Supporting Information for:

A fluorescent sensor for GABA and synthetic $GABA_B$ receptor ligands

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Chemistry

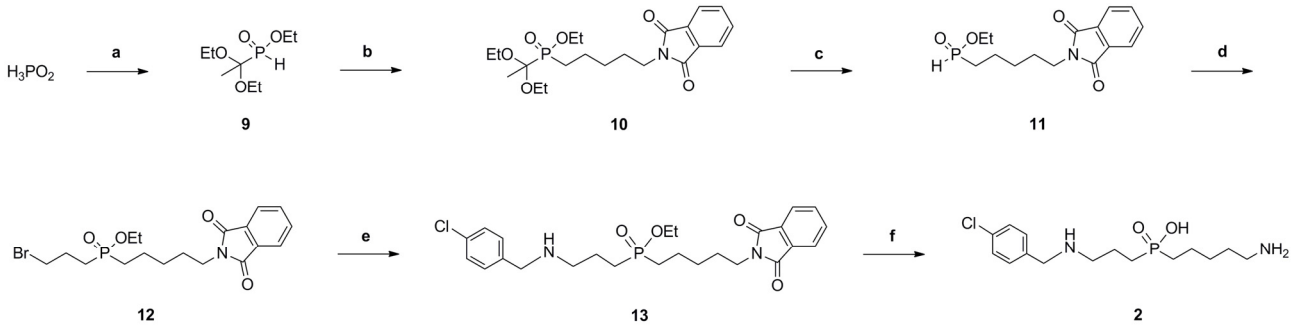
General considerations.

All reactions were carried out in oven-dried glassware under an atmosphere of nitrogen, unless stated otherwise. THF and CH_2Cl_2 were dried by passage over activated alumina under nitrogen atmosphere (H_2O content < 30 ppm, *Karl-Fischer* titration). Chemicals were purchased from Sigma-Aldrich, Fisher Scientific, Merck, Alfa Aesar, Acros, Axon Lab or Reactolab and used without further purification. LHMDS solution was freshly prepared following the description for LDA preparation by Leonard et al.[58] using hexamethyldisalazane instead of diisopropylamine.

Anhydrous solvents from Acros were used without further treatment and distillation. Flash column chromatography was performed with Merck silica gel (230-400 mesh). Reverse-phase analytical high-pressure liquid chromatography (RP-HPLC) was run on a Dionex system equipped with a P680 pump, an ASI 100 automatic sample injector and an UltiMate 3000 diode array detector for product visualization using a Waters symmetry C18 column (5μm, 3.9 x 150 mm). Buffer A: 0.1% w/v TFA in H_2O Buffer B: acetonitrile. Typical gradient was from 0% to 100% B within 15 minutes with 1 ml/min flow. Reverse-phase semi-preparative high-pressure liquid chromatography was performed on a Dionex system equipped with an an UltiMate 3000 pump and an UVD 170U UV-Vis detector for product visualization on a Waters SunFire™ Prep C18 column (5 μm, 10×150 mm). Buffer A: 0.1% w/v TFA in H_2O Buffer B: acetonitrile. Typical gradient was from 0% to 100% B within 30 minutes with 4 ml/min flow.

Reverse-phase preparative high-pressure liquid chromatography was performed on a Dionex system equipped with an an UltiMate 3000 pump and an UVD 170U UV-Vis detector for product visualization on a Waters SunFire™ Prep C18 OBD™ 5 μm 19×150 mm Column. Buffer A: 0.1% w/v TFA in H_2O Buffer B: acetonitrile. Typical gradient was from 0% to 100% B within 30 minutes with 7 ml/min flow.

Proton and carbon nuclear magnetic resonance (NMR) spectra were recorded at room temperature on a Bruker Avance-III 400 or on a Bruker DRX-600 equipped with a cryoprobe, with chemical shifts (δ) reported in ppm relative to the solvent residual signals. $CDCl_3$: δ_H 7.26 ppm, δ_C 77.16 ppm; DMSO-d_6: δ_H 2.5 ppm, δ_C 39.5 ppm; CD_3OD: δ_H 3.31 ppm, δ_C 49.0 ppm. Proton-decoupled phosphorous NMR spectra were recorded on a Bruker Avance-III 400 at room temperature using H_3PO_4 as external standard. Coupling constants are reported in Hz. Mass spectra were recorded on a Thermo Finnigan TSQ 7000 (ESI). High resolution mass spectra (HRMS) were measured on a Micromass Q-TOf Ultima spectrometer with electrospray ionization (ESI).



Scheme 1. Synthesis of compound **2**. a) BF_3, $CH_3C(OEt)_3$; b) LHMDS, *N*-(5-bromopentyl)phtalimide, THF c) TMSCl, EtOH/CH_2Cl_2; d) LHMDS, 1,3-dibromopropane, THF; e) 4-chlorobenzylamine, Et_3N, DMF, reflux; f) $MeNH_2$, NaOH, reflux.

Compound 9. Following a slight modification of the reported procedure[45], boron trifluoride (3.79 ml, 30 mmol, 0.15 equiv.) was slowly added to triethyl orthoacetate (80.65 ml, 440 mmol, 2.2 equiv.) at 0°C. After the addition of concentrated hypophosphorous acid (13.2 g, 200 mmol, 1 equiv.) at 0°C the reaction was stirred at room temperature for 1 ½ hours. Proceeding of the reaction was followed by ^{31}P-NMR. The work up was performed after all starting material had reacted by slowly adding saturated aqueous $NaHCO_3$ at 0°C, extracting the product with CH_2Cl_2 and drying over $MgSO_4$. Solvent evaporation resulted in a colorless oil (18 g crude yield containing 40% of **9** estimated by ^{31}P-NMR spectrum). The product was used without further purification.

Compound 10. The synthesis and work up of **10** were performed following a slight modification of the reported procedure [59]. 3.6 g of crude extract containing compound **9** was dissolved in dry and degased THF and cooled down to -78°C. Freshly prepared 0.5 M LHMDS solution in THF (24 ml, 12.1 mmol) was added dropwise under argon. After 15 minutes *N*-(5-bromopentyl)phtalimide (3.6 g, 12.1 mmol) in dry and degased THF was added to the reaction at -78°C. The reaction mixture was warmed to room temperature and stirred overnight. Proceeding of the reaction was followed by ^{31}P-NMR and work up was performed after all starting material had reacted. The reaction was quenched with NH_4Cl/brine 1:1 on ice and the product was extracted with ethyl acetate, dried over $MgSO_4$ and concentrated resulting in a yellow oil (6.2 g crude yield). The conversion was estimated by P^{31}-NMR spectrum to 76%. The product was used without further purification.

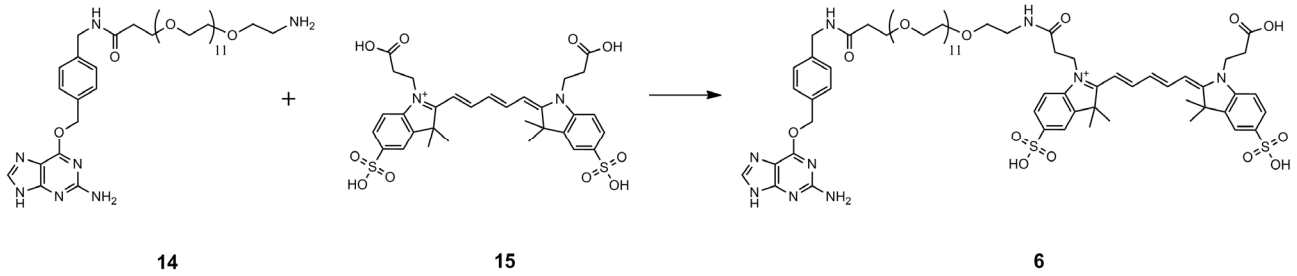
Compound 11. The synthesis and purification of **11** was performed following a slight modification of the reported procedure [60]. 15 g of crude extract containing compound **10** was dissolved in dry EtOH:CH_2Cl_2 (1:9) (110 ml) and trimethylchlorosilane (11.2 ml, 70.5 mmol) was added slowly under argon at 0°C and the reaction mixture was stirred overnight at room temperature. The reaction was quenched by addition of half saturated NH_4Cl and the solvent was evaporated. Purification by flash chromatography on silica with acetone:toluene:AcOH 50:50:1 resulted in **11** as a yellow oil (3.52 g, 21% based on H_2PO_3). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.83-7.89 (m, 2 H), 7.71-7.79 (m, 2 H), 7.13 (dt, 1 H, *J* = 530, 1.8 Hz), 4.04-4.25 (m, 2 H), 3.71 (t, 2 H, *J* = 7.1 Hz), 1.61-1.85 (m, 6 H), 1.43-1.53 (m, 2 H), 1.37 (t, 3 H, *J* = 7.0 Hz). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 168.45, 133.99, 132.09, 123.26, 62.68, 62.61, 37.60, 28.94, 28.17, 28.01, 27.72, 27.56, 20.38, 20.35, 16.31, 16.24. **^{31}P NMR** (161.98 MHz, $CDCl_3$) δ 38.87 (s). **HRMS** (ESI, pos. mode) m/z calc. for $C_{15}H_{21}NO_4P^+$ 310.1203, found 310.1198 $[M+H]^+$.

Compound 12. 0.5 M LHMDS solution in THF (2.48 ml, 1.24 mmol, 1 equiv.) was added dropwise to a solution of **11** (0.38 g, 1.24 mmol, 1 equiv.) in dry and degased THF (4 ml) at -78°C. After 15 minutes 1,3-dibromopropane (1.26 ml, 12.4 mmol, 10 equiv.) was added to the mixture at -78°C, the reaction was warmed to room temperature and stirred overnight. Proceeding of the reaction was followed by ^{31}P-NMR. After all starting material had reacted saturated aqueous $NaHCO_3$ was added, the product was extracted with ethyl acetate, washed with brine, dried over $MgSO_4$ and concentrated. The product was purified by RP-HPLC and lyophilized to afford **12**, as a yellow oil (35 mg, 6%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.82-7.88 (m, 2 H), 7.73 (m, 2 H), 4.02-4.12 (m, 2 H), 370 (t, 2 H, *J* = 7.1 Hz), 3.49 (t, 2 H, *J* = 6.4 Hz), 2.09-2.19 (m, 2 H), 1.85-1.93 (m, 2 H), 1.57-1.80 (m, 6 H), 1.32 (t, 3 H, *J* = 7.0 Hz).**^{13}C NMR** (101 MHz, $CDCl_3$) δ 168.4, 134.0, 132.1, 123.2, 60.6, 60.5, 37.6, 34.0, 33.8, 28.5, 28.1, 27.9, 27.6, 27.0, 26.1, 25.3, 21.5, 16.7. **^{31}P NMR** (161.98 MHz, $CDCl_3$) δ 56.54 (s). **HRMS** (ESI, pos. mode) m/z calc. for $C_{18}H_{26}BrNO_4P^+$ 430.0777, found 430.0769 $[M+H]^+$.

Compound 13. To a solution of **12** (29 mg, 67 µmol, 1 equiv.) in DMF (0.5 ml), first triethylamine (19 µl, 134 µmol, 2 equiv.), then 4-chlorobenzylamine (32 µl, 261 µmol, 3.9 equiv.) were added and the reaction was stirred at 80°C overnight under reflux. Proceeding of the reaction was followed by ^{31}P-NMR and MS-spectrometry. After all starting material had reacted, saturated aqueous $NaHCO_3$ was added and the product was extracted with ethyl acetate. Drying under high vacuum overnight resulted in **5** as pinkish crystals (52 mg crude yield). The conversion was estimated by P^{31}-NMR spectrum to 90%. The product was used without further purification.

Compound 2. 10 M methylamine in EtOH:H_2O 1:1 (4 ml, 40 mmol, 610 equiv.) was added to compound **13** (32 mg, 65 µmol, 1 equiv.). After 1 hour at 45°C the solvents were evaporated. Then, following a slight modification of the reported procedure [60] a mixture of 5 N NaOH (1 ml), EtOH (0.96 ml) and water (1 ml) was added and the reaction was stirred at 80°C for 1 hour using reflux. Proceeding of the reaction was followed by MS-spectrometry. After all starting material had reacted 5 N HCl (1 ml) was added to the reaction at 0°C and the solvent was evaporated under reduced pressure. The product was purified by RP-HPLC and lyophilized to afford **2** as a colorless clear oil (20 mg, 92%). **^{1}H NMR** (400 MHz, MeOD) δ 7.50 (m, 4 H), 4.21 (s, 2 H), 3.16 (t, 2 H, *J* = 7.3 Hz), 2.95 (t, 2 H, *J* = 7.5 Hz), 2.02 (m, 2 H), 1.60-1.86 (m, 8 H), 1.52 (q, 2 H, *J* = 7.5 Hz).**^{13}C NMR** (101 MHz, MeOD) δ 135.31, 131.29, 130.03, 128.99, 50.08, 47.71, 39.05, 28.89, 27.97, 27.23, 27.08,

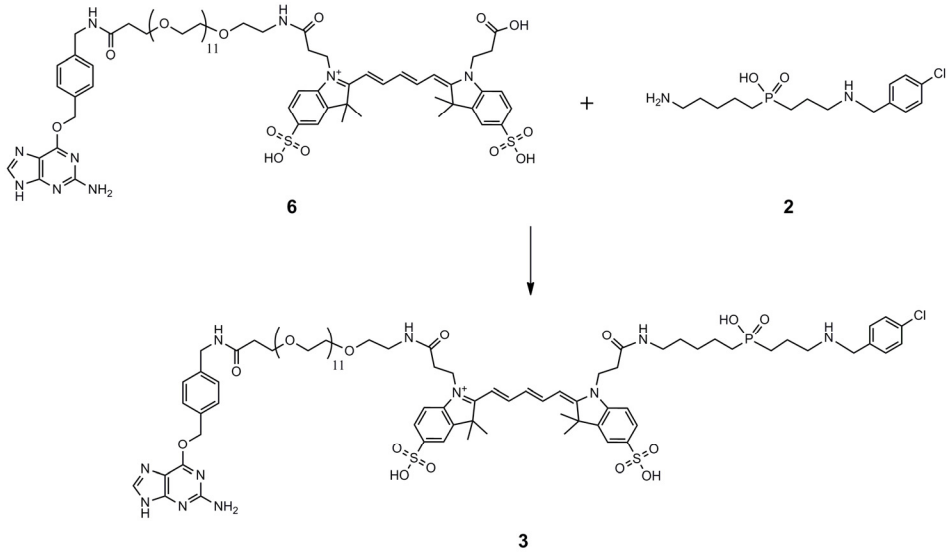
26.72, 26.37, 25.46, 21.16, 21.12, 18.60, 18.56. **^{31}P NMR** (161.98 MHz, $CDCl_3$) δ 50.76 (s). **HRMS** (ESI, pos. mode) m/z calc. for $C_{15}H_{27}ClN_2O_2P^+$ 333.1493 , found 333.1497 $[M+H]^+$.



Scheme 2. Synthesis of compound **6**.

Compound 6. To a solution of BG-$(PEG)_{11}$-NH_2, **14** (11 μmol, 1 equiv.) and Cy5 derivative **15** (11 μmol, 1 equiv.) in 200 μl DMSO were added EDC·HCl (2.1 mg, 11 μmol, 1 equiv.), HOBt (1.5 mg, 11 μmol, 1 equiv.) and DIPEA (15 μl, 90 μmol, 7.9 equiv.). After stirring the reaction at room temperature overnight 200 μl H_2O were added. After 15 minutes 30 μl AcOH were added and the crude product was purified by RP-HPLC and lyophilized to afford **6** (5.1 mg, 30 %) as blue powder. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.74 (br, s), 8.33-8.44 (m, 3 H), 8.18 (t, 1 H, *J* = 5.5 Hz), 7.81 (dd, 2 H, *J* = 7.2, 1.1 Hz), 7.64 (m, 2 H), 7.51 (d, 4 H, *J* = 8.1 Hz), 7.27-7.34 (m, 4 H), 6.55 (t, 1 H, *J* = 12.5 Hz), 6.39 (d, 1 H, *J* = 13.7 Hz), 6.30 (d, 1 H, *J* = 13.8 Hz), 5.56 (s, 2 H), 4.27-4.35 (m, 6 H), 3.63 (t, 2 H, *J* = 6.3 Hz), 3.38-3.52 (m, 42 H), 3.26 (t, 2 H, *J* = 6.1 Hz), 3.12 (q, 2 H, *J* = 5.5 Hz), 2.70 (t, 2 H, *J* = 7.0 Hz), 2.55 (m, 2 H), 2.39 (t, 2 H, *J* = 6.3 Hz), 1.69, 1.68 (2s, 12 H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 173.94, 173.32, 172.35, 170.69, 169.88, 159.02, 158.87, 158.62, 155.00, 154.89, 153.01, 145.84, 145.64, 142.19, 141.02, 140.84, 140.73, 133.97, 129.51, 127.69, 126.59, 126.45, 120.35, 116.79, 110.78, 110.62, 104.49, 104.11, 70.23, 70.17, 70.12, 70.07, 70.01, 69.33, 69.17, 67.33, 49.49, 49.28, 42.23, 41.10, 40.52, 39.17, 36.61, 33.78, 31.94, 27.53, 27.49. **HRMS** (ESI, pos. mode) m/z calc. for $C_{71}H_{101}N_9O_{23}S_2^{2+}$ 755.8220, found 755.8208 $[M+H]^{2+}$.

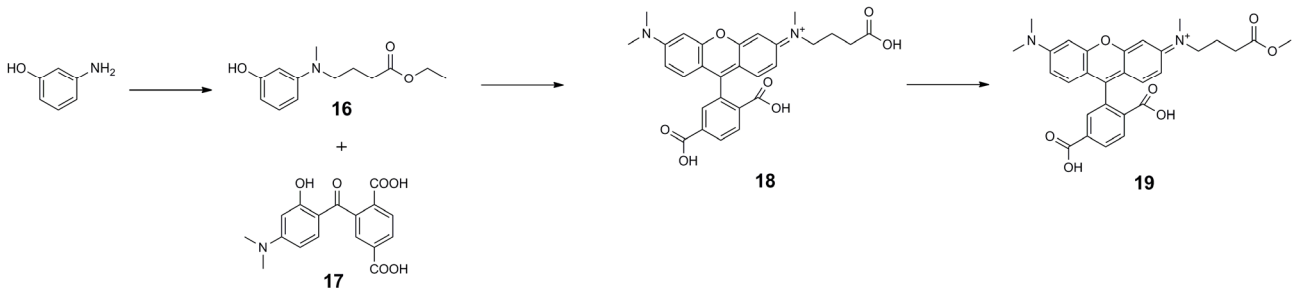
BG-$(PEG)_{11}$-NH_2, (**14**) and compound **15** were prepared as previously reported.[19,61]



Scheme 3. Synthesis of compound **3**.

Compound 3. To a 4.8 mM solution of **6** in dry DMSO (250 μl, 1.2 μmol, 1 equiv.) were added successively DIPEA (15 μl mol, 90 μmol, 75 equiv.) and a 48 mM TSTU solution in DMSO (25 μl, 1.2 μmol, 1 equiv.). After 5 minutes, a 48 mM solution of **2** in dry DMSO (25 μl, 1.2 μmol, 1 equiv.) was added. After 15 minutes at room temperature the reaction was quenched by addition of 100 μl H_20 and 10 μl AcOH. The product was purified by RP-HPLC and lyophilized to afford **3** as blue powder (0.8 mg, 37 %) which was dissolved in DMSO. **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.94 (br, s, 1 H), 8.49 (br, s, 1 H), 8.34-8.41 (m, 3 H), 8.18 (m, 1 H), 8.02 (m, 1 H), 7.82 (d, 2 H, *J* = 9.4 Hz), 7.64

(t, 2 H, *J* = 9.3 Hz), 7.47-7.59 (m, 6 H), 7.27 (m, 4 H), 6.53 (t, 1 H, *J* = 11.5 Hz), 6.32 (d, 2 H, *J* = 13.7 Hz), 5.54 (s, 2 H), 4.26-4.37 (m, 6 H), 4.18 (m, 2 H), 3.63 (m, 2H), 3.47-3.51 (m, 42H), 3.42 (m, 2 H), 3.28 (t, 2 H, *J* = 5.5 Hz), 3.13 (m, 2 H), 3.04 (m, 2 H), 2.92 (m, 2 H), 2.39 (m, 2 H), 1.84 (m, 2 H), 1.69 (s, 12 H), 1.44 (m, 2 H), 1.30 (m, 2 H), 1.15 (m, 2 H), 1.06 (m, 2 H, *J* = 2.7 Hz). **^{31}P NMR** (161.98 MHz, $CDCl_3$) δ 49.4 (s). **HRMS** (ESI, pos. mode) m/z calc. for $C_{86}H_{125}ClN_{11}O_{24}PS_2^{2+}$ 912.8878, found 912.8877 $[M+H]^{2+}$.

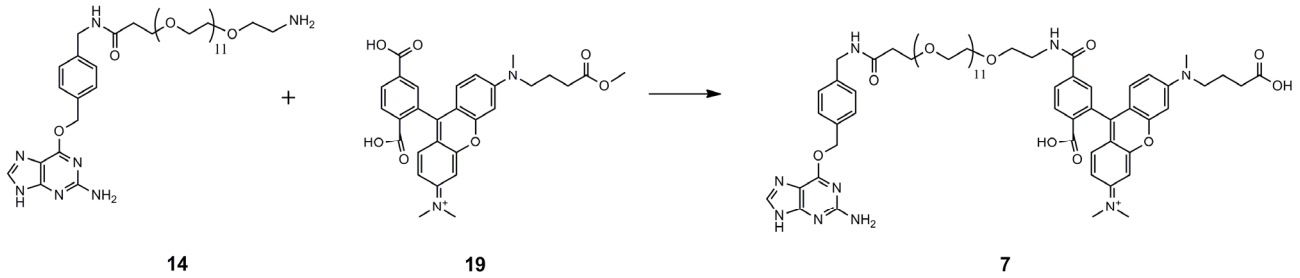


Scheme 4. Synthesis of compound **19**.

Compound 16. 3-aminophenol (1.3 g, 12 mmol, 1.0 equiv.) and 4-bromobutyrate ethylester (2.37 ml, 12 mmol, 1.0 equiv.) were dissolved in DMF, and DIPEA (2ml, 12 mmol, 1.0 equiv.) was added. The mixture was heated to 100°C for 1.5h. It was cooled to r.t., then iodomethane (0.95 ml, 16 mmol, 1.3 equiv.) and DIPEA (2 ml, 12 mmol, 1.0 equiv.) were added to the mixture. The reaction was heated to 80° for 2h, cooled and poured in a well stirred mixture of AcOEt/Hexane 1:1 and H_2O (150 ml each). The organic phase was washed with 150 ml H_2O, dried and evaporated. The oily residue was purified by flash chromatography (40 g SiO_2, isocratic AcOEt:Hexane 2:8) to afford 1.1 g of **16** (39%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.08 (t, 1 H, *J* = 8.1 Hz), 6.31 (m, 1 H), 6.23 (m, 2 H), 5.89 (s, 1 H), 4.17 (q, 2 H, *J* = 7.1 Hz), 3.34 (t, 2 H, *J* = 7.3 Hz), 2.91 (s, 3 H), 2.37 (t, 2 H, *J* = 7.2 Hz), 1.92 (quint, 2 H, *J* = 7.2 Hz), 1.28 (t, 3 H, *J* = 7.1 Hz). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.9, 156.9, 150.8, 130.1, 104.9, 103.5, 99.4, 60.7, 51.9, 38.4, 31.6, 22.1, 14.2. **HRMS** (ESI) calcd for $C_{13}H_{20}NO_3^+$ $[M+H]^+$ 238.1438; found 238.1432.

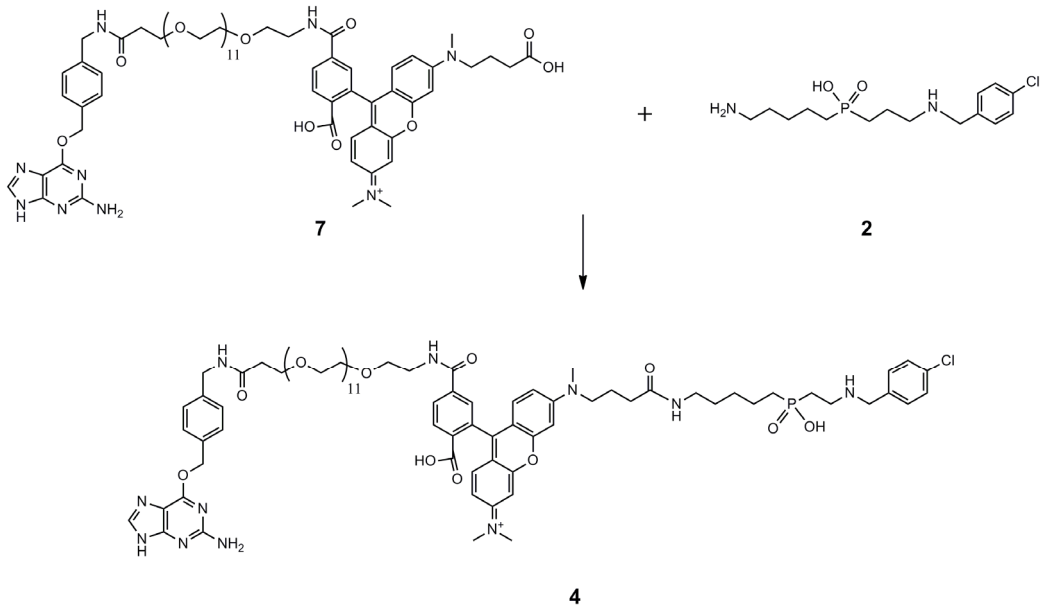
Compound 18. Compound **16** (430 mg, 1.8 mmol, 1.2 equiv.) and **17** (517 mg, 1.5 mmol, 1 equiv.), prepared as previously reported [62], were dissolved in DMF and TMS-polyphosphate (0.3 ml) was added. The mixture was heated to 100 °C for 6 h, cooled to r.t., poured in 50 ml 1 M NaOH and stirred for 1h at r.t. 37% HCl was added to adjust the pH to 1 (precipitation). The precipitate was centrifuged and the supernatant was discarded. The solid residue washed with water and dissolved in MeOH (ca. 25 ml). 5 g of Lichroprep (RP-C18 SiO_2) were added and the solvent was evaporated. The dark violet powder was added on top of a 20 g Lichroprep (RP-C18 SiO_2) column equilibrated with 0.1% TFA in water. Elution was performed by applying 100 ml steps of eluent with 5% increment of MeCN in 0.1% TFA. After evaporation of the solvents, 220 mg (25%) of **18** were obtained. **^{1}H NMR** (400 MHz, MeOD) 8.43 (d, 1 H, *J* = 8.1 Hz), 8.40 (dd, 1 H, *J* = 8.2, 1.5 Hz), 8.00 (d, 1 H, *J* = 0.8 Hz), 7.17 (dd, 2 H, *J* = 9.6, 1.0 Hz), 7.13 (dd, 1 H, *J* = 9.5, 2.0 Hz), 7.08 (dd, 2 H, *J* = 9.5, 2.4 Hz), 7.00 (d, 1 H, *J* = 2.4 Hz), 3.71 (t, 2 H, *J* = 7.7 Hz), 3.33 (s, 6 H), 3.31 (s, 3 H), 2.45 (t, 2 H, *J* = 6.9 Hz), 1.99 (quint, 2H, *J* = 6.9 Hz) **^{13}C NMR** (101 MHz, MeOD) δ 175.06, 166.38, 166.16, 159.03, 157.81, 157.70, 157.58, 156.98, 134.94, 134.50, 133.89, 131.37, 130.94, 130.88, 130.73, 130.62, 114.23, 113.57, 113.56, 96.22, 96.10, 51.87, 39.56, 38.14, 29.91, 21.80.**HRMS** (ESI) calcd for $C_{28}H_{27}N_2O_7^+$ 503.1813; found 503.1807 $[M^+]$

Compound 19. Compound **18** (25 mg, 49 µmol, 1.0 equiv.) was dissolved in DMSO (0.6 ml) and DIPEA (50 µl, 290 µmol, 6 equiv.) was added. Then a solution of HBTU (18.5 mg, 49 µmol, 1.0 equiv.) in DMSO (0.1 ml) was added. After 5 min at r.t., MeOH (0.3 ml) was added to the mixture. After 15 minutes the reaction was quenched with H_2O (0.3 ml). The crude product was purified by RP-HPLC and lyophilized to afford 19.6 mg of **19** (77%) as a pink powder. NOE-NMR confirmed the presence of the methyl ester only at the aliphatic carboxylic acid group. **^{1}H NMR** (400 MHz, MeOD) δ 8.43 (d, 1 H, *J* = 8.1 Hz), 8.39 (dd, 1 H, *J* = 8.2, 1.5 Hz), 8.00 (d, 1 H, *J* = 1.1 Hz), 7.16 (dd, 2 H, *J* = 9.5, 1.8 Hz), 7.02-7.12 (m, 3 H), 6.97 (d, 1 H, *J* = 2.4 Hz), 3.65-3.71 (m, 5 H), 3.31 (s, 6 H), 3.28 (s, 3 H), 2.47 (t, 2 H, *J* = 6.8 Hz), 1.99 (quint, 2 H, *J* = 7.0 Hz). **^{13}C NMR** (101 MHz, MeOD) δ 173.67, 166.26, 165.94, 158.95, 157.74, 157.67, 157.57, 156.92, 134.60, 134.49, 133.93, 131.47, 130.97, 130.85, 130.71, 130.48, 114.26, 114.07, 113.55, 113.42, 96.24, 96.13, 51.77, 50.86, 39.59, 38.12, 29.84, 21.73., **HRMS** (ESI) calcd for $C_{29}H_{29}N_2O_7^+$ 517.1975; found 517.1965 $[M]^+$.



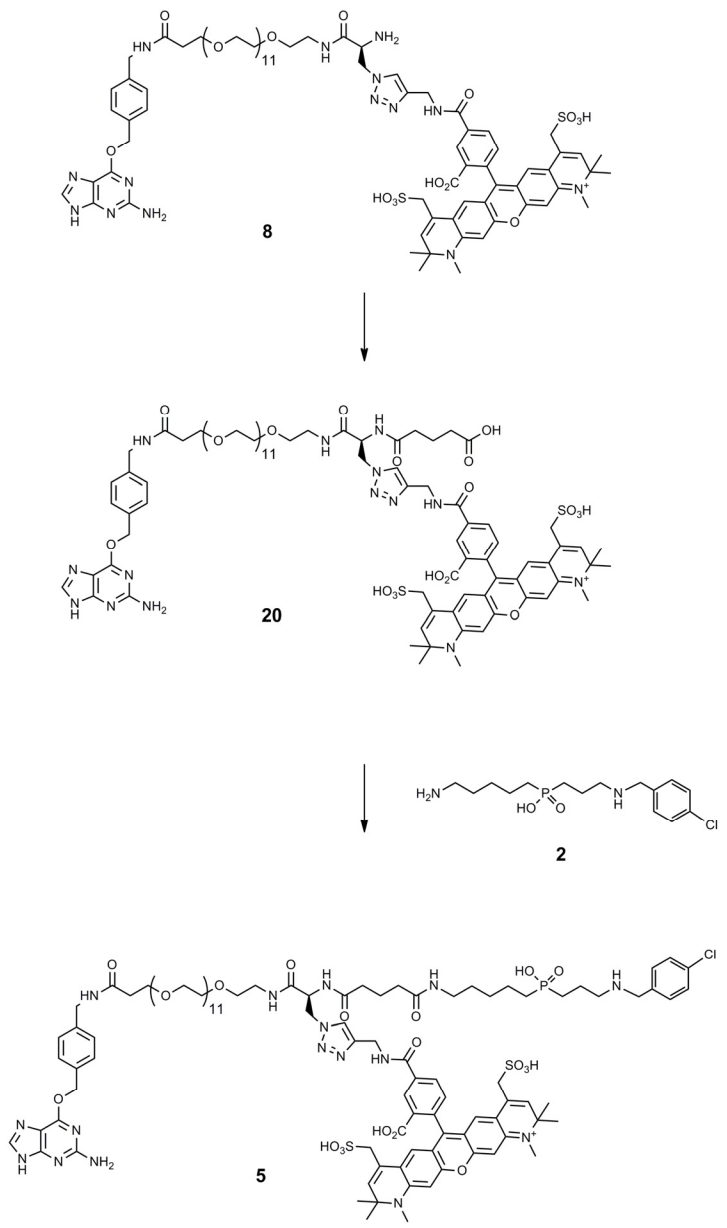
Scheme 5. Synthesis of compound **7**.

Compound 7. To a 52 mM solution of **19** in DMSO (100 µl, 5.2 µmol, 1.1 equiv.) were successively added DIPEA (10 µl, 60 µmol, 12.5 equiv.) and 100 mM TSTU solution (60 µl, 6 µmol, 1.2 equiv.). After 1 min, BG-$(PEG)_{11}$-NH_2 (**14**) in DMSO (120 µl, 4.8 µmol, 1 equiv.) was added. After 15 minutes at room temperature 100 µl 1M NaOH were added. After 10 min at room temperature, 20 µl AcOH were added and the crude product was purified by RP-HPLC, lyophilized and dissolved in 400 µl DMSO to afford **7** (4.0 µmol, 83%).**^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.85 (t, 1 H, *J* = 5.4 Hz), 8.39 (t, 1 H, *J* = 5.9 Hz), 8.28-8.35 (m, 2 H), 8.25 (dd, 6 H, *J* = 8.2, 1.6 Hz), 7.90 (d, 1 H, *J* = 1.4 Hz), 7.48 (d, 2 H, *J* = 8.0 Hz), 7.29 (d, 2 H, *J* = 8.0 Hz), 7.02-7.15 (m, 6 H), 6.98 (d, 1 H, *J* = 1.9 Hz), 5.51 (s, 2 H), 4.28 (d, 2 H, *J* = 5.6 Hz), 3.61-3.68 (m, 4 H), 3.42-3.55 (m, 48 H), 3.28 (s, 6 H), 3.25 (s, 3 H), 2.33-2.41 (m, 4 H), 1.83 (m, 2 H). **^{13}C NMR** (151 MHz, DMSO-d_6) δ 174.49, 170.63, 167.06, 166.35, 165.00, 159.46, 158.58, 158.35, 157.31, 157.18, 156.69, 140.42, 137.87, 134.75, 133.55, 131.52, 131.20, 129.50, 129.23, 127.68, 120.11, 117.96, 115.99, 115.17, 115.00, 114.03, 113.31, 96.74, 70.23, 70.17, 70.02, 69.20, 68.13, 67.34, 52.16, 42.26, 41.02, 40.52, 36.60, 30.86. **HRMS** HRMS (ESI) calcd for $C_{68}H_{93}N_9O_{20}^{2+}$ 677.8263; found 677.8249 $[M+H]^{2+}$. BG-$(PEG)_{11}$-NH_2 (**14**) was prepared as previously reported.[19]



Scheme 6. Synthesis of compound **4**.

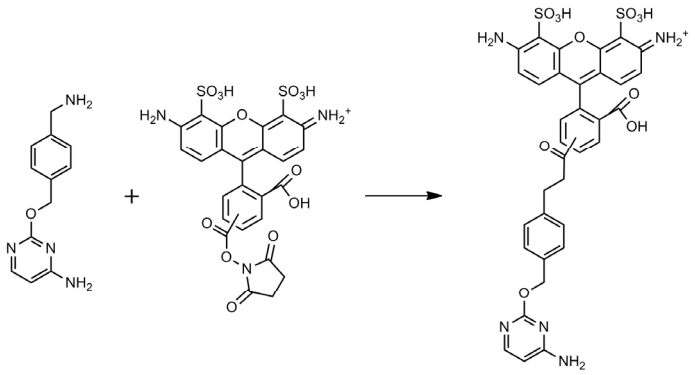
Compound 4. To a 10 mM solution of **7** in dry DMSO (100 µl, 1 µmol, 1 equiv.) were added successively DIPEA (15 µl mol, 90 µmol, 90 equiv.) and a 50 mM TSTU solution in DMSO (20 µl, 1 µmol, 1 equiv.). After 5 minutes, **2** in dry DMSO (22 µl, 1 µmol, 1 equiv.) was added. The reaction was quenched by addition of 100 µl of H_20 and 10 µl AcOH. The product was purified by RP-HPLC and lyophilized to afford **4** as pink powder (0.73 mg, 44 %) which was dissolved in DMSO-d_6. **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.97 (br, s, 2 H), 8.85 (t, 1 H, *J* = 4.6 Hz), 8.37 (t, 1 H, *J* = 5.8 Hz), 8.21-8.30 (m, 2 H), 7.80-7.99 (m, 3 H), 7.52 (m, 4 H), 7.44 (d, 2 H, *J* = 7.8 Hz), 7.27 (d, 2 H, *J* = 7.8 Hz), 6.88-7.10 (m, 4 H), 5.46 (s, 2 H), 4.28 (d, 2 H, *J* = 5.6 Hz), 4.16 (s, 2 H), 3.63 (t, 2 H, *J* = 6.1 Hz), 3.46-3.54 (m, 48 H), 3.22 (m, 9 H), 3.02 (m, 4 H), 2.47 (s, 2 H), 2.38 (m, 2 H), 2.29 (t, 1 H, *J* = 7.3 Hz), 2.16 (s, 2 H), 1.84 (m, 4 H), 1.67 (m, 2 H), 1.49-1.60 (m, 4 H), 1.36-1.49 (m, 4 H), 1.29-1.35 (m, 2 H), 0.86 (t, 2 H, *J* = 6.7 Hz). **^{31}P NMR** (161.98 MHz, $CDCl_3$) δ 49.4 (s). **HRMS** (ESI) calcd for $C_{83}ClH_{117}N_{11}O_{21}P^{2+}$ 834.8920; found 834.8909 $[M+H]^{2+}$.



Scheme 7. Synthesis of compound **5**.

Compound 20. To a 2,7 mM solution of **8** in dry DMSO (100 µl, 0.27 µmol, 1.0 equiv.) were added successively DIPEA (1.5 µl mol, 9 µmol, 33 equiv.) and a 50 mM glutaric anhydride solution in DMSO (11 µl, 0.54 µmol, 2.0 equiv.). After 30 minutes at room temperature the reaction was quenched by addition of 100 µl H_20 and 10 µl AcOH. The product was purified by RP-HPLC and lyophilized to afford **20** as pink powder (0.2 µmol, 74%) which was dissolved in DMSO. **HRMS** (ESI, pos. mode) m/z calc. for $C_{86}H_{116}N_{14}O_{28}S_2^{2+}$ 928.3757; found 928.3735 $[M+H]^{2+}$. Compound **8** was prepared as previously reported. [20]

Compound 5. To a 2 mM solution of **20** in dry DMSO (45 µl, 90 nmol, 1 equiv.) were added successively DIPEA (1.5 µl mol, 9 µmol, 100 equiv.) and a 48 mM TSTU solution in DMSO (15 µl, 0.72 µmol, 8 equiv.). After 5 minutes a 1.3 mM solution of **2** in dry DMSO (70 µl, 90 nmol, 1 equiv.) was added. After 60 minutes at room temperature the reaction was quenched by addition of 100 µl H_20 and 10 µl AcOH. The product was purified by RP-HPLC and lyophilized to afford **5** as pink powder (30 nmol, 33%) which was dissolved in DMSO. **HRMS** (ESI, pos. mode) m/z calc. for $C_{101}ClH_{140}N_{16}O_{29}PS_2^{2+}$ 1085.4414; found 1085.4420 $[M+H]^{2+}$.

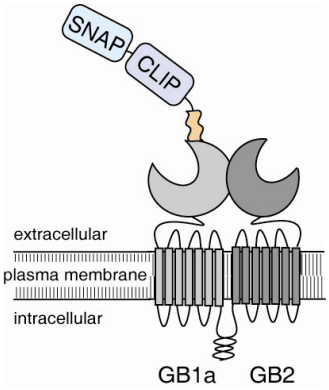


Scheme 8. Synthesis of compound **23**.

Compound 23. To a solution of **22** (Molecular Probes, 1.4 mg, 2.1 µmol, 1 eq.) in dry DMSO (100 µl) were added successively **21** (0.7 mg, 3.0 µmol, 1.5 eq.) and DIPEA (10 µl, 58 µmol, 27 eq.) After 5 minutes room temperature the reaction was quenched by addition of 50 µl H_20 and 5 µl AcOH. The product was purified by RP-HPLC and lyophilized to afford **23** as red powder (1.12 µmol, 53%) which was dissolved in DMSO. ^{1}**H NMR** (400 MHz, DMSO-d_6) δ 9.46 (t, 1 H, J = 5.0 Hz), 8.70 (s, 1 H), 8.32 (d, 1 H, J = 8.0 Hz), 8.02 (d, 1 H, J = 6.6 Hz), 7.50 (d, 3 H, J = 7.6 Hz), 7.42 (d, 2 H, J = 7.4 Hz), 6.89 (br, s, 4 H), 6.34 (d, 1 H, J = 6.4 Hz), 5.45 (s, 2 H), 4.57 (d, 2 H, J = 5.2 Hz). **HRMS** (ESI, neg. mode) m/z calc. for $C_{33}H_{25}N_6O_{11}S_2^-$ 745.1023; found 745.1009 $[M]^-$.

Biology

Sensor protein sequences



pRK5-HA-SNAP-CLIP-GB1a

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EGGLAVKEWLLAHEGHRLGKPGLGTRDIDKDCEMKRTTLDSPLGKLELSGCEQGLHRIIFLGKGTSAADAVEVPAPAAVLGGPEPL
IQATAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISESHLAALVGNPAATAAVNTALDGNPVPILIPCH
RVVQGDSDVGPYLGGLAVKEWLLAHEGHRLGKPGLGTRGGAQTPNATSEGCQIIHPPWEGGIRYRGLTRDQVKAINFLPVDYEIE
YVCRGEREVVGPKVRKCLANGSWTDMDTPSRCVRICSKSYLTLENGKVFLTGGDLPALDGARVEFRCDPDFHLVGSSRSVCSQGQ
WSTPKPHCQVNRTPHSERRAVYIGALFPMSGGWPGGQACQPAVEMALEDVNSRRDILPDYELKLIHHDSKCDPGQATKYLYELLY
NDPIKIILMPGCSSVSTLVAEAARMWNLIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPTRVKLFEKWGWKKIATIQQTTEVFTST
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DFNYNNQTITDQIYRAMNSSSFEGVSGHVVFDASGSRMAWTLIEQLQGGSYKKIGYYDSTKDDLSWSKTDKWIGGSPPADQTLVI
KTFRFLSQKLFISVSVLSSLGIVLAVVCLSFNIYNSHVRYIQNSQPNLNNLTAVGCSLALAAVFPLGLDGYHIGRSQFPFVCQARLWLL
GLGFSLGYGSMFTKIWWVHTVFTKKEEKKEWRKTLEPWKLYATVGLLVGMDVLTLAIWQIVDPLHRTIETFAKEEPKEDIDVSILP
QLEHCSSKKMNTWLGIFYGYKGLLLLLGIFLAYETKSVSTEKINDHRAVGMAIYNVAVLCLITAPVTMILSSQQDAAFAFASLAIVFSS
YITLVVLFVPKMRRLITRGEWQSETQDTMKTGSSTNNNEEEKSRLLEKENRELEKIIAEKEERVSELRHQLQSRQQLRSRRHPPTPP
DPSGGLPRGPSEPPDRLSCDGSRVHLLYK

green : amino acid sequence of HA tag

red: amino acid sequence of SNAP-tag

blue : amino acid sequence of CLIP-tag

violet: amino acid sequence of GB1a

pRK5-Flag-GB2

DYKDDDDKTRWTRGAPRPPPSSPPLSIMGLMPLTKEVAKGSIGRGVLPAVELAIEQIRNESLLRPYFLDLRLYDTECDNAKGLKAF
YDAIKYGPNHLMVFGGVCPSVTSIIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLLKHFRWRRVGTLTQ
DVQRFSEVRNDLTGVLYGEDIEISDTESFSNDPCTSVKKLKGNDVRIILGQFDQNMAAKVFCCAFEESMFGSKYQWIIPGWYEPAW
WEQVHVEANSSRCLRRSLLAAMEGYIGVDFEPLSSKQIKTISGKTPQQYEREYNTKRSGVGPSKFHGYAYDGIWVIAKTLQRAMETL
HASSRHQRIQDFNYTDHTLGKIILNAMNETNFFGVTGQVVFRNGERMGTIKFTQFQDSREVKVGEYNAVADTLEIINDTIRFQGS
EPPKDKTIILEQLRKISLPLYSILSALTILGMIMASAFLFFNIKNRNQKLIKMSSPYMNNLIILGGMLSYASIFLFGLDGSFVSEKTFETLC
TVRTWILTVGYTTAFGAMFAKTWRVHAIFKNVKMKKKIIKDQKLLVIVGGMLLIDLCILICWQAVDPLRRTVERYSMEPDPAGRDI
SIRPLLEHCENTHMTIWLGIVYAYKGLLMLFGCFLAWETRNVSIPALNDSKYIGMSVYNVGIMCIIGAAVSFLTRDQPNVQFCIVAL
VIIFCSTITLCLVFVPKLITLRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLQSENHRLRMKITELDKDLEEVT
MQLQDTPEKTTYIKQNHYQELNAAASLGNFTESTDGGKAILKNHLDQNPQLQWNTTEPSRTCKDPIEDINSPEHIQRRLSLQLPI
LHHAYLPSIGGVDASCVSPCVSPTASPRHRHVPPSFRVMVSGL

green : amino acid sequence of FLAG tag

red: amino acid sequence of GB2

Methods

Sensor plasmid preparation. HA-SNAP-CLIP-GB1a is composed of a HA-tag, SNAP-tag, CLIP-tag and the wild-type rat GB1a receptor. As coding sequences for SNAP- and CLIP-tag E30R mutants are used that exhibit a faster reaction rate for their substrates. The SNAP-tag sequence was amplified by PCR and subcloned into pRK5-HA-$SNAP_{26}$-GB1a that was previously described [28] replacing $SNAP_{26}$. Subsequently the PCR amplified CLIP-tag sequence was subcloned into pRK5-HA-SNAP-GB1a resulting in pRK5-HA-SNAP-CLIP-GB1a. Plasmid pRK5-FLAG-GB2 containing a FLAG-tag and the wild-type rat GB2 receptor was previously described.[63] Neither HA- nor FLAG-tag were used in this study but were retained in the plasmids.

Calcium data analysis. For calcium measurements data were analyzed with the program Soft Max Pro (Molecular Devices, Sunnyvale, CA, USA). Dose-response curves were fitted using Origin 7.5 (OriginLab Corporation, Northhampton, MA, USA) following equation 1 to obtain the half maximal effective concentration EC_{50}:

$$\Delta y = \Delta y_{min} + \frac{\Delta y_{max} - \Delta y_{min}}{1 + \left(\frac{[GABA]}{EC_{50}}\right)^{nH}} \qquad (1)$$

With Δy being the experimental calcium response, [GABA] the concentration of GABA, Δy_{min} and Δy_{max} being the calcium response in absence and presence of GABA, respectively and nH being the Hill coefficient.

Calculating the intensity ratio changes. The maximum ratio change is calculated using equation 2:

$$\Delta R_{max} = r_{zero}^{F_{donor}/F_{acceptor}} / r_{sat}^{F_{donor}/F_{acceptor}} \quad (2)$$

With ΔR_{max} being the maximum ratio change, $r_{zero}^{Fdonor/Facceptor}$ the intensity ratio of donor fluorescence (F_{donor}) and acceptor fluorescence ($F_{acceptor}$) when no GABA is present and $r_{sat}^{Fdonor/Facceptor}$ the intensity ratio of donor fluorescence and acceptor fluorescence at saturating GABA concentrations.

Calculating the environmental effect. The environmental effect on the fluorophore attached to the intramolecular ligand is calculated using equation 3:

$$R_{non-FRET} = F_{zero} / F_{sat} \quad (3)$$

With $R_{non\text{-}FRET}$ being the change in fluorescence intensity of the fluorophore coupled to the intramolecular ligand upon GABA-Snifit opening, F_{zero} the fluorescence intensity of the fluorophore in absence of GABA and F_{sat} the fluorescence intensity of the fluorophore at saturating GABA concentrations.

Fitting of the fluorescence intensity ratios. The data were fit to binding isotherm 4 to obtain the binding constant for competing ligands $K_d^{comp,ligand}$:

$$\Delta r = \Delta r_{zero} + \frac{\Delta r_{sat} - \Delta r_{zero}}{1+(\frac{[ligand]}{K_d^{comp,ligand}})^{nH}} \quad (4)$$

with Δr being the experimental intensity ratio (DY-547/Cy5) change, [ligand] the concentration of competitive ligands (GABA, *R,S*-baclofen, APPA or CGP 52432), Δr_{zero} and Δr_{sat} the intensity ratio (DY-547/Cy5) changes in absence and presence of ligand, respectively and nH being the Hill coefficient. The life-time of sensor opening ($\tau_{opening}$) was determined by fitting the intensity ratio time course to the following equation

$$r = r_{sat} + r_{i-sat} \times e^{-t/\tau_{opening}} \quad (5)$$

with r being the experimental intensity ratio of donor vs acceptor, r_{sat} the intensity ratio of donor vs acceptor in presence of ligand, $r_{i\text{-}sat}$ the value of the intensity ratio at t = 0 s minus the constant r_{sat}, and t the time. The life-time of sensor closing ($\tau_{closing}$) was determined by fitting their intensity ratio time course to the following equation

$$r = r_{zero} + r_{i-zero} \times e^{-t/\tau_{closing}} \quad (6)$$

with r being the experimental intensity ratio of donor vs acceptor, r_{zero} the intensity ratio of donor vs acceptor in absence of ligand, $r_{i\text{-}zero}$ the value of the intensity ratio at t = 0 s minus the constant r_{zero}, and t the time. Fits were performed with Origin 7.5 (OriginLab Corporation) with Δr_{zero} set to 1 and with free fit parameters $K_d^{comp,ligand}$ and Δr_{sat} for equation (4) and with free fit parameters $r_{i\text{-}sat}$, and $\tau_{opening}$ for equation (5) and $r_{i\text{-}sat}$, and $\tau_{closing}$ for equation (6).

K_d^{comp} Analysis of different $GABA_B$ receptor ligands. For the K_d^{comp} analysis of individual cells 18-25 cells were analyzed. The sensor protein density on the sensor surface varied between 2000 and 18000 fluorescent arbitrary units. The K_d^{comp} values for different cells were within the experimental error. In experiments including the allosteric modulators CGP 7930 and rac-BHFF their concentration was kept constantly at 30 µM and 10 µM, respectively during the perfusion.

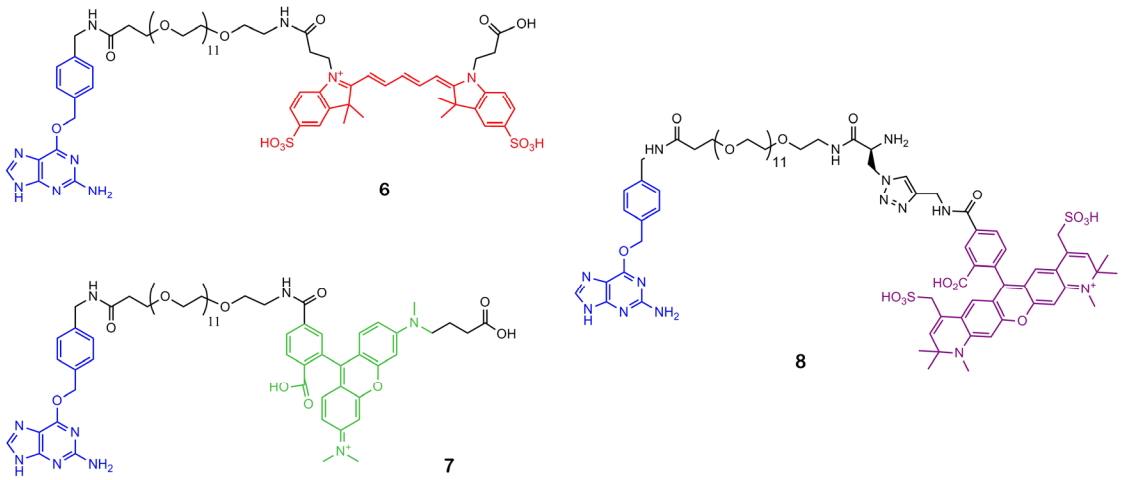

Figure S1. Substrates for labeling of GABA-Snifit for control experiments. Molecules contain the SNAP-tag substrate benzylguanine (BG) (blue) and different fluorophores but lack the $GABA_B$ receptor antagonist. Top: BG-Cy5 **6** (red), bottom: BG-TMR **7** (green), right: BG-AlexaFluor594 **8** (violet).

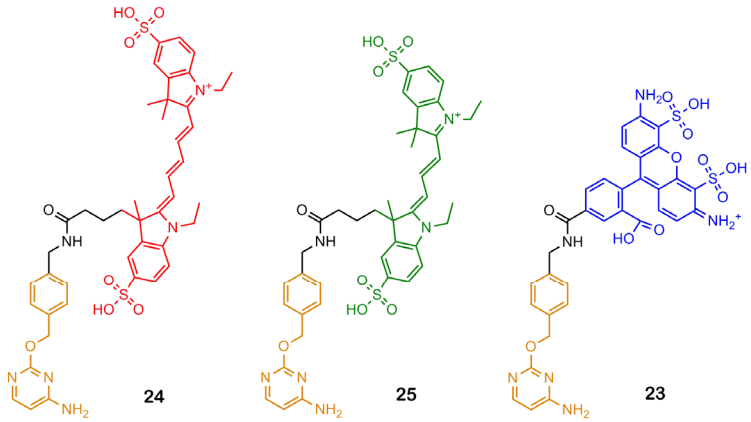

Figure S2. Synthetic fluorophores used for CLIP-tag labeling containing the CLIP-tag substrate benzylcytosine (BC) (yellow) and different fluorophores. Left: BC-DY-647 **24** (red), middle: BC-DY-547 **25** (green), right: BC-AlexaFluor488 **23** (blue). Compounds **24** and **25** are commercially available from New England Biolabs, Inc. (Ipswich, MA, USA) under the names CLIP Surface 547 (Cat.Number S9233S) and CLIP Surface 647 (Cat.Number S9234S).

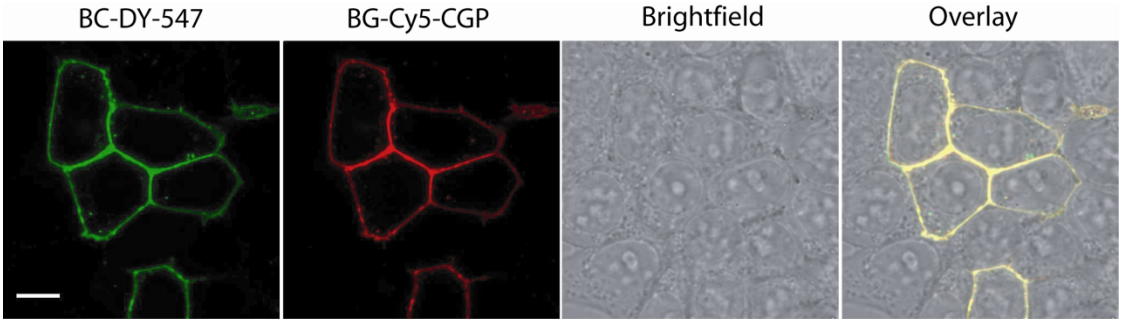

Figure S3. GABA-Snifit labeled exclusively on the surface of HEK 293 cells. The labeling was performed with BC-DY-547 **25** and BG-Cy5-CGP **3**. Images were taken using a confocal Zeiss LSM 700 microscope. Scale bar 10 µm.

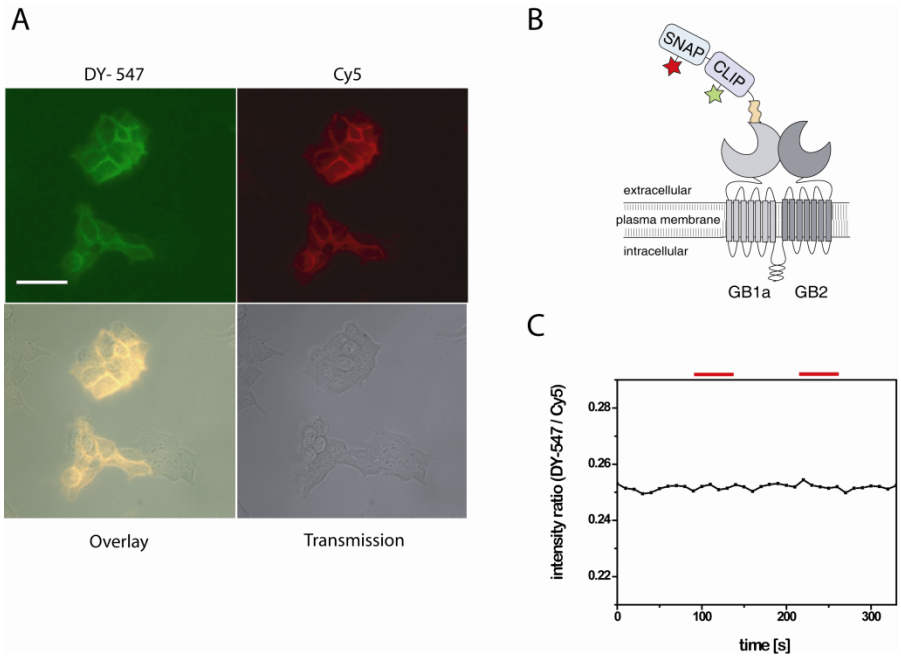

Figure S4. Control perfusion experiments on the surface of HEK 293 cells. (A) Donor channel (DY-547), FRET channel (Cy5) and transmission channel of the labeled sensor protein on HEK 293 cells with BC-DY-547 **25** and BG-Cy5 **6**. Scale bar 50 µm. (B) GABA-Snifit for control experiments. (C) Time course of the intensity ratio of donor emission vs acceptor emission upon addition and removal of 1 mM GABA. The red bar indicates the time span of GABA perfusion.

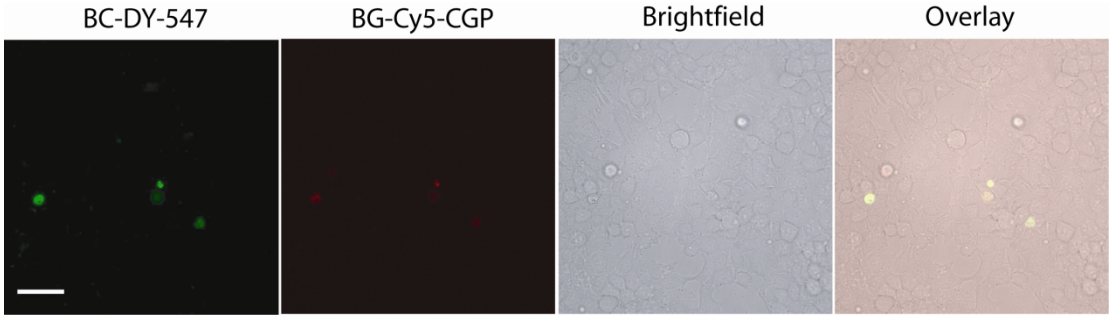

Figure S5. Labeling control with mock-transfected HEK 293 cells. The cells were incubated with BC-DY-547 **25** and BG-Cy5-CGP **3**, washed with HBSS and imaged. No unspecific fluorescent labeling was observed. Scale bar 50 µm.

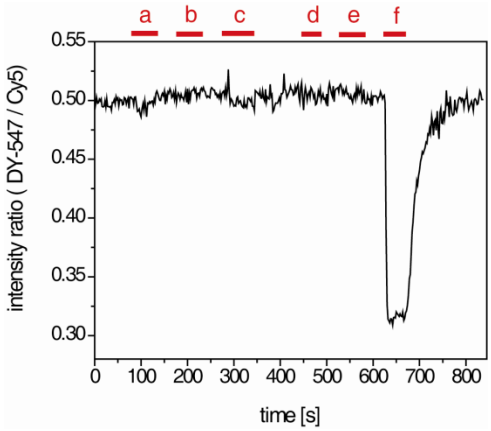

Figure S6. Control perfusion experiments of GABA-Snifit on the surface of HEK 293 cells with unspecific $GABA_B$ ligands. GABA-Snifit was labeled with BC-DY-547 **25** and BG-Cy5-CGP **3** and perfused with (a) 2 mM glycine, (b) 1 mM aspartic acid, (c) 1 mM glutamic acid, (d) 1 mM kynurenic acid, (e) 10 mM γ-hydroxy butyric acid (GHB) and (f) 10 mM GABA. Time course of the intensity ratio of donor emission vs acceptor emission is shown and the red bar indicates the time span of ligand perfusion.

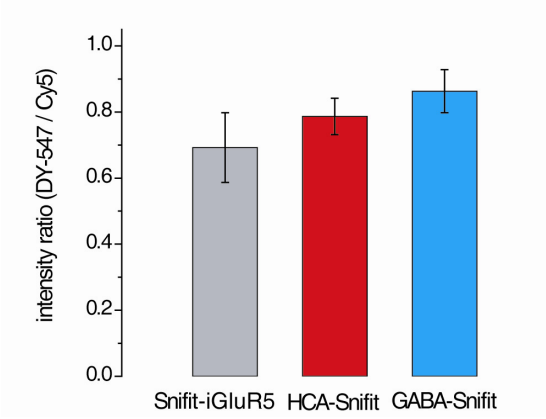

Figure S7. Comparison of the FRET efficiency of different Snifits. The Snifit for glutamate based on the ionotropic glutamate receptor iGluR5 (Snifit-iGluR5), the Snifit for sulfonamides based on human carbonic anhydrase (HCA-Snifit) and GABA-Snifit were expressed in HEK 293 cells and labeled with BG-Cy5 **6** and BC-547 **26**. Snifits labeled with fluorophores lacking a ligand for the receptor protein mimic the open state of the sensors. Measurements were performed under identical instrument settings and the mean intensity ratio ± s.d. of $n \geq 70$ is plotted for Snifit-iGluR5 (grey), HCA-Snifit (red) and GABA-Snifit (blue).

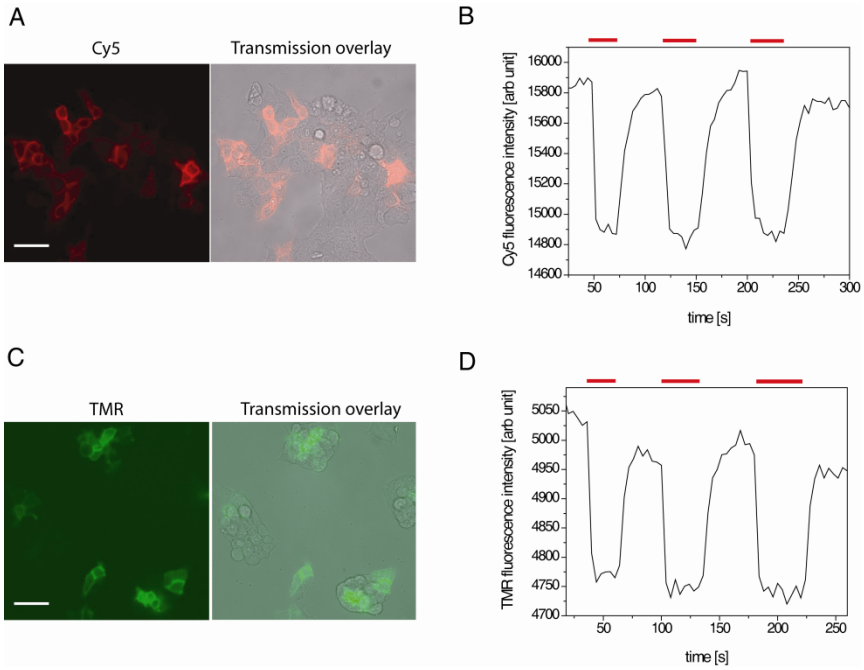

Figure S8. Environmental effect on the fluorophore coupled to the intramolecular ligand. (A) Cy5 channel and transmission overlay of the labeled sensor protein on HEK 293 cells with BG-Cy5-CGP **3**. Scale bar 50 µm. (B) Time course of the fluorescence emission of Cy5 upon addition and removal of 1 mM GABA in HEK 293 cells shown in (A). (C) TMR channel and transmission overlay of the labeled sensor protein on HEK 293 cells with BG-TMR-CGP **4**. Scale bar 50 µm. (D) Time course of the fluorescence emission of TMR upon addition and removal of 1 mM GABA in HEK 293 cells shown in (C). The red bar indicates the time span of GABA perfusion.

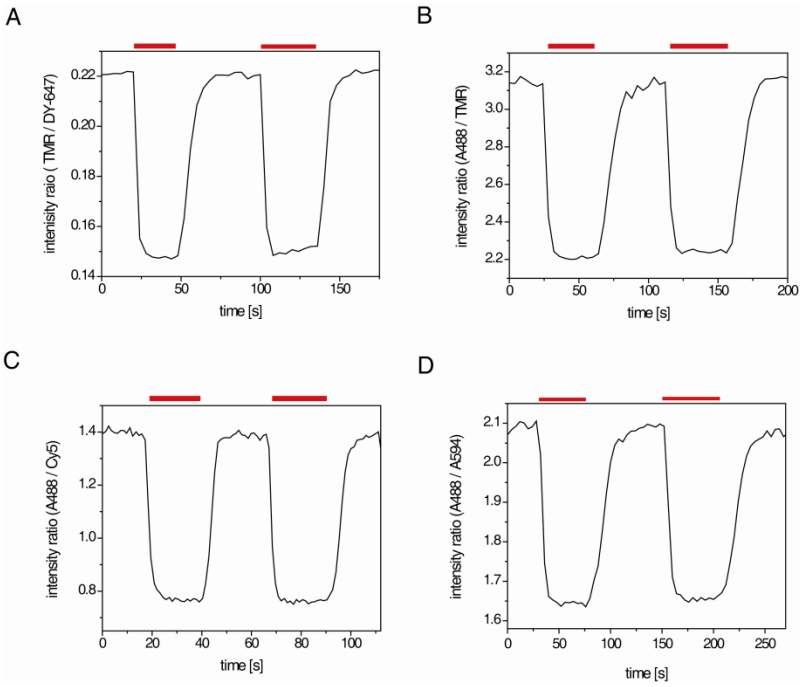

Figure S9. Perfusion experiments with GABA-Snifit labeled with different FRET fluorophore pairs for GABA detection on the surface of HEK 293 cells. Time course of the intensity ratio of donor emission vs acceptor emission for (A) TMR/DY-647, (B) Alexa Fluor 488/TMR, (C) Alexa Fluor 488/Cy5 and (D) Alexa Fluor 488/Alexa Fluor 594 upon addition and removal of 1 mM GABA. The red bar indicates the time span of GABA perfusion.

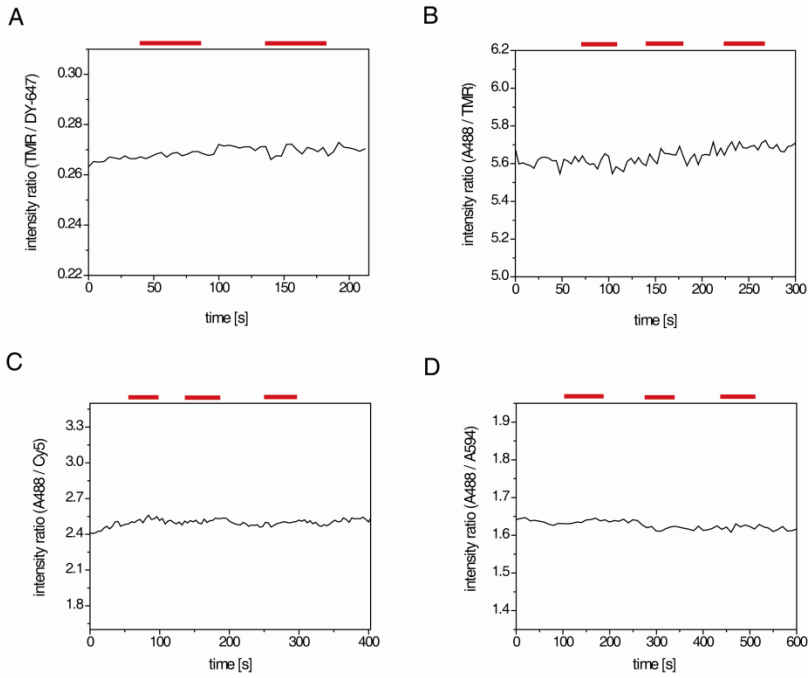

Figure S10. Control perfusion experiments with the sensor protein labeled with substrates containing different FRET fluorophore pairs but lacking the intramolecular ligand on the surface of HEK 293 cells. Time course of the intensity ratio of donor emission vs acceptor emission for (A) TMR/DY-647, (B) Alexa Fluor 488/TMR, (C) Alexa Fluor 488/Cy5 and (D) Alexa Fluor 488/Alexa Fluor 594 upon addition and removal of 1 mM GABA. The red bar indicates the time span of GABA perfusion.

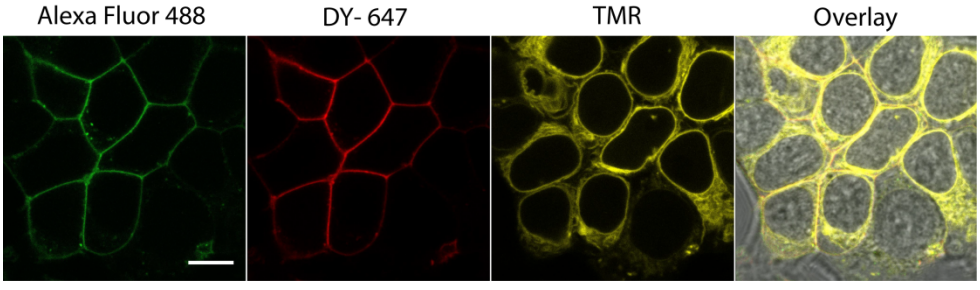

Figure S11. Intracellular pool of GABA-Snifit. The GABA-Snifit was labeled first with cell non-permeable dyes BC-Alexa Fluor 488 **23** and BG-DY-647 (SNAP Surface 647, Cat. Number S9137S, NEB) to visualize the extracellular sensor pool and was subsequently labeled with the cell-permeable CP-TMR (SNAP-Cell TMR-Star, Cat.Number S9105S, NEB) to demonstrate the intracellular SNAP-CLIP-GB1a pool in HEK 293 cells. Images were taken using a confocal Zeiss LSM 700 microscope. Scale bar 10 µm.

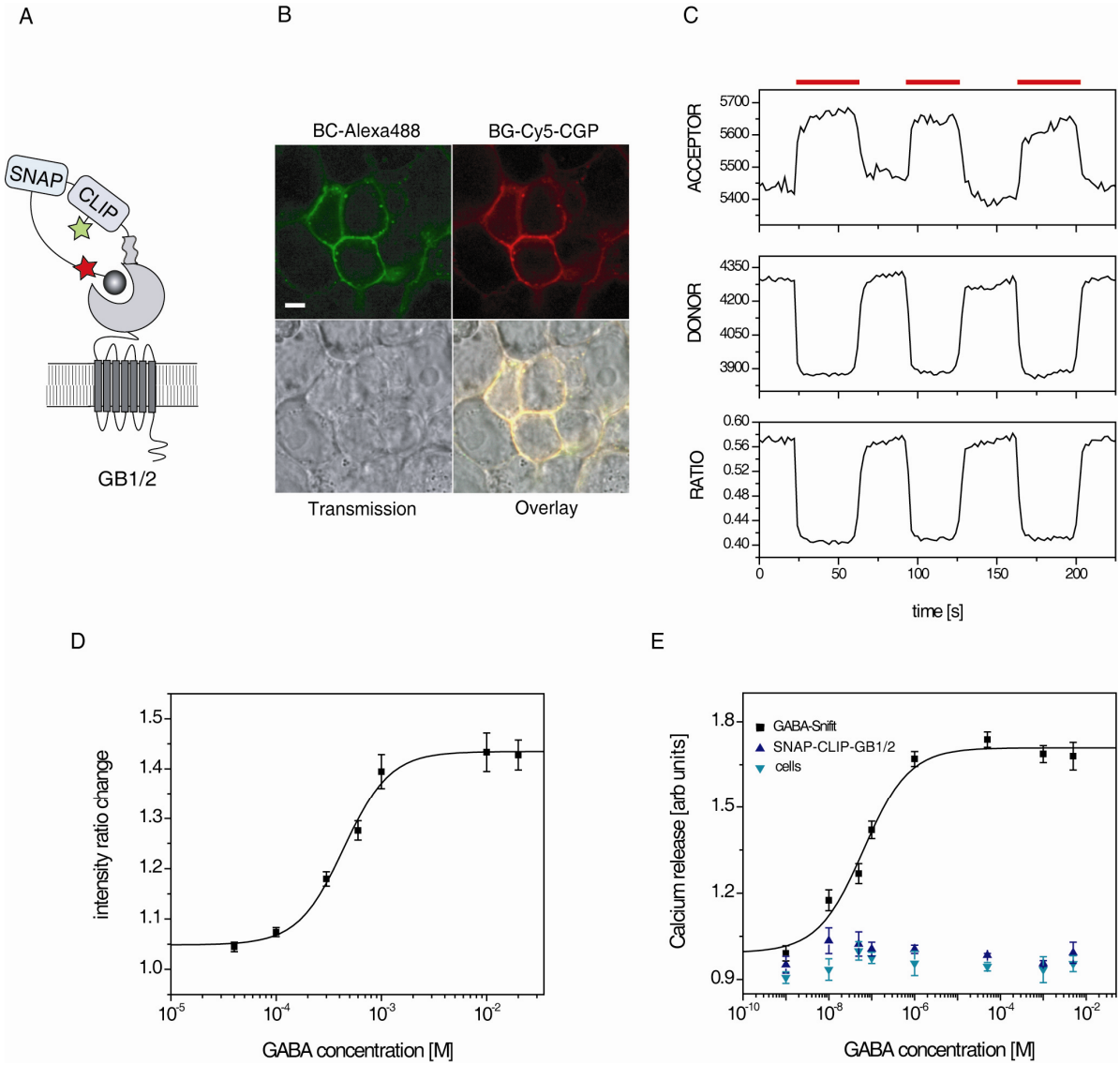

Figure S12. The receptor chimera GB1/2 as part of the GABA-Snifit. (A) Snifit model. (B) SNAP-CLIP-GB1/2 expressed in HEK 293 cells and labeled on cell surface with BC-Alexa488 **23** and BG-Cy5-CGP **3**. Images were taken using a confocal Leica SP5 WL microscope. Scale bar 5 µm. (C) Perfusion experiment for GABA detection on the surface of HEK 293 cells. Shown is the time course of the acceptor channel (Cy5; top), of the donor channel (DY-547; middle) and of the intensity ratio of donor emission vs. acceptor emission (bottom) upon addition and removal of 5 mM GABA. The red bar indicates the time span of GABA perfusion. (D) GABA titration curve of SNAP-CLIP-GB1/2 on the surface of HEK 293 cells. Shown is the intensity ratio change ΔR for different GABA concentrations. Data are means ± s.d. of three independent experiments; (n = 54). Intensity ratio changes were fitted to eq. 4. (E) Calcium dose response generated by increasing concentrations of GABA in HEK 293 cells expressing the original GABA-Snifit (black squares) or SNAP-CLIP-GB1/2 (blue triangles) together with the chimeric G protein Gqi9. As control non-transfected cells were tested (cyan triagles). Data are means ± s.d. of triplicate determinations and representative of three independent experiments. Calcium release values were fitted to eq. 1.

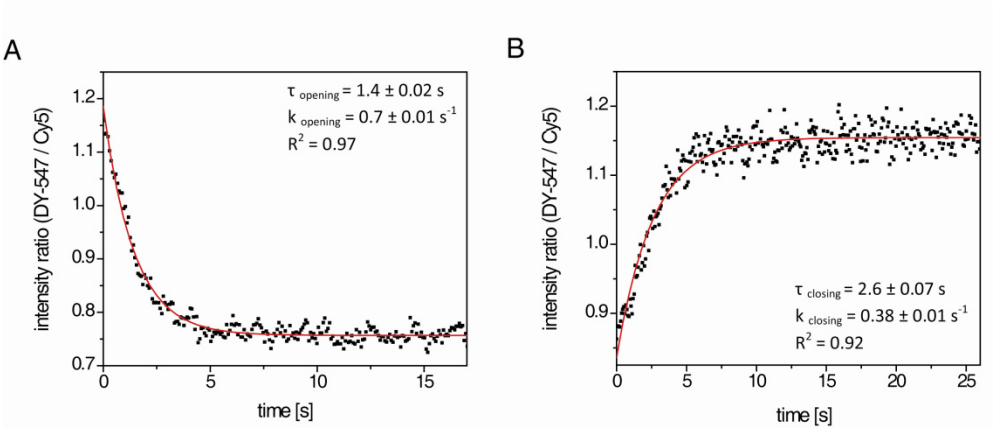

Figure S13. Kinetics of GABA-Snifit. (A) Fitting of the time course of GABA-Snifit opening upon addition of 300 µM GABA to equation 5. (B) Fitting of GABA-Snifit closing upon removal of 300 µM GABA to equation 6.

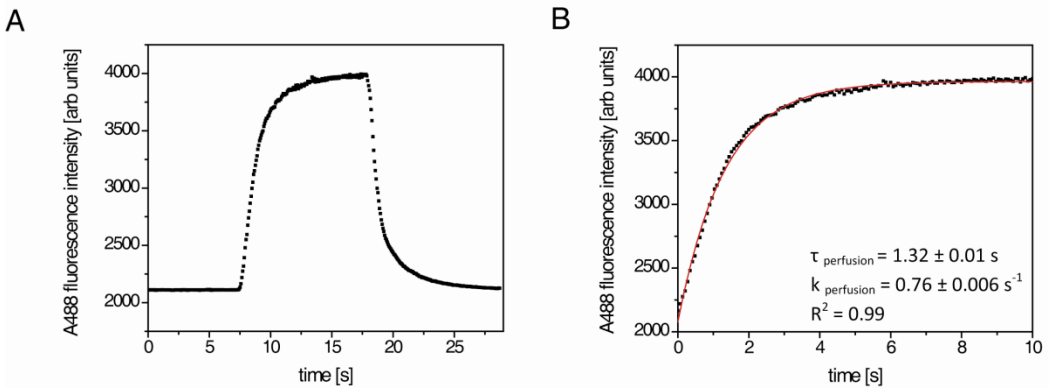

Figure S14. Analysis of the kinetic resolution of the used perfusion system (imaging chamber from Warner Instruments). (A) Time course of a perfusion experiment where HEK 293 cells were perfused with HBSS (0 - 7.5 s and 18 - 27 s) or 50 nM Alexa Fluor 488 dye in HBSS (7.5 s – 18 s) to monitor the speed of complete liquid exchange in the chamber. (B) Fitting of the time course upon Alexa Fluor 488 dye addition to equation 6.

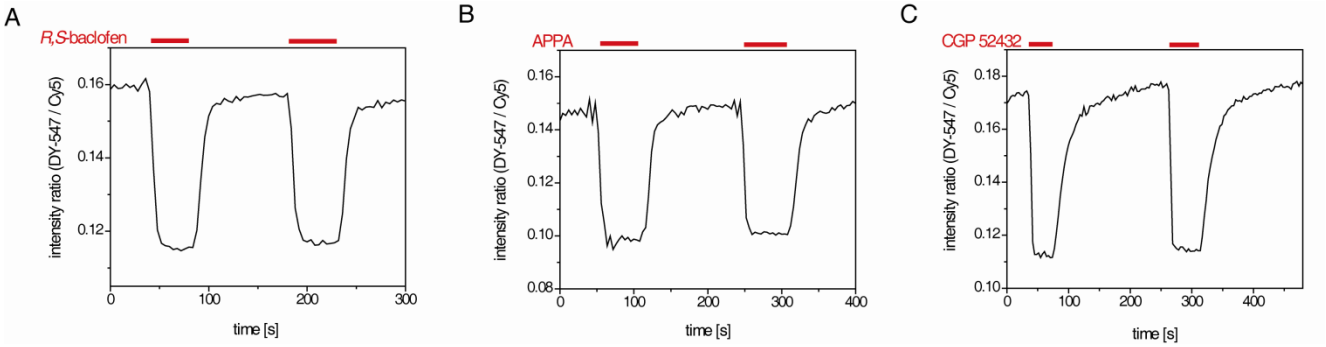

Figure S15. Perfusion experiments with different $GABA_B$ receptor ligands for opening GABA-Snifit on the surface of HEK 293 cells. GABA-Snifit was labeled with BC-DY-547 **25** and BG-Cy5-CGP **3** and perfused with (A) 1 mM *R,S*-baclofen, (B) 1 mM APPA and (C) 10 μM CGP 52432. Time course of the intensity ratio of donor emission vs acceptor emission is shown and the red bar indicates the time span of ligand perfusion.

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