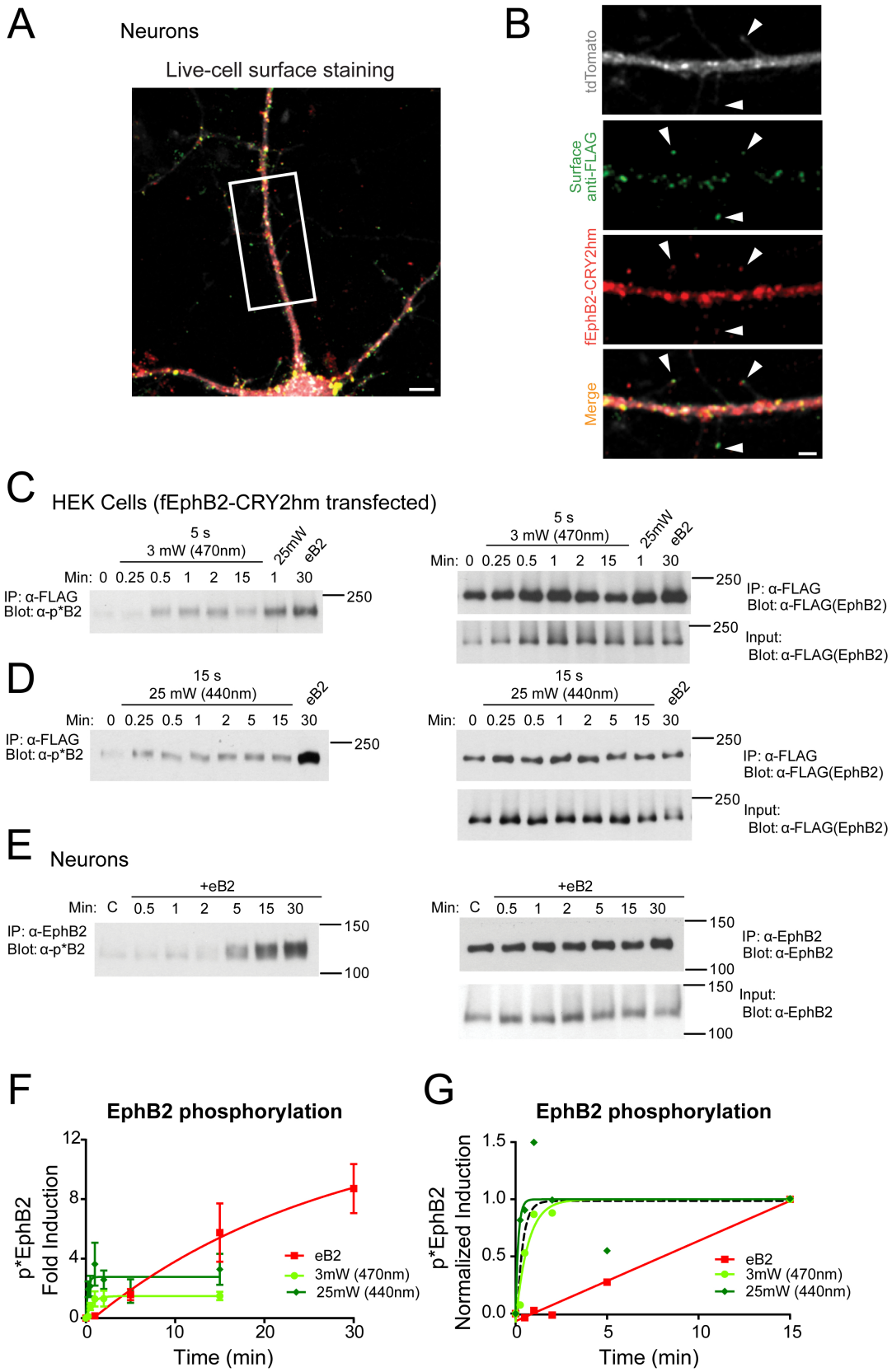
A
Neurons
Live-cell surface staining
B
tdTomato
Surface anti-FLAG
fEphB2-CRY2hm
Merge
C
HEK Cells (fEphB2-CRY2hm transfected)
5 s
3 mW (470nm)
25mW
eB2
Min: 0 0.25 0.5 1 2 15 1 30
IP: α-FLAG
Blot: α-p*B2
250
IP: α-FLAG
Blot: α-FLAG(EphB2)
Input:
Blot: α-FLAG(EphB2)
D
15 s
25 mW (440nm)
eB2
Min: 0 0.25 0.5 1 2 5 15 30
E
Neurons
+eB2
Min: C 0.5 1 2 5 15 30
IP: α-EphB2
Blot: α-p*B2
150
100
IP: α-EphB2
Blot: α-EphB2
Input:
F
EphB2 phosphorylation
p*EphB2 Fold Induction
Time (min)
eB2
3mW (470nm)
25mW (440nm)
G
EphB2 phosphorylation
p*EphB2 Normalized Induction
Time (min)

Figure S7. Validation of the photoactivatable EphB2 and characterization of EphB2 activation kinetics. Related to Figure 7.

(A) Representative pseudocolored images of live-cell surface staining for fEphB2-CRY2hm (anti-FLAG, green, indicates surface staining) in DIV 7-10 neurons transfected with tdTomato (gray) and fEphB2-CRY2hm (red, indicates total EphB2-CRY2hm). Merged images show the ROI and neuronal morphology. Scale bars = 5 μm.(B) Arrowheads indicate anti-FLAG puncta colocalized with transfected fEphB2-CRY2hm at the tips of filopodia. Scale bars = 2 um. (C) HEK293T cells transfected with fEphB2-CRY2hm were left unstimulated (0) or stimulated with 470 nm LED light (3 mW) for 5s and lysed at 0.25, 0.5, 1, 2, 5, 15 minutes, To determine the maximal activation, cells were treated with 440 nm for 1 minute or activated ephrin-B2 for 30 minutes. EphB2 was immunoprecipitated with anti-FLAG antibody. The IP was probed for EphB2 phosphorylation using an anti-p*EphB2 antibody (left blot) and for anti-FLAG to determine IP efficiency (top right blot). Input was probed for anti-FLAG (Bottom Right). (D) As in C, but HEK293T cells are stimulated with 440 nm laser light (25 mW) for 15 s. (E) DIV7-10 neurons were treated with activated ephrin-B2 for 0.5, 1, 2, 5, 15, 30 minutes. The controls were lysed without addition of ephrin-B2. Blots shown are as in C. (F) Quantification of the fold induction of EphB phosphorylation (p*EphB2) activated by either light (Green) or activated ephrin-B2 (Red). P*EphB2 induction was determined relative to t = 0 and normalized to the intensity of FLAG (HEK293T cells) or EphB2 (neurons). The phosphorylation of endogenous EphB2 shows slow activation whereas that of the fEphB2-CRY2hm exhibits fast activation (≤ 30s). Higher intensity light (25 mW) induced higher levels of EphB phosphorylation (Dark green vs light green lines, p = 0.0005, F-test). Error bars indicate SEM. (G) Scaled plot of EphB phosphorylation indicates that the kinetics of kinase responses to low (3mW) and high intensity light (25mW) do not differ significantly (p = 0.26, extra-sum-of-squares F test compared the goodness-of-fit of both curves). Scaling the data of activated ephrin-B2 induced EphB phosphorylation in neuron reveals that the kinetics of endogenous EphB activation significantly slower, compared to light-induced EphB activation (p = 0.0005 eB2 vs. 3mW, p = 0.0053 eB2 vs. 25mW, F-test).

Figure S8:

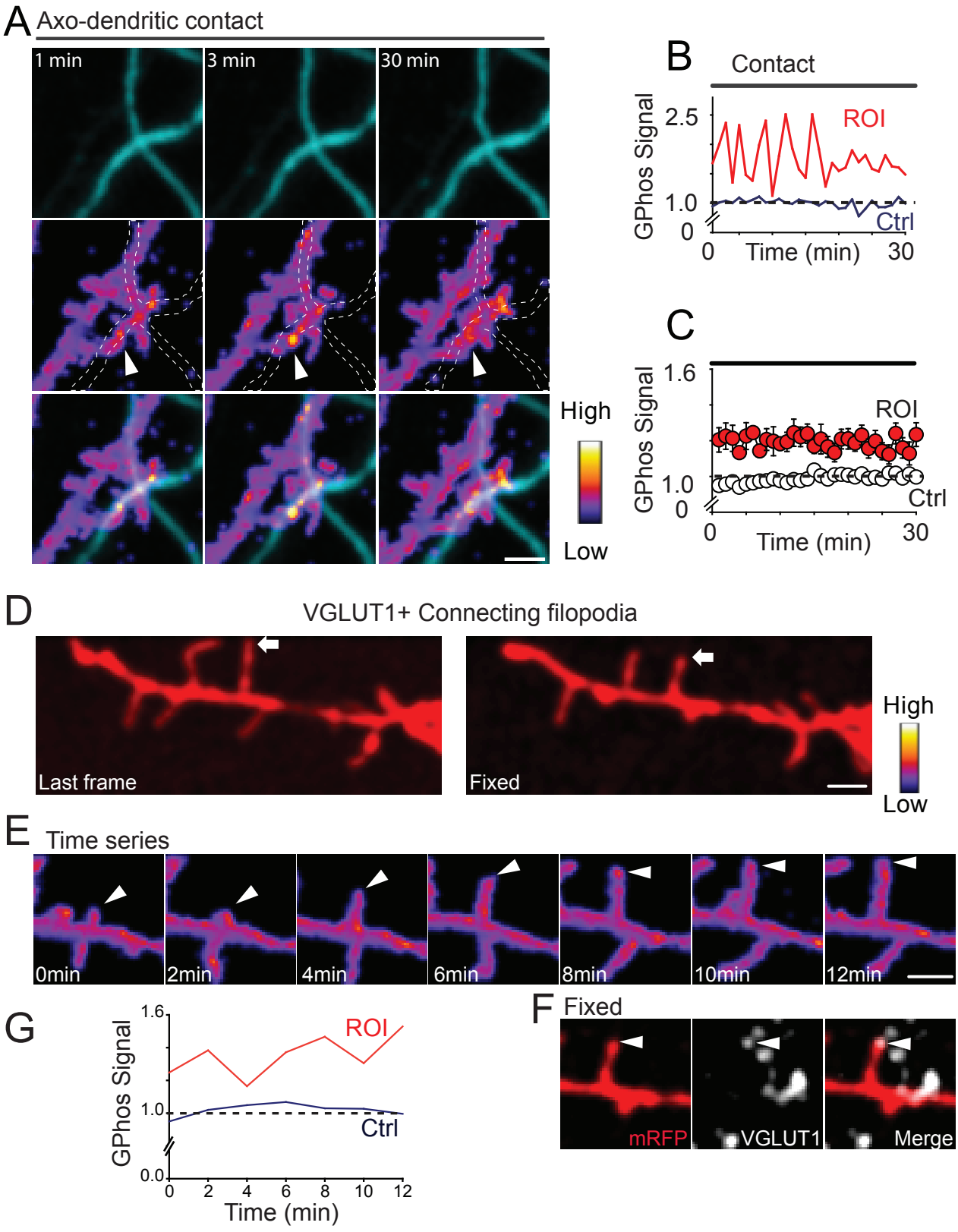
A
Axo-dendritic contact
1 min
3 min
30 min
High
Low
B
Contact
GPhos Signal
2.5
1.0
0
ROI
Ctrl
0
Time (min)
30
C
GPhos Signal
1.6
1.0
0
ROI
Ctrl
0
Time (min)
30
D
VGLUT1+ Connecting filopodia
Last frame
Fixed
High
Low
E
Time series
0min
2min
4min
6min
8min
10min
12min
F
Fixed
mRFP
VGLUT1
Merge
G
GPhos Signal
1.6
1.0
0.0
ROI
Ctrl
0
2
4
6
8
10
12
Time (min)

Figure S8. Elevated EphB activity at axo-dendritic contact and an example of a Connecting filopodia that is colocalized with VGLUT1 puncta. Related to Figure 8.

(A) An example of persistent signal when dendrites are transfected with GPhosEphB and contacting axons transfected with mTurquoise2, showing EphB activity upon activation of endogenous ephrin-Bs. Ratiometric images show pseudocolored GPhosEphB signal at contact with mTurquoise2 axon. Scar bar = 2 μm. (B) Quantification of GPhos signal in A. (C) Quantification of GPhos signal in the pooled data set (ROI: n = 20, indicated by arrowheads; Ctrl: n = 11, $p < 0.001$, K-S Test). (D) An example of Connecting filopodia. Images of RFP channel at the last frame of the 30-minute movie and RFP image after fixation was shown. Arrows point to an example of connecting filopodia. Scale bar = 2 μm. (E) Time series of the connecting filopodium. (F) After fixation, immunostaining images show the colocalization of this filopodium and VGLUT1 puncta. (G) Quantification of GPhos signal in the filopodium.