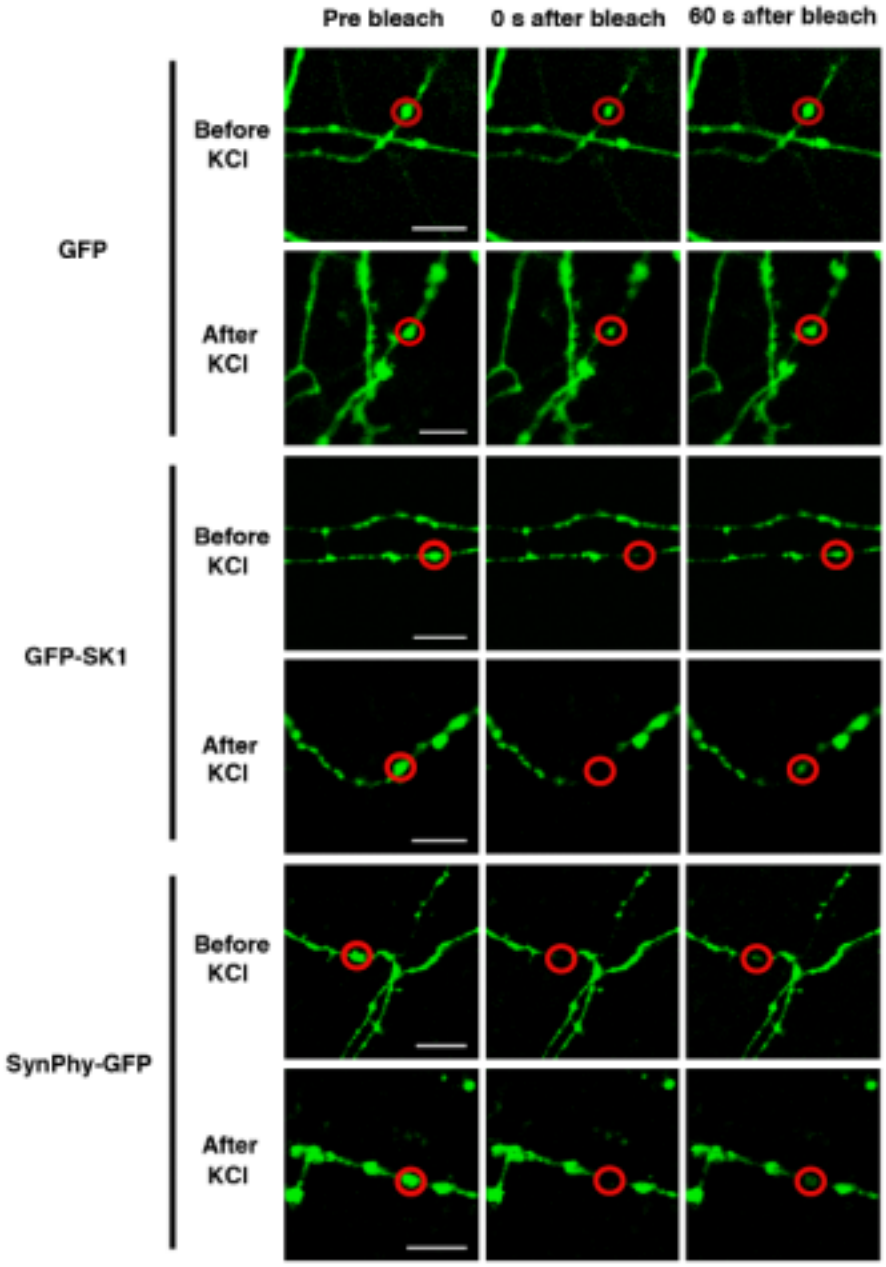
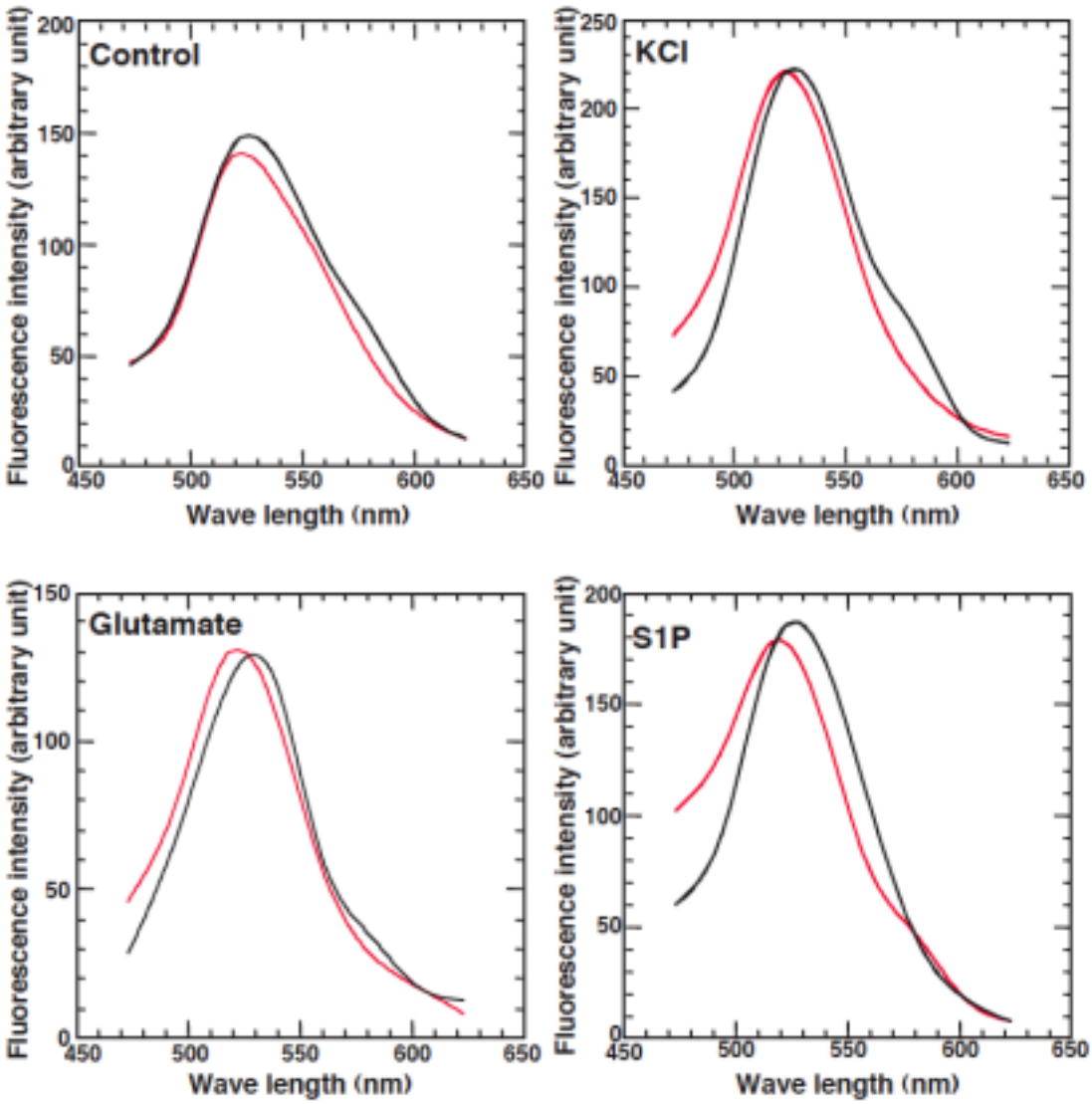
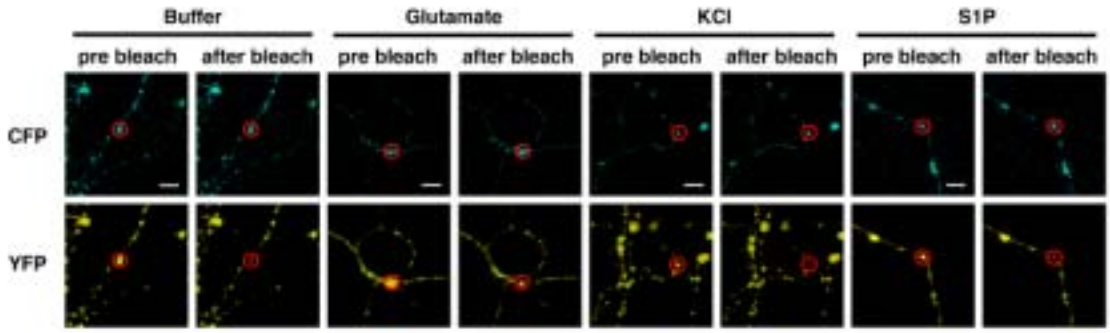


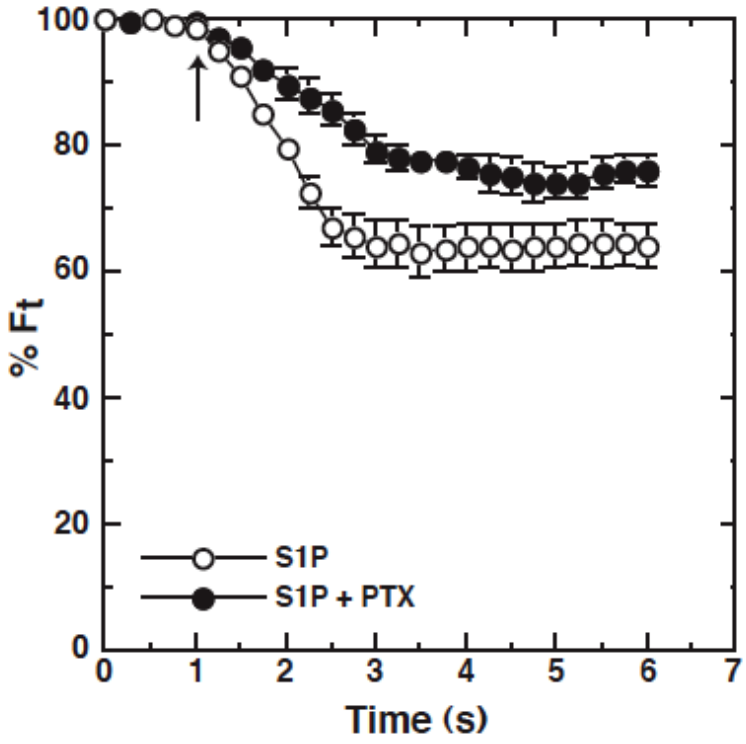
Supplemental figure 1. Effects of depolarization on fluorescence recovery of GFP-SK1, GFP, or SynPhy-GFP after photobleaching in axonal puncta. Hippocampal neurons were transiently transfected with expression vectors encoding GFP-SK1, free GFP, or SynPhy-GFP as indicated. FRAP analysis was performed before or 1 min after stimulation with 50 mM KCl. The images were obtained at pre bleaching and 0 s or 60 s after photobleaching. The bleached areas are shown in red circles. Bars, 5 μm.



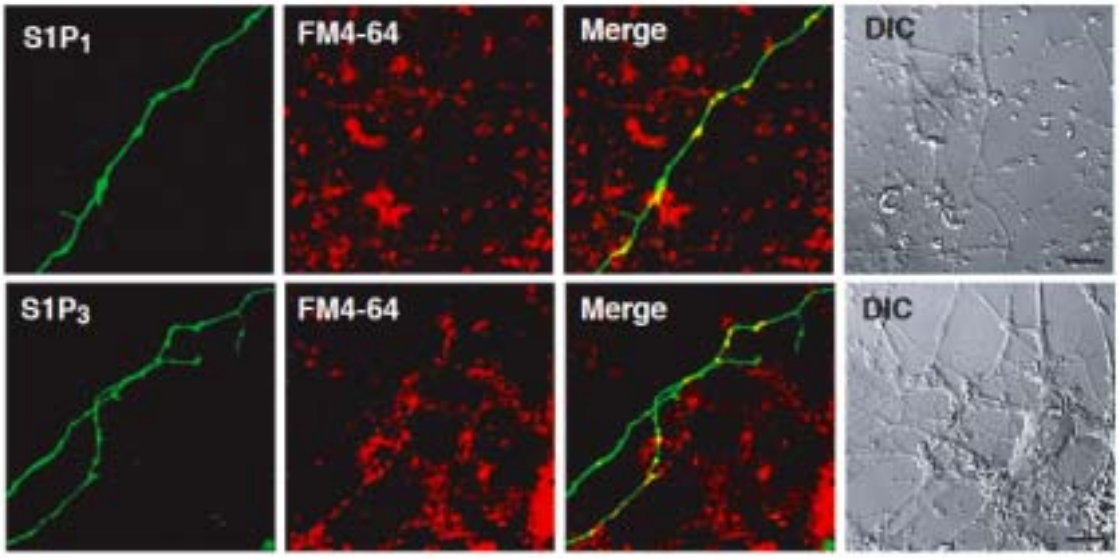
Spplemental figure 2. Hippocampal neurons cotransfected with expression plasmids encoding S1P1-CFP and YFP-β-arrestin were treated without (buffer) or with 50 mM KCl, 100 μM glutamate, or 10 nM S1P, and were analyzed for FRET in living cells. Emission spectrum detected from an increase in donor fluorescence after acceptor photobleaching of puncta of interest was measured. Emission spectrum before (black line) or after (red line) acceptor photobleaching is shown. Note that fluorescence intensity of short wave length (~500 nm) is increased only in stimulated puncta after photobleaching.



Supplemental figure 3. Visualization of S1P receptor activation using the spectral unmixing technique with FRET by acceptor photobleaching. Hippocampal neurons cotransfected with S1P1-CFP and YFP-β-arrestin plasmids were stimulated without (buffer) or with 100 μM glutamate, 50 mM KCl, or 10 nM S1P and fixed. Then the mixed spectral images using 458-nm excitation were obtained at pre and after photobleaching of YFP. 2 channel (CFP, YFP) images were generated by applying Linear Unmixing to the Lambda Stacks. The bleached areas are shown in red circles. Bars, 10 μm.



Supplemental figure 4. Primary rat hippocampal neurons were prelabeled with FM4-64. Neurons were then preincubated without or with either 100 ng/ml pertussis toxin for 24 hours. Fluorescence of the dye was monitored after treatment with10 nM S1P. Arrow indicates the addition of S1P. Note that PTX treatment caused about 50% inhibition of S1P-induced transmitter release.



Supplemental figure 5. Localization of S1P receptors in axonal puncta in hippocampal neurons. Primary rat hippocampal neurons transiently expressing $S1P_1$-GFP or $S1P_3$-GFP receptor were labeled with membrane dye FM4-64 and analyzed for fluorescence localization in living cells. DIC and merged images are also presented. Scale bars, 10 µm.