Supporting Information

SPLICEIT Fluorescent Sensor for Integrating Dopamine Release with Cellular Resolution

Steven M. Havens, Brian Georgis, Jian Weng, Jessi Rodriguez, Hannah Rivett, Shadan Alrawi, Peng Li, and Wenjing Wang*

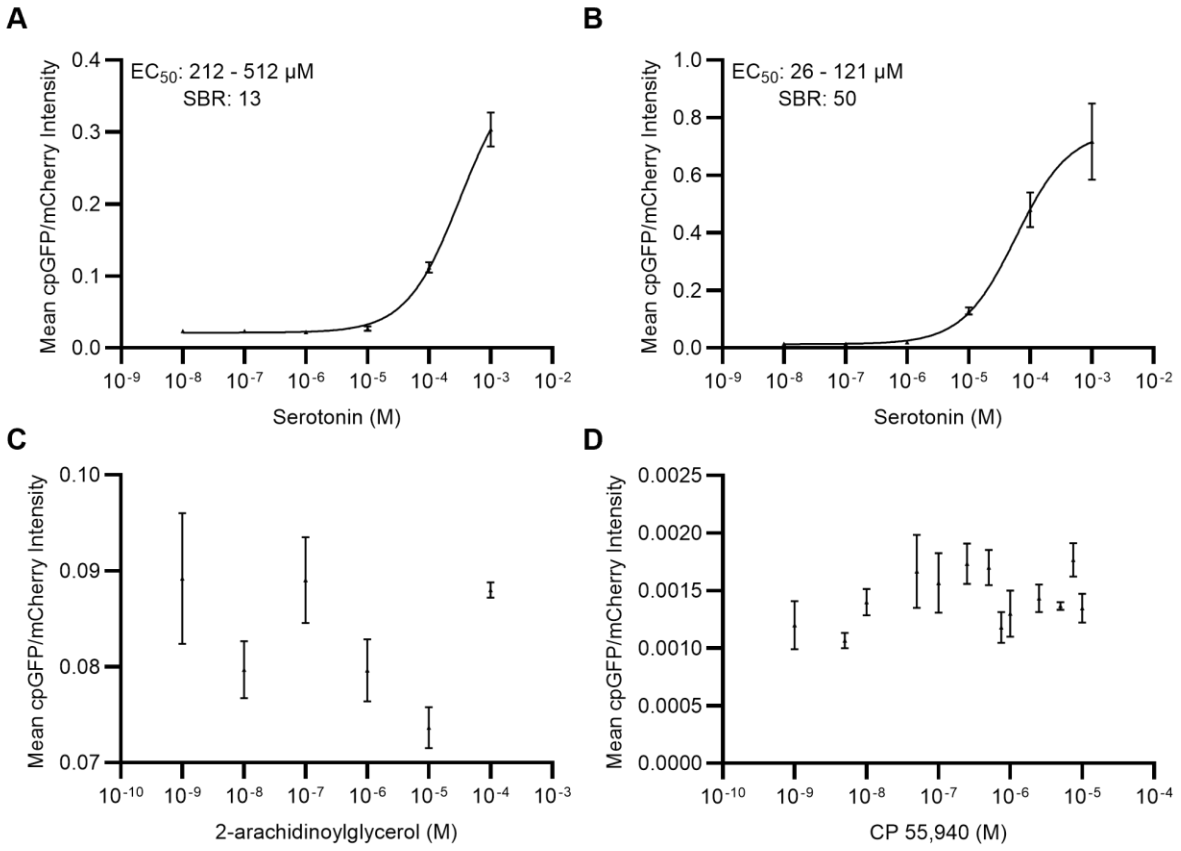


Figure SI1. Dose response curves for c5HT-1D, c5HT-1E, and cCB2. (A) c5HT-1D dose response curve against serotonin (B) c5HT-1E dose response curve against serotonin. (C) cCB2 dose response curve against the endocannabinoid 2-arachidoylglycerol (D) cCB2 dose response curve against the synthetic cannabinoid CP 55,940. Cells were transfected, stimulated, imaged, and analyzed as in Figure 6. SBR, ratio of the mean ratiometric response at the highest concentration of ligand to the ratiometric response in the absence of ligand.

Materials and Methods

Plasmids, cloning, and concentrated AAV1/2 virus production.

Constructs for HEK293T/17 cell expression were cloned in an ampicillin-resistant adeno-associated viral vector containing a CAG promoter. AAV-CAG-M-SPOTIT2 (addgene plasmid #191489) was used as the starting backbone vector, where standard cloning procedures such as NEB restriction enzyme digest, PCR amplification with Q5 polymerase, and Gibson assembly were used to replace the MOR sequence with chimeric receptor sequences synthesized as a gene block from TwistBio. Plasmids were transformed into XL1-blue competent cells using heat shock transformation. Full sequencing of these plasmids showed that a deletion in the 3'ITR region could lower viral titers, and the full DRD3-SPLICEIT2.0 sequence was re-cloned into an AAV vector custom ordered from VectorBuilder with a CAG promoter, wPRE3, and BGH pA prior to AAV production. Concentrated AAV1/2 virus was prepared as previously described.[30]

Structural prediction through AlphaFold2.

All structures were predicted through AlphaFold2,[19a] using a local Colabfold[19b] installed on the University of Michigan Great Lakes high-performance computing cluster. Five models were generated for each potential structure, utilizing mmseq2, an early stop tolerance of 0.05, and up to 20 recycles allowed, with the highest ranked model relaxed before use.

Binding energy calculation through Rosetta.

Each chimeric structure was predicted through AlphaFold2, as described above. Although present in the MOR/Nb39 crystal structure (PDB: 5C1M),[13] the MOR structure was also predicted through AlphaFold2 up to the palmitoylation site for consistent comparison to the chimeric structure models. Each structure was then aligned with the crystal structure of MOR in Pymol for approximate binding distance with Nb39, before performing local docking through RosettaDock to generate 1000 potential bound structures. The top 10% of total scoring designs for each pair were analyzed with Rosetta InterfaceAnalyzer and the top 10 designs taken for each pair.

HEK293T/17 cell culture.

HEK293T/17 cells were cultured in a 1:1 mixture of DMEM (Dublecco's Modified Eagle medium, GIBCO): MEM (Modified Eagle medium, GIBCO), supplemented to 10% FBS (Fetal Bovine Serum, Sigma), 1% penicillin/streptomycin (Gibco). Cells were lifted for passaging or transfection through incubation with 0.25% trypsin (Gibco) at 37 °C for 1-3 min. 48-well surface treated plates (Fisherbrand) were pretreated with 200 µL of 10 µg/mL human fibronectin (Millipore Sigma), or 20 µg/mL for 24-well glass bottom plates (Corning), for 10 min at 37 °C under 5% CO_2.

HEK293T/17 cell transfection with PEI MAX.

During plating, cells were transfected with a homemade PEI solution of 1 mg/mL PEI MAX (polyethyleneimine, Polysciences) in H_20 for all transfection experiments. For transfection per well of a 48-well plate, 150 ng DNA and 1 µL PEI max solution were incubated in 10 uL DMEM for 10 min at room temperature. After incubation, the solution was added to lifted cells prior to pipetting into each well. Cells were plated at a density to reach 90% confluence for stimulation 18-24 hours later.

HEK293T/17 cell fixation and immunostaining.

For each well, cell media was aspirated and a fixation solution of 4% formaldehyde in PBS (Thermo Scientific Chemicals) was added for incubation at room temperature for 15 minutes. The fixative was then removed, -20 °C methanol added, and the plate incubated at -20 °C for 5 min to permeabilize the cell membranes before washing with PBS three times. For immunostaining, mouse anti-FLAG (Sigma, F3165) and anti-mouse 647 (Life Technologies, A21235), or chicken anti-GFP (Abcam, ab13970) and anti-chicken 647 (Abcam, ab150171), antibodies were each diluted 1000x in PBS containing 1% BSA and 200 µL added per well. The cells were incubated with mouse anti-FLAG antibody at room temperature for 30 min on a rocker, before washing with PBS three times and incubating with anti-mouse 647 antibody for 20 min. The cells were then washed and fixed again, before adding pH 11 CAPS buffer for imaging. For ratiometric designs containing a fluorescent protein expression marker, immunostaining was not performed, unless otherwise noted, and pH 11 CAPS buffer was added after the first fixation step.

Confocal microscopy.

For all fixed cell imaging, except for the pH titration experiment, cells were imaged in a pH 11 CAPS buffer. Confocal imaging was performed with a Nikon inverted confocal microscope with a 10x air objective, 20x air objective, 60x oil immersion objective, Yokogawa CUX-X1 5000RPM spinning disk confocal head, Ti2-ND-P perfect focus system 4, and a 4-line laser source containing: 405 nm (100 mW), 488 nm (100 mW), 561 nm (100 mW), and 640 nm (75 mW) lasers. The acquisition time for all HEK293T/17 cell images was 500 ms with 30% laser power intensities, and images were taken with the 20x objective lens unless otherwise noted. All images were collected using Nikon NIS-Elements hardware control.

Analysis of confocal images.

NIS-Elements General Analysis 3 software was used to analyze all images. For all HEK293T/17 and neuronal culture images, 10 fields of view per well were imaged and three technical replicates were performed. For each experiment, the mean intensity for each emission wavelength in an area devoid of cells was used to determine the background for the respective excitation laser against the plate. A threshold was used at twice this level of background intensity to generate a mask of cell areas with real fluorescence for each emission wavelength and the mean intensity value and total object area were determined. The total object area was multiplied by the mean intensity after subtracting the background intensity to calculate the sum intensity value for each wavelength.

For HEK293T/17 and neuronal culture experiments, the sum intensity value of all fields of view within a well were averaged and then each technical replicate was plotted as one point in the graph.

For all comparison experiments, Prism GraphPad software was used to perform unpaired two-sided Student's *t* tests, or one-way ANOVA with comparison of the mean of each condition to the mean of the control condition, to calculate significance and plot data. Dose response curves are modeled in Prism with three parameter "[Agonist] vs. response" nonlinear fit, and EC_{50} displayed as 95% confidence interval. All images were included in analysis, except for images containing auto-fluorescent artifacts or lower than 50% cell density.

Background subtraction for immunostaining images.

Immunostaining images in Figures 1 and 4 are displayed with rollingball background correction, for visual clarity. This was performed in FIJI, utilizing a 75 pixel radius rollingball subtraction. Raw images were instead used for any analysis, as described above.

SPLICEIT stimulation in HEK cells.

HEK293T/17 cells were cultured in 48-well plates and transfected with AAV plasmids as described above. The cells were stimulated in triplicate the next day with described concentration of agonist, or the equivalent volume of fresh growth media for control conditions. After 18-24 hours, the cells were fixed, immunostained, imaged, and analyzed as described above.

SPLICEIT response in neuronal culture.

E18 whole rat brain (Transnetyx) was dissociated with papain (Transnetyx), and cultured in 48-well plates as previously described. Neuronal cultures were infected with concentrated DRD3-SPLICEIT2.0 AAV1/2 for six days, before stimulation with ligand or additional neuronal growth media for 24 hours. Cells fixed on ice, imaged, and analyzed as above.

SPLICEIT response in mouse brain.

All procedures were carried out in accordance with the animal care standards in NIH guidelines and approved by the University Committee on Use and Care of Animals at the University of Michigan. All mice strains utilized were C57BL/6J background and were maintained on a 12 hour light/dark cycle with ad libitum access to food and water. Both male and female mice were used.

Adult mice were anesthetized with isoflurane (4-5% for induction, 1.5% for maintenance) for stereotactic injection. Animals received 5 mg/kg of preemptive analgesic carprofen prior to placement in the stereotactic frame (David Kopf Instruments) and body temperature was maintained at 35 to 37 °C using a feedback-controlled heating pad (Physitemp). 1 µL of the AAV1/2-CAG-DRD3-SPLICEIT2.0 virus was injected into the lateral hypothalamic area (LHA): medial-lateral +/- 1.0 mm, anterior-posterior -1.2 mm, dorsal-ventral -5.2 mm to bregma, at a rate of 60 nL/min. After injection, the pipette was left in the brain for 10 min to allow for pressure to equalize.

Six days after injection of the viral vector, 1 µmol/kg of dopamine or saline (Hospira) was administered through intracerebroventricular injection at the following coordinates: medial-lateral +1.0 mm, anterior-posterior -0.5 mm, dorsal-ventral -2.5 mm to bregma. One day after drug administration, the mice were deeply anesthetized by isoflurane and transcardially perfused with 10 mL PBS, followed by 10 mL 4% paraformaldehyde (PFA) (Electron Microscopy Science). The brain tissue was removed and postfixed overnight at 4 °C in 4% PFA and then cryopreserved in 30% sucrose in PBS for 48 hours. The processed tissue was then embedded into optimum cutting temperature compound and sectioned into serial slices through the LHA region at 40 microns with a Leica cryostat CM3050 S cryostat.

Sections were then washed in 0.1% PBS Tween 20 three times before staining with DAPI dihydrochloride (Invitrogen) diluted 1:10,000 in PBS. After washing in 0.1% PBS Tween 20 three times, sections were imaged on a confocal microscope with pH 11 buffer.

SPLICEIT FACS analysis.

DRD3-SPLICEIT2.0 was transfected into HEK cells, allowing 24 hours of expression, and then stimulated with either 100 µM dopamine or fresh cell growth media. One day later, the cells were lifted with cell dissociation buffer (Gibco) and suspended in fresh cell growth media for FACS analysis with a BD Discovery S8 Cell Sorter. The mCherry fluorescence served as an expression marker, and the cpGFP fluorescence as the sensor activation signal. HEK cells expressing only cpGFP or mCherry were used as single-color controls, and untransfected HEK cells as a zero color control. Each population was analyzed for 10,000 events.

DRD3-SPLICEIT sequence(LUCY tag, FLAG tag, DRD3, MOR loops, c-terminal version 1.1, KOR tail leading into M-SPOTIT2[12]**).**

MRPQILLLLALLTLGLADYKDDDDAASMYALSYCALILAIVFGNGLVMYVIVRYTKMKTATNIYIFNLAVADLLVATLVMPWVVYLEVTGGV
WNFSRICCDVFVTLDVMMCTASILNLCAISVDRYIAVCHPVKALDFRTPRNAKIVNVCNWVLAFAVSCPLLFGFNTTGDPTVCSISNPDF
VIYSSVVSFYLPFGVTVLVYGLMILRLKSVRMLSGSKEKDRNLRRITRMVAIVLGAFIVCWLPFFLTHVLNTHCQTCHVSPELYSATTWL
GYVNSALNPVIYAFLDENFKRCFRCFPLKM…

Chimeric receptor sequences used for computational Nb39 binding analysis.

cβ_1AR:
GTWDAYAASQQWTAGMGLLMALIVLLIVAGNVLVMYVIVRYTKMKTATNIYIFNLALADLVMGLLVVPFGATIVVWGRWEYGSFFCELW
TSVDVLCVTASIETLCTMSVDRYIAVCHPVKALDFRTPRNAKIVVCTVWAISALVSFLPILMHWWRAESDEARRCYNDPKCCDFVTNRA
YAIASSVVSFYVPLCIMAFVYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFTLCWLPFFLANVVKAFHRELVPDRLFVFFNWL
GYANSAFNPVLY

cβ_2AR:
DVTQQRDEVWVVGMGIVMSLIVLAIVFGNVLVMYVIVRYTKMKTATNIYIFNLACADLVMGLAVVPFGAAHILMKMWTFGNFWCEFWT
SIDVLCVTASIETLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWIVSGLTSFLPIQMHWYRATHQEAINCYAEETCCDFFTNQAYAI

ASSIVSFYVPLVIMVFVYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFIVCWLPFFIVNIVHVIQDNLIRKEVYILLNWIGYVNS
GFNPVLYAFLDENFKRCF

cβ_3AR:
GVPWEAALAGALLALAVLATVGGNFLVMYVIVRYTKMKTATNIYIFNLALADLVMGLLVVPPAATLALTGHWPLGATGCELWTSVDVLCV
TASIETLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWVVSAAVSFAPIMSQWWRVGADAEAQRCHSNPRCCAFASNMPYVLLS
SSVSFYLPLLVMLFVYALMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFIVCWLPFFLANVLRALGGPSLVPGPAFLALNWLGYAN
SAFNPVLYAFLDENFKRCF

cα_2AR:
SLQVTLTLVCLAGLLMLLTVFGNVLVMYVIVRYTKMKTATNIYIFNLASADILVATLVIPFSLANEVMGYWYFGKAWCEIYLALDVLFCTSS
AWHLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWVISAVISFPPLISIEPAEPRCEINDQKWYVISSCIGSFFAPCLIITVCYGLMIL
RLKSVRMLSGSKEKDRNLRRITRMVLVVIGVFVVCWFPFFFTYTLTAVGCSVPRTLFKFFFWFGYCNSSLNPVLYAFLDENFKRCF

c5-HT$_{1A}$:
DVTVSYQVITSLLLGTLIFCAVLGNFLVMYVIVRYTKMKTATNIYIFNLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVLCCT
SSILHLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWLIGFLISIPPMLGWRTPEDRSDPDACTISKDHGYTIYSTFGAFYIPLLLML
VLYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFILCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVLYAFLDE
NFKRCF

c5-HT$_{1B}$:
DYIYQDSISLPWKVLLVMLLALITLATTLSNFLVMYVIVRYTKMKTATNIYIFNLAVTDLLVSILVMPISTMYTVTGRWTLGQVVCDFWLSS
DITCCTASILHLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWVFSISISLPPFFWRQAKAEEEVSECVVNTDHILYTVYSTVGAFIM
PVLIITVCYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFIVCWLPFFIISLVMPICKDACWFHLAIFDFFTWLGYLNSLINPVLYA
FLDENFKRCF

c5-HT$_{1D}$:
AWDPRTLQALKISLAVVLSVITLATVLSNFLVMYVIVRYTKMKTATNIYIFNLATTDLLVSILVMPISIAYTITHTWNFGQILCDIWLSSDITCC
TASILHLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWAISICISIPPLFWRQAKAQEEMSDCLVNTSQISYTIYSTCGAFIMPVLIITV
CYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFIVCWLPFFVVSLVLPICRDSCWIHPALFDFFTWLGYLNSLINPVLYAFLDEN
FKRCF

c5-HT$_{1E}$:
PKTITEKMLICMTLVVITTLTTLLNLLVMYVIVRYTKMKTATNIYIFNLAVTDLLVAVLVMPLSIIYIVMDRWKLGYFLCEVWLSVDMTCCTCS
ILHLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWTISIFISMPPLFWRSHRRLSPPPSQCTIQHDHVIYTIYSTLGAFIMPVLIITVCY
GLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFILSWLPFFIKELIVGLSIYTVSSEVADFLTWLGYVNSLINPVLYAFLDENFKRCF

c5-HT$_{1F}$:
LTSEELLNRMPSKILVSLTLSGLALMTTTINSLVMYVIVRYTKMKTATNIYIFNLAVTDFLVAVLVMPFSIVYIVRESWIMGQVVCDIWLSVDI
TCCTCSIFTLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWIISVFISMPPLFWRHQGTSRDDECIIKHDHIVSTIYSTFGAFYIPLLII
TVCYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFVICWLPFFVKELVVNVCDKCKISEEMSNFLAWLGYLNSLINPVLYAFLD
ENFKRCF

cCB2:
MKEYMILSSGQQIAVAVLCTLMGLLSALENMAVLYIIVRYTKMKTATNIYIFNLAGADFLASVIFACNFVIFHVFHGVDSNAIFLLKIGSVTM
TFTASVGSLLLTAVDRYIAVCHPVKALDFRTPRNAKIVNVCNWILSALISYLPLMGWTCCPSPCSELFPLIPNDYLLGWLLFIAILFSGIIITV
CYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVLLICWFPALALMGHSLVTTLSDQVKEAFAFCSMLCLVNSMVNPVLYAFLDE
NFKRCF

cChrm2:
TSPYKTFEVVFIVLVAGSLSLVTIIGNILVMYVIVRYTKMKTATNIYIFNLACADLIIGVFSMNLYTLYTVIGYWPLGPVVCDLWLALDYYNMF
TSIFTLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCNWILSFILWAPAILFWQFIVGVRTVEDGECYIQFFSNAAVTFGTAIAAFYLPVII
MTVCYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFIITWAPYNVMVLINTFCAPCIPNTVWTIGYWLCYINSTINPVLYAFLDE
NFKRCF