A genetically encoded single-wavelength sensor for imaging cytosolic and cell surface ATP

[1]Mark Lobas, [1]Jun Nagai, [1]Mira T. Kronschläger, [3]Philip Borden, [3]Jonathan S. Marvin, [3]Loren L. Looger & [1,2]Baljit S. Khakh

[1]Departments of Physiology and [2]Neurobiology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA 90095-1751, USA

Janelia Research Campus, 19700 Helix Drive, Ashburn Virginia 20147

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Materials and methods

Cloning

The QUEEN-7μ coding sequence was purchased as a gBlock Gene Fragment from Integrated DNA Technologies with a *BglII* restriction site preceding the coding sequence of QUEEN-7μ and a *PstI* restriction site following the coding sequence. This gBlock was cloned into an in-house modified pRSET-A vector (ThermoFisher: V35120), termed pHHM.pHHM contained an N-terminal His6Gly tag utilized for purification, followed by a hemaglutinin (HA) epitope tag and a multiple cloning site before the gene of interest, as well as a myc tag at the C-terminus of the gene. This vector was used for screening, because it has the HA and myc tags present in pDisplay (ThermoFisher: V66020), which is the vector we planned to use for surface expression in mammalian cells[1,2]. Cloning from pHHM to pDisplay or into pDisplayMini, an in-house vector that lacks the N-terminal HA epitope present in pDisplay, was achieved via a restriction digest of both the iATPSnFR and pDisplay plasmids with *BglII* and *PstI*. Cloning into the pZAC-AAV-GfaABC$_1$D was done using PCR amplification to add an *NheI* restriction site to the N-terminus of iATPSnFR and a *NotI* restriction site to the C-terminus of iATPSnFR coding sequence. Cloning was performed using routine subcloning techniques. Note that the mRuby we used was mRuby3 minus MDELYK from the C-terminus.

Protein expression and purification

Vectors containing iATPSnFR variants were transformed into *E. coli* BL21(DE3) cells (lacking pLysS). Proteins were expressed by growing the *E. coli* in liquid auto-induction media [3] supplemented with 100 μg/ml ampicillin at 30°C for at least 18 hours. Following expression, the bacteria were harvested at 1500 g for 20 min. The pellet was resuspended in PBS with 1 M NaCl and frozen. Protein was extracted using freeze-thaw lysis and sonication. Upon thawing, 1 μM PMSF was added to the solution before sonication. Following sonication, the crude lysate was initially clarified at 4000 g for 20 min. The supernatant was then isolated and further clarified by centrifugation at 35,000 g for 45 min. Proteins were purified using Ni-NTA agarose affinity

chromatography (5 ml column). The iATPSnFR proteins were eluted with a 120 ml gradient from 0 to 200 mM imidazole at 2 ml/min. A blue light box was used to determine which fractions were fluorescent. The fluorescent fractions were combined and concentrated down to 2-3 ml. The protein was then dialyzed with a minimum of 1 l PBS for at least 24 hours. Auto-induction media consisted of ~900 ml of ZY media (1% tryptone and 0.5% yeast extract) with 20 ml of 50x M (25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl and 5 mM Na_2SO_4), 20 ml of 50x 5052 (0.5% glycerol, 0.05% glucose, 0.2% α-lactose), 2 ml 1 M $MgSO_4$, and 200 µL of 1000x trace metals (50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM $CoCl_2$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM Na_2MoO_4, 2 mM Na_2SeO_3, 2 mM H_3BO_3).

Protein titrations with ATP

After protein expression, 100 µl of protein was transferred to flat-bottom black 96-well plates (Greiner). Initial fluorescence was assayed in a Safire2 plate-reading fluorimeter with a stacker (Tecan). Excitation/emission wavelengths were 485/515 nm. Gain was between 80-120 V and bandpass filters were 5 nm. Fluorescence was assayed again after the addition of 10 µl ATP solution containing between 315 nM-100 mM ATP for final ATP concentrations of 31.5 nM-10 mM. After a second reading, dF/F was calculated.

For experiments performed with clarified HEK-293 lysate, 10 cm dishes were transfected with the cytoplasmic gene of interest. 24 hours following transfection the cells were washed twice with PBS. A third wash of PBS was used to detach the cells. The cells were then pelleted and protein were extracted by freeze/thaw lysis. The pH of the lysate was adjusted appropriately and the lysate was concentrated to 1.5 mL using an Amicon centrifugal filter. After the lysate was concentrated does response curves were generated at above.

For determining the pH dependence of fluorescence, buffered solutions of specific pH were made (Hydrion Chemvelope) and concentrated, purified protein was added to a final concentration of 0.2 µM. Fluorescence of 100 µL triplicate samples was measured as above. ATP was added (from a 200 mM stock of Mg•ATP) to a final concentration of 6 mM and fluorescence was measured again.

Stopped-flow kinetics

Equal volumes of 0.2 µM purified protein in Mg-containing buffer (10 mM HEPES, 145 mM NaCl, 2.5 mM KCl, 10 mM Glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) and ATP-containing buffer (various concentrations) were mixed in an SX-20 stopped-flow fluorimeter (Applied Photophysics), with data points collected every millisecond. Excitation was from a 490 nm LED. Emission was detected with a photomultiplier tube and a 510 nm long pass filter. Mixing reactions were performed in quintuplicate and averaged. Data was fit to an equation containing two exponential rises:

$$F = F_0 + \Delta F_{max1}*(1 - \exp(-k_{obs1}*\text{time})) + \Delta F_{max2}*(1 - \exp(-k_{obs2}*\text{time}))$$

Mutagenesis

Linker variants and point mutations were generated by site-directed Kunkel mutagenesis[4] with a uracil template. Uracil template generation: CJ236 cells were transformed with promising vectors and grown on plates containing chloramphenicol overnight at 37°C.The following day a 1 ml culture of LB with only ampicillin was inoculated with a colony and grown at 37°C with 215 rpm until cloudy – usually about 6 hours. Once the culture was cloudy, 1 µl of M13KO7

helper phage (NEB: N0315S) was added. After incubating for an additional hour, the culture was expanded to a 40 ml culture of LB with ampicillin (no chloramphenicol) in a 250 ml flask and incubated overnight at 37°C with 215 rpm shaking. The following day, the bacteria were pelleted at 4000 g for 20 min at 4°C. The supernatant was transferred to a new 50 ml Falcon tube containing 10 ml of 20% PEG in 2.5 M NaCl. After thorough mixing, the solution was incubated on ice for 15 min. The phage was then pelleted at 4000 g for 20 min. The supernatant was removed and the phage was resuspended in 2 ml of PBS. The 2 ml of PBS were divided into two different Eppendorf tubes and centrifuged at maximum speed for 5 min to pellet the remaining bacteria. The supernatant was transferred to two new Eppendorf tubes containing 300 µl of 20% PEG in 2.5 M NaCl. After vortexing, the solution was incubated on ice for 10 min. The phage was pelleted at maximum speed for 5 min. The supernatant was removed. Another quick spin was done to remove any residual supernatant. The phage was then resuspended in 1 ml of PBS. This was centrifuged at maximum speed for 5 min and the supernatant was transferred to a new Eppendorf tube. The uracil template was then purified using the Qiagen QIAprep Spin M13 Kit (Cat. No. 27704) using the manufacturer's protocol. 11 µl of MP buffer was added to the 1 ml PBS/template solution. After vortexing, the mixture was incubated at room temperature for 5 min. This mixture was then applied to the QIAprep spin column and centrifuged at 7000 rpm. The flowthrough was discarded. After the entire mixture was applied, 700 µl of PB buffer was applied to the column. After one minute of incubation, the column was centrifuged at 7000 rpm for 15 seconds. This was repeated before washing the column three times with 700 µl of PE buffer, and again centrifuging at 7000 rpm for 15 seconds. After ensuring the column was dry, 25 µL of EB was applied to the column which was then centrifuged at 7000 rpm. This was done three times for a total of 75 µl of EB. DNA quality was checked via using agarose gel electrophoresis. High quality preparations run as a single band about 1/3 the size of the plasmid. Kinasing primers: degenerate primers were designed to mutate two amino acids at a time. This was done by flanking the degenerate/mutated sequence of DNA with about 15 bases of unmutated DNA on either side. The primers were phosphorylated using T4 kinase. The reaction was performed in an Eppendorf tube containing 18 µl H_2O, 3 µl T4 Kinase buffer, 1 µl 10 µM ATP, 1 µl T4 Kinase (NEB M0201L), and 7 µl 100 µM of the oligo template. The reaction was then incubated for 30 min at 37°C. Annealing primers: to determine the optimal template to primer ratio, four annealing reactions were initially set up using four different primer dilutions of the kinased primer; 1:1, 1:5, 1:10 and 1:100. 1 µl of diluted primer, 1 µl of template and 1 µl of 10x T4 DNA ligase buffer, and 7 µl of nuclease-free water was combined in a PCR tube. The reaction was heated to 95°C for two minutes using a PCR machine. The reaction was then slowly brought back to 25°C using 5°C steps for 30 seconds each. Polymerizing DNA: To polymerize the DNA, 9.5 µl water, 1.5 µl 10x T4 DNA ligase buffer, 1 µl 25 mM dNTPs, 1 µl 10 mM ATP, 1 µl T7 polymerase unmodified (NEB: M0274L), 1 µl T4 ligase (NEB: M0202M), and the entire 10 µl annealing reaction was added to a PCR tube and was allowed to incubate at room temperature for an hour. This reaction was transformed directly into *E. coli* BL21(DE3) cells (lacking pLysS) and plated on 24 cm x 24 cm LB-Agar plates with 100 µg/ml ampicillin. After determining the optimal concentration of kinased oligos, that dilution can be used for subsequent reactions with that particular uracil template.

High-throughput screening

Individual colonies were selected using a QPix2^{xt} colony-picking robot (Genetix). Colonies were deposited into deep 96-well plates filled with 800 µl auto-induction media with

100 µg/ml ampicillin. Cultures were grown with vigorous (300 RPM) shaking overnight at 30°C. The bacteria were harvested by centrifugation (3000g) and washed three times with 900 µl PBS before freezing. Protein was extracted by freeze-thaw lysis with 900 µl phosphate-buffered saline. Rapid shaking was used to homogenize the solution. Crude lysate was clarified by centrifugation at 4000 g for 30 min. 100 µl of clarified lysate was transferred to flat-bottom black 96-well plates (Greiner) using a liquid-handling robot. Initial fluorescence was assayed in a Safire2 plate-reading fluorimeter with stacker (Tecan). Excitation/emission wavelengths were 485/515 nm. Gain was between 80-120 V and bandpass filters were 5 nm. Fluorescence was assayed again after the addition of 10 µl 100 mM ATP (final ATP concentration 10 mM). After calculating dF/F, new uracil templates were made for promising candidates and were subject to subsequent rounds of mutagenesis. The most promising sensors were sequenced and cloned into either pDisplay or pDisplayMini for further screening in mammalian cells.

Transfection of HEK-293 and U373 cells

HEK-293 and U373MG cells were maintained in DMEM/F-12, supplemented with GlutaMAX (Gibco: 10565042), 10% FBS (Gibco 10082147), and 5 ml Penicillin-Streptomycin (10,000 U/ml) (Gibco: 15140-122) at 37ºC with 5% CO_2. Cells were trypsinized once they reached confluence and were diluted at a ratio of 1:10. HEK-293 and U373MG cells were transfected with either Lipofectamine 2000 (ThermoFisher 11668027) or Effectene (Qiagen ID: 301425) following the manufacturer's guidelines. The following is the procedure for the transfection of a single well of a 6-well dish. Lipofectamine 2000 transfections: 100 µl of OptiMEM (Gibco: 31985062) was placed in two tubes. 2.5 µg of DNA was added to one tube and 6.25 µl of Lipofectamine 2000 was added to the other. The content of the two tubes was combined, thoroughly mixed, and incubated for 20 min at room temperature before adding the mixture to cells that were 70-90% confluent with fresh media. The media was changed 6-8 hours later. Effectene transfections: 0.4 µg DNA was added to a tube, followed by 8 µl of enhancer. 100 µl of pipetted Buffer EC was used to mix the contents by pipetting. The mixture was then incubated for 2 min before thoroughly mixing 8 µl of Effectene into the mixture. The solution was then incubated for 10 min. 600 µl of fresh media was added before adding the mixture to cells that were 70-90% confluent with fresh media. The media was changed the following day.

Lysis of HEK-293 cells

HEK-293 cells were washed with cold PBS three times prior to lysing cells. Cells were lysed on ice using RIPA buffer: 150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1% triton, 0.1% SDS, and 0.5% sodium deoxycholate at pH 8 supplemented with protease inhibitors (ThermoFisher). Following the 10 min incubation on ice, the mixture was centrifuged at max speed for 10 min. The supernatant was taken and diluted with an equal volume of 2x Laemmli buffer for western blot analysis.

Western Blotting

Precast gels were purchased from BioRad and were run for 110 min at 100 V. 1x running buffer was composed of 3.0 g Tris base, 14.4 g glycine and 1.0 g SDS in 1 l water. Gels were transferred to nitrocellulose for 1 hour at 100 V in 1x transfer buffer. 10x transfer buffer was composed of 30.0 g Tris and 144.0 g glycine in 1 l water. 1x Transfer buffer is composed of 10% 10x running buffer, 20% methanol, and 70% water. Following transfer, blots were blocked with 5% bovine serum albumin (BSA) (Sigma: A9647-100G) in TBS with 0.05% Tween-20 (TBST)

(Sigma: P2287-100ML) for 1 hour. Antibodies were made with 5% BSA in TBST. Primary antibodies: GAPDH 1:5000 mouse (Thermofisher MA5-15738). GFP 1:1000 rabbit (Invitrogen A11122). Secondary antibodies: IRDye 680RD anti-mouse 1:20,000 (Licor 926-68170). IRDye 800CW anti-rabbit 1:20,000 (Licor 925-68024). Primary antibodies were incubated overnight at 4°C. Following three washes with TBST of at least 10 min each, secondary antibodies were incubated for 1 h at room temperature. Three washes with TBST of at least 10 min each were sufficient to remove the secondary antibodies. Membranes were imaged using a Licor Odyssey infrared imager.

AAV2/5 microinjections *in vivo*

Animals were initially deeply anesthetized in a chamber with 5% isofluorane. The head of the animal was then shaved to remove the fur around the surgical site. The animal had its head fixed in a stereotaxic setup using ear bars. A nose cone was placed over the nose for continuous administration of isofluorane throughout the procedure. There was continuous monitoring of the animal's breathing during the procedure and anesthesia was adjusted accordingly (0.5-3%). The animal was administered buprenorphine (0.1 mg/kg Buprenex) and the surgical site was cleaned three times with 10% povidone iodine and 70% ethanol prior to the surgery. A small incision was made to expose the skull. A ~2 mm hole was drilled into the skull with a high-speed drill at 2 mm posterior to bregma and 1.5 mm left of bregma for hippocampus CA1, and at 0.8 mm anterior to bregma and 2 mm left of bregma and for dorsolateral striatum. After removing a bit of the skull, saline was applied to the surgical site to remove any debris. The CA1 region of the hippocampus was injected with 1 µl of virus (titer 1.5E13) over the course of 5 min. The stereotaxic coordinates used are 2 mm posterior from bregma, 1.5 mm left of the midline, and 1.6 mm from the pial surface for hippocampus CA1, and 0.8 mm anterior to bregma, 2 mm left of the midline, and 2.4 mm from the pial surface for dorsolateral striatum. Once the injection was complete, the needle remained in place for 10 min to allow the virus to diffuse into the tissue. The needle was gradually removed over the course of 2 min. The surgical site was sutured. Animals recovered in a cage placed on a heating pad. Animals were given 0.1 mg/kg Buprenex the following day for pain. Animals were monitored after recovery to make sure they were healthy, and they received trimethoprim sulfamethoxiazole in their food for a week following the operation. Targeting of MSNs was achieved using a FLEX-dependent iATPSnFR$^{1.0}$ AAV with D1 and D2 selective Cre mouse lines[5].

Imaging

Live cell imaging: HEK-293 cells were transiently transfected with a vector encoding the IgK leader sequence followed by iATPSnFR, GGTGGS linker, a myc tag and the transmembrane domain of the PDGF receptor. After 24 hours of incubation at 37°C with 5% CO_2 in 6-well plates, cells were trypsinized and plated onto coverslips that were pre-coated with poly-lysine. After another 24 hour incubation at 37°C with 5% CO_2, the coverslips were mounted to a perfusion chamber. Images were acquired with an Olympus IXS 71 using an UplansApo 40x 0.90 NA oil immersion lens or an Olympus BX51WI confocal microscope with a LUMPlanFL/IR 40x 0.80 NA water immersion lens. Once mounted in the perfusion chamber, a constant flow of fresh HEK buffer was perfused over the cells before and after the application of ligands. ATP solutions were made up fresh daily. The steady-state fluorescence level was calculated as an average of the images acquired in the first 30 seconds. Fast-solution changes were achieved using SF-77B perfusion fast step controller from Warner Instruments. A 3-

barrelled glass square (Warner cat 64-0119) was placed as close to the cell as possible for imaging purposes. Steps of 800 µm were used to move the barrels onto and off of the cells. On and off rates were determined using pClamp 10.6. HEK buffer: 140 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose adjusted to pH 7.4 with 5 mM NaOH.

Immunohistochemistry (IHC): Animals were deeply anesthetized with pentobarbital (i.p.) prior to transcardial perfusion. Once no reflexes were observed, the chest cavity was opened. The left ventricle was impaled with a needle connected to a perfusion pump and the right was punctured to permit transcardial perfusion. The animals were perfused with at least 30 ml of ice cold PBS to completely flush the blood, followed by at least 30 ml of 10% buffered formalin to fix the tissues. Tissues were gently dissected out and fixed overnight in 10% buffered formalin at 4^{o}C. Tissues were then cryoprotected with 30% sucrose in PBS before being frozen in O.C.T. compound (Fisher Scientific: 23-730-571). 40 µm sections were harvested using a cryostat microtome (Leica). Sections were harvested into PBS and blocked with 10% normal goat serum and 0.5% Triton-X 100 in PBS for 1 hour at room temperature prior to staining. Primary antibody was applied overnight at 4^{o}C in PBS with 10% normal goat serum and 0.5% Triton-X 100. Sections were washed three times with PBS following primary application. The secondary antibody was incubated at room temperature for 1 hour followed by three washes with PBS before mounting on to slides. Sections were allowed to completely dry on the slide before applying fluoromount-G (SouthernBiotech: 0100-01). Primary antibodies: S100β rabbit 1:1000 (Abcam ab41548). NeuN rabbit 1:1000 (Cell Signaling D3S3I). GFP chicken 1:1000 (Abcam ab13970). Secondary antibodies: goat anti-chicken IgG Alexa 488 1:2000 (Invitrogen A11039). Goat anti-rabbit Alexa 546 1:2000 (Invitrogen A11010). A 488 nm argon laser adjusted to 5-10% maximum output intensity was used for imaging Alexa 488 dyes. A 543 HeNe laser adjusted to 20-30% maximum output intensity was used for imaging Alexa 546 dyes.

Puffer application of ATP: A Model P-97 pipette puller from Sutter Instrument Co. was used to pull capillaries purchased from VWR (1B100-4). Patch pipettes were filled with 3 mM ATP and Alexa fluor 568 hydrazide (1:200) (ThermoFisher Scientific: A10441). Micromanipulators were used to place the tip of the pipette 10-20 µm above the cell being imaged. A constant flow of fresh buffer perfused the imaging chamber at all times. After 30 seconds of imaging, a 5 second puff of 3 mM ATP was administered via the PicoSpritzer III from Intracel.

Live slice imaging: Animals were deeply anesthetized with isofluorane prior to decapitation. The brain was removed as gently and as quickly as possible, and then cooled as quickly as possible. The brain was mounted with superglue to a cutting platform and was bathed in ice-cold hippocampal slicing buffer while 300 µm sections were harvested using a vibratome. For spinal cord slices, a laminectomy was performed. The dura mater and all roots were removed and the spinal cord was subsequently mounted using superglue on an agarose block for transversal sectioning (300 µm). These sections were then placed in 32^{o}C hippocampal imaging buffer. All solutions were bubbled with 95% O_2 and 5% CO_2 for at least 30 min prior to slicing. Following sectioning, the slices were placed in a perfusion chamber for imaging with a constant flow of 2-3 ml/min of hippocampal imaging buffer. Images were taken using an Olympus BX51WI confocal microscope with a LUMPlanFL/IR 40x 0.80 NA water immersion lens. A 488 nm argon laser adjusted to 10-15% maximum output intensity for imaging GFP. Hippocampal slicing buffer: 87 mM NaCl, 25 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 75 mM sucrose,

7 mM $MgCl_2$, and 0.5 mM $CaCl_2$. Hippocampal imaging buffer: 126 mM NaCl, 25 mM $NaHCO_3$, 10 mM glucose, 2.5 mM KCl, 1.24 mM NaH_2PO_4, 1.30 mM $MgCl_2$, and 2.4 mM $CaCl_2$.

Data analyses and statistics

Data from every experiment represents a minimum of n animals. In all the imaging experiments, n was > 3 mice. Sample sizes were not calculated *a priori*. In the figure panels, n is defined as the number of cells. For AAV injections, mice were randomly assigned to each experimental group. No experimental data points were excluded. Statistical tests were run in Origin 9. Summary data are presented as mean ± s.e.m. Note that in some of the graphs, the bars representing the s.e.m. are smaller than the symbols used to represent the mean. For each set of data to be compared, we determined within Origin or GraphPad InStat whether the data were normally distributed or not using the Shapiro-Wilk test. If they were normally distributed, we used parametric tests. If the data were not normally distributed, we used non-parametric tests. Paired and unpaired Student's two-tailed *t* tests (as appropriate) and two tailed Mann–Whitney tests were used for most statistical analyses with significance declared at $P < 0.05$. When a statistical test was employed that was not a Student's *t* test or a Mann-Whitney test for a specific case, then it is stated as such in the text. If the *P* value was greater than 0.05, then it is stated as $P > 0.05$. Throughout the manuscript, the results of statistical tests (*P* values and n numbers) are reported in full on the figure panels to save space in the main body of the manuscript. However, where appropriate, key statistics are also reported in the text. Images were analyzed using ImageJ. The GECIquant program[6] plugin was used to subtract the background from images as well as to select regions of interest. Mean grey value measurements were imported into Excel for dF/F analysis. The final results were graphed in OriginPro 2016 and the figures assembled in CorelDraw 2017.

Ig-kappa leader sequence | GFP | PDGFR 513-561
epsilon subunit | GGTGGS linker | **Mutations**
Linkers 1 and 2 | Myc epitope | **ATP-binding pocket**

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                    M  E  T  D  T  L  L  L  W  V  L  L  L  W  V  P  G  S  T  G
iATPSnFR1.0  1      ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGT  60
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  1      ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGT  60

                    D  R  S  M  K  T  I  H  V  S  V  V  T  P  D  G  P  V  Y  E
iATPSnFR1.0  61     GACAGATCTATGAAGACTATTCACGTGAGTGTCGTAACTCCCGACGGGCCTGTATATGAG  120
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  61     GACAGATCTATGAAGACTATTCACGTGAGTGTCGTAACTCCCGACGGGCCTGTATATGAG  120

                    D  D  V  E  M  V  S  V  K  A  K  S  G  E  L  G  I  L  P  G
iATPSnFR1.0  121    GATGACGTTGAAATGGTGAGCGTCAAAGCAAAAAGTGGCGAGCTCGGTATTCTCCCAGGC  180
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  121    GATGACGTTGAAATGGTGAGCGTCAAAGCAAAAAGTGGCGAGCTCGGTATTCTCCCAGGC  180

                    H  I  P  L  K  A  P  L  E  I  S  A  A  R  L  K  K  G  G  K
iATPSnFR1.0  181    CACATTCCCTTGAAAGCTCCCCTAGAGATCAGTGCCGCACGCCTGAAGAAAGGGGGCAAA  240
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  181    CACATTCCCTTGAAAGCTCCCCTAGAGATCAGTGCCGCACGCCTGAAGAAAGGGGGCAAA  240

                    T  Q  Y  I  A  V  S  G  G  N  L  E  V  R  P  D  K  V  T  I
iATPSnFR1.0  241    ACACAGTATATCGCTGTGTCAGGCGGCAACCTCGAAGTGCGGCCTGACAAGGTGACCATC  300
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  241    ACACAGTATATCGCTGTGTCAGGCGGCAACCTCGAAGTGCGGCCTGACAAGGTGACCATC  300

                    Y  A  Q  A  A  E  R  A  E  D  I  D  V  L  R  A  K  A  A  K
iATPSnFR1.0  301    TATGCCCAGGCAGCCGAGAGGGCTGAGGATATCGATGTCCTGCGCGCCAAGGCCGCCAAG  360
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  301    TATGCCCAGGCAGCCGAGAGGGCTGAGGATATCGATGTCCTGCGCGCCAAGAAAGCCAAG  360
                                                                        K

                       E  R  A  E  R  R  L  Q  S  Q  V  L  S  H  N  V  Y  I  T  A
iATPSnFR1.0  361    GAGAGAGCCGAGCGCCGACTCCAATCACAGGTCCTGAGCCACAACGTCTATATCACCGCC  420
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  361    GAGAGAGCCGAGCGCCGACTCCAATCACAGGTCCTGAGCCACAACGTCTATATCACCGCC  420

                    D  K  Q  K  N  G  I  K  A  N  F  K  I  R  H  N  V  E  D  G
iATPSnFR1.0  421    GACAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACGTGGAGGACGGC  480
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  421    GACAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACGTGGAGGACGGC  480

                    S  M  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L
iATPSnFR1.0  481    AGCATGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG  540
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  481    AGCATGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG  540

                    L  P  D  N  H  Y  L  S  T  Q  S  V  L  S  K  D  P  N  E  K
iATPSnFR1.0  541    CTGCCCGACAACCACTACCTGAGCACCCAGTCCGTGCTGAGCAAAGACCCTAACGAGAAG  600
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  541    CTGCCCGACAACCACTACCTGAGCACCCAGTCCGTGCTGAGCAAAGACCCTAACGAGAAG  600


                    R  D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  L  G  M  D
iATPSnFR1.0  601    CGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGAC  660
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
iATPSnFR1.1  601    CGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGAC  660
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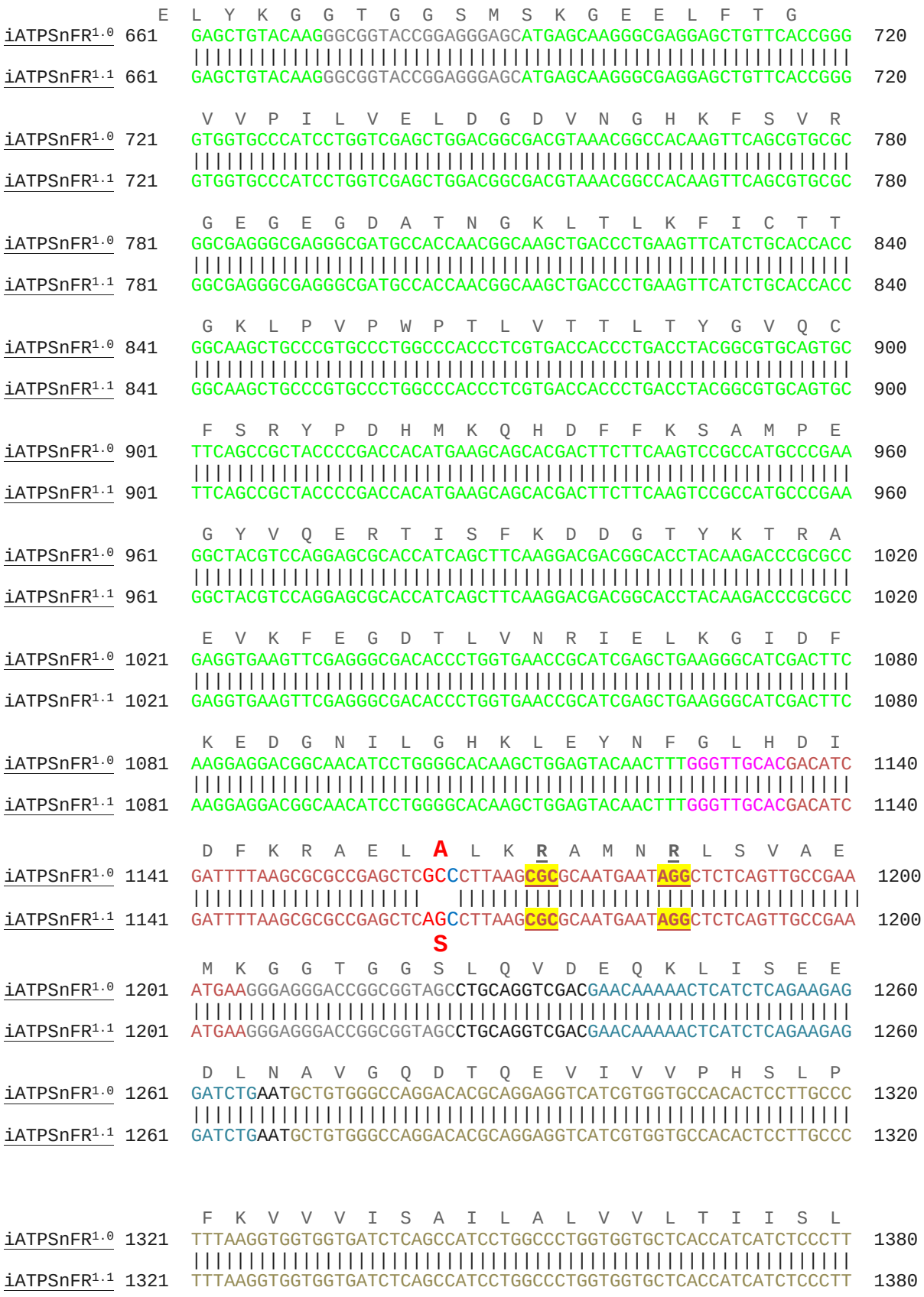
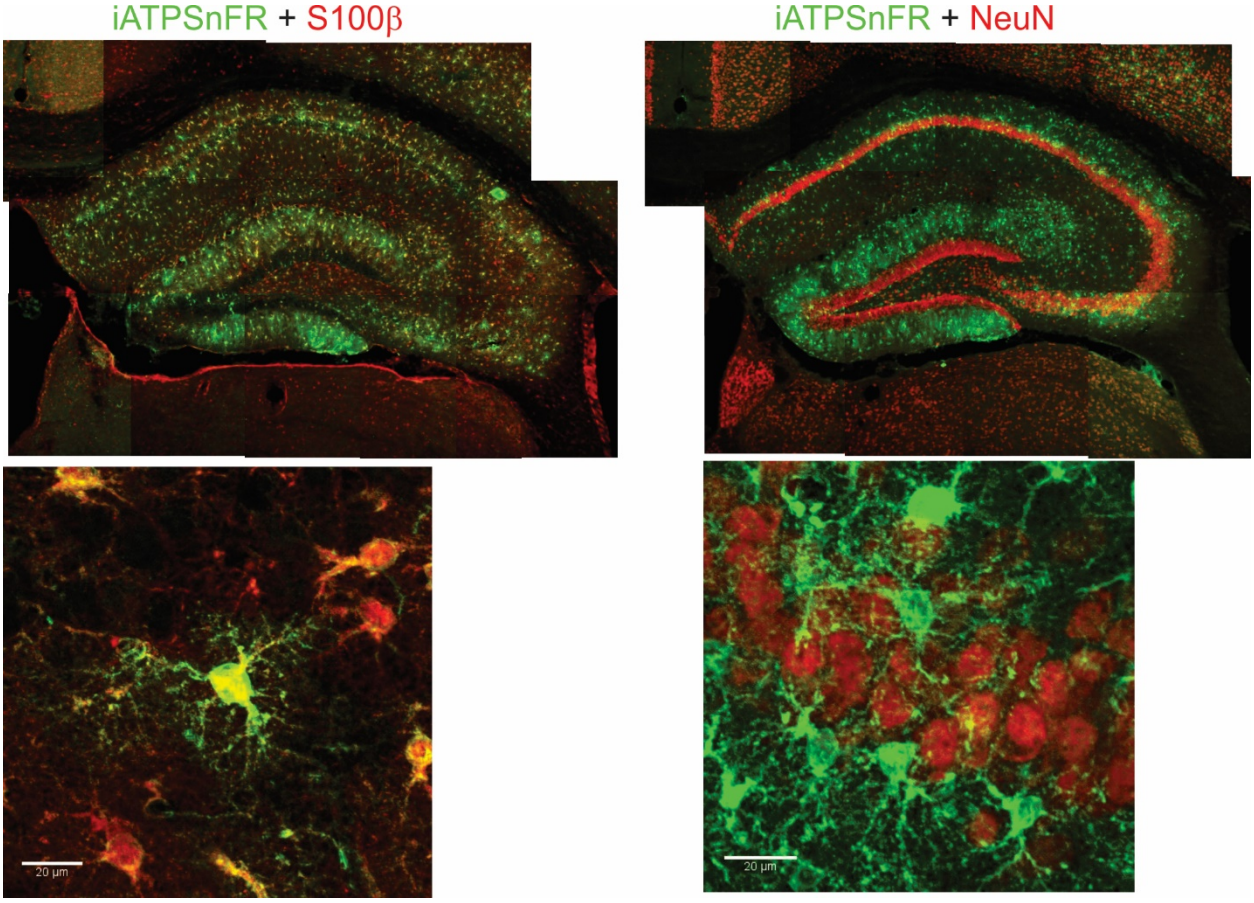
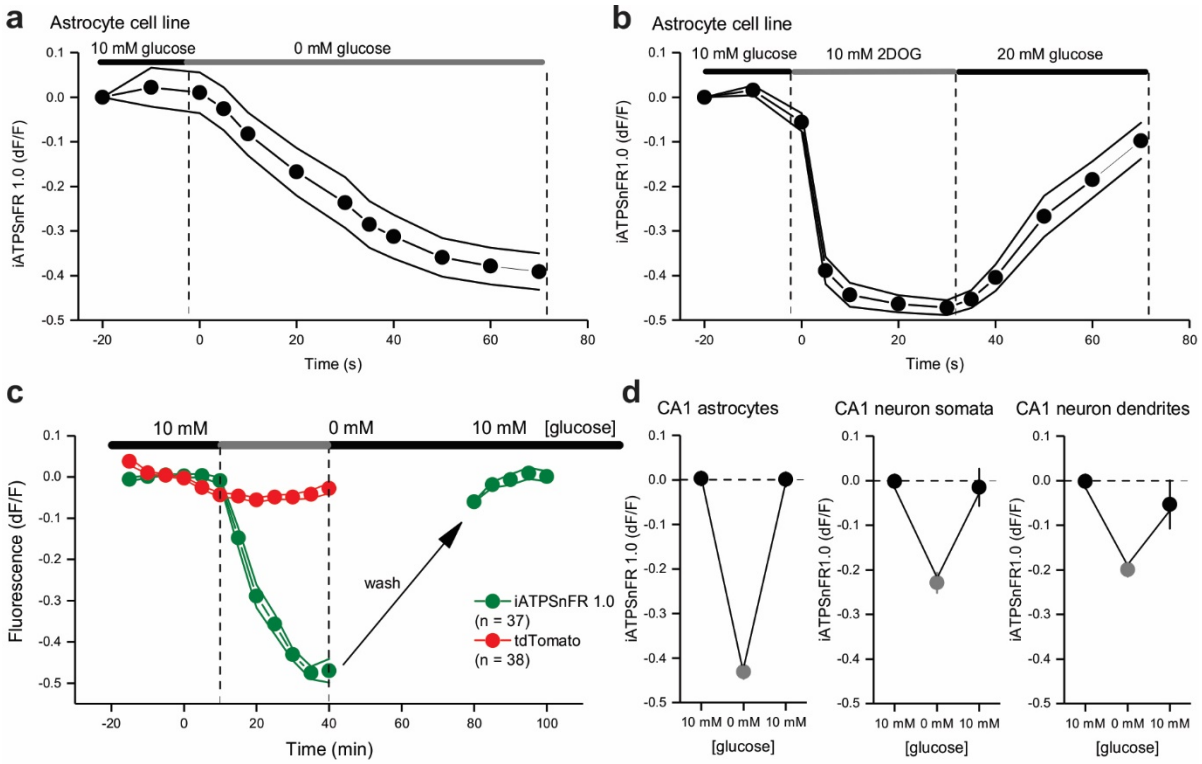

E L Y K G G T G G S M S K G E E L F T G
iATPSnFR1.0 661 GAGCTGTACAAGGGCGGTACCGGAGGGAGCATGAGCAAGGGCGAGGAGCTGTTCACCGGG 720
iATPSnFR1.1 661 GAGCTGTACAAGGGCGGTACCGGAGGGAGCATGAGCAAGGGCGAGGAGCTGTTCACCGGG 720
V V P I L V E L D G D V N G H K F S V R
iATPSnFR1.0 721 GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGCGC 780
iATPSnFR1.1 721 GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGCGC 780
G E G E G D A T N G K L T L K F I C T T
iATPSnFR1.0 781 GGCGAGGGCGAGGGCGATGCCACCAACGGCAAGCTGACCCTGAAGTTCATCTGCACCACC 840
iATPSnFR1.1 781 GGCGAGGGCGAGGGCGATGCCACCAACGGCAAGCTGACCCTGAAGTTCATCTGCACCACC 840
G K L P V P W P T L V T T L T Y G V Q C
iATPSnFR1.0 841 GGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGC 900
iATPSnFR1.1 841 GGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGC 900
F S R Y P D H M K Q H D F F K S A M P E
iATPSnFR1.0 901 TTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAA 960
iATPSnFR1.1 901 TTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAA 960
G Y V Q E R T I S F K D D G T Y K T R A
iATPSnFR1.0 961 GGCTACGTCCAGGAGCGCACCATCAGCTTCAAGGACGACGGCACCTACAAGACCCGCGCC 1020
iATPSnFR1.1 961 GGCTACGTCCAGGAGCGCACCATCAGCTTCAAGGACGACGGCACCTACAAGACCCGCGCC 1020
E V K F E G D T L V N R I E L K G I D F
iATPSnFR1.0 1021 GAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTC 1080
iATPSnFR1.1 1021 GAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTC 1080
K E D G N I L G H K L E Y N F G L H D I
iATPSnFR1.0 1081 AAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTTTGGGTTGCACGACATC 1140
iATPSnFR1.1 1081 AAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTTTGGGTTGCACGACATC 1140
D F K R A E L A L K R A M N R L S V A E
iATPSnFR1.0 1141 GATTTTAAGCGCGCCGAGCTCGCCCTTAAGCGCGCAATGAATAGGCTCTCAGTTGCCGAA 1200
iATPSnFR1.1 1141 GATTTTAAGCGCGCCGAGCTCAGCCTTAAGCGCGCAATGAATAGGCTCTCAGTTGCCGAA 1200
S
M K G G T G G S L Q V D E Q K L I S E E
iATPSnFR1.0 1201 ATGAAGGGAGGGACCGGCGGTAGCCTGCAGGTCGACGAACAAAAACTCATCTCAGAAGAG 1260
iATPSnFR1.1 1201 ATGAAGGGAGGGACCGGCGGTAGCCTGCAGGTCGACGAACAAAAACTCATCTCAGAAGAG 1260
D L N A V G Q D T Q E V I V V P H S L P
iATPSnFR1.0 1261 GATCTGAATGCTGTGGGCCAGGACACGCAGGAGGTCATCGTGGTGCCACACTCCTTGCCC 1320
iATPSnFR1.1 1261 GATCTGAATGCTGTGGGCCAGGACACGCAGGAGGTCATCGTGGTGCCACACTCCTTGCCC 1320
F K V V V I S A I L A L V V L T I I S L
iATPSnFR1.0 1321 TTTAAGGTGGTGGTGATCTCAGCCATCCTGGCCCTGGTGGTGCTCACCATCATCTCCCTT 1380
iATPSnFR1.1 1321 TTTAAGGTGGTGGTGATCTCAGCCATCCTGGCCCTGGTGGTGCTCACCATCATCTCCCTT 1380

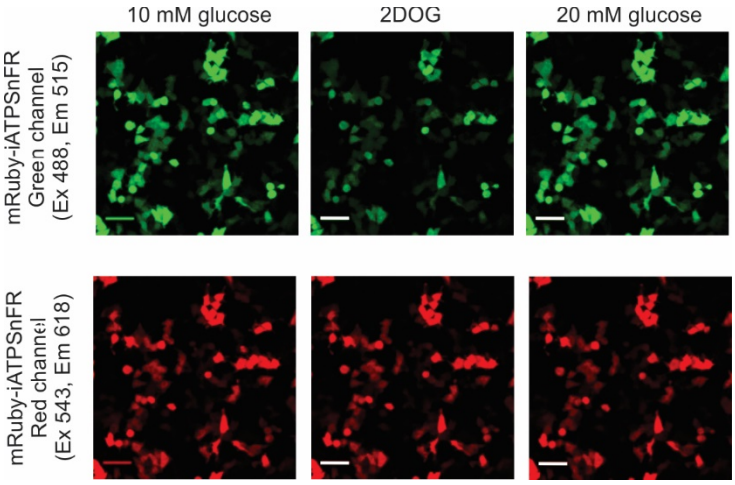
Supplementary Figure 1: Sequence alignment of the iATPSnFR$^{1.0}$ and iATPSnFR$^{1.1}$ cDNAs. The sequences were aligned with Vector NTI and have been deposited at Addgene with the plasmids (see Supp Table 1 for plasmid IDs).



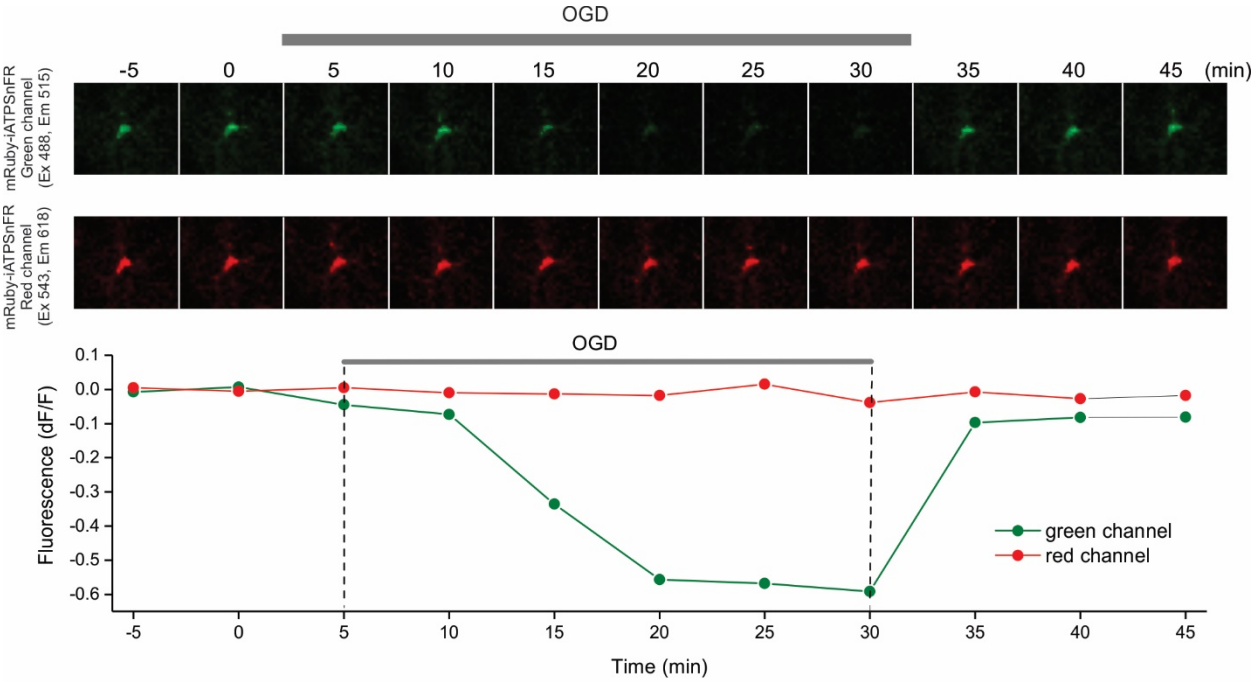
Supplementary Figure 2: iATPSnFR$^{1.0}$ was expressed in S100β-positive astrocytes, but not neurons following *in vivo* expression with AAV2/5 and the GfaABC$_1$D promoter. The representative images are from 4 similar mice. Note also that iATPSnFR$^{1.0}$ was found throughout the astrocyte's processes.



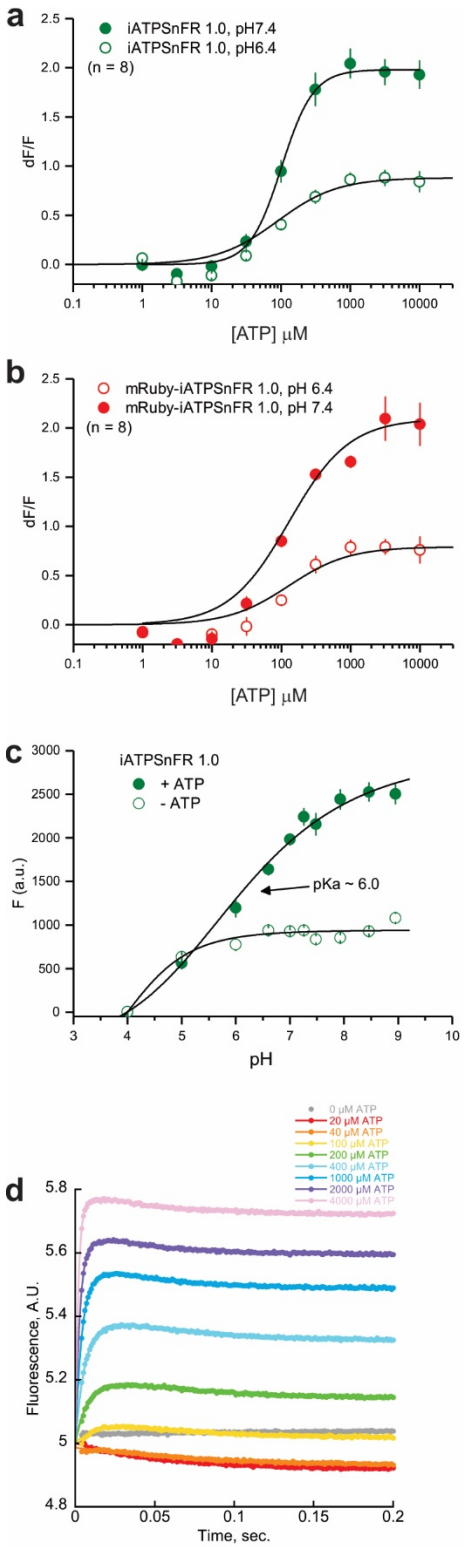
Supplementary Figure 3: Responses of cytosolic iATPSnFR$^{1.0}$. **(a)** Drop in fluorescence in U373MG astroglia during 0 mM glucose applications. **(b)** Drop in fluorescence in U373Mg astroglia during 2DOG to reduce glycolysis. **(c)** As in (a), but for expression of iATPSnFR$^{1.0}$ in hippocampal astrocytes with AAV. **(d)** Summary from experiments such as those in (c) for hippocampal astrocytes, CA1 pyramidal neurons, and dendrites.



Supplementary Figure 4: Representative images for mRuby-iATPSnFR[1.0] expressed in HEK-293 cells and subjected to 2DOG (glycolysis inhibitor).



Supplementary Figure 5: Representative traces and images of an astrocyte expressing mRuby-iATPSnFR$^{1.0}$ in hippocampal brain slices following *in vivo* delivery with AAV2/5. Oxygen-glucose deprivation caused clear decreases in iATPSnFR$^{1.0}$ fluorescence, but left mRuby fluorescence intact.



Supplementary Figure 6: (a-b) ATP concentration-effect curves for iATPSnFR$^{1.0}$ and mRuby-iATPSnFR$^{1.0}$ at pH 7.4 and 6.4. **(c)** iATPSnFR$^{1.0}$ fluorescence as a function of pH for ATP bound and unbound states (6 mM ATP). **(d)** Stop flow kinetics for iATPSnFR$^{1.0}$. Experiments shown here were with iATPSnFR$^{1.0}$ in solution from HEK-293 cell lysates (a,b) and bacteria (c,d).

Plasmid name	Expresses in	Vector	Addgene ID
HHM-iATPSnFR$^{1.0}$	Bacteria	HHM modified pRSET	102546
HHM-iATPSnFR$^{1.1}$	Bacteria	HHM modified pRSET	102547
pm-iATPSnFR$^{1.0}$	Mammalian cells	pDisplay	102548
pm-iATPSnFR$^{1.1}$	Mammalian cells	pDisplay	102549
cyto-iATPSnFR$^{1.0}$	Mammalian cells	modified pDisplay	102550
cyto-iATPSnFR$^{1.0}$-mScarlet	Mammalian cells	modified pDisplay	
cyto-iATPSnFR$^{1.0}$-P2A-mScarlet	Mammalian cells	modified pDisplay	
cyto-Ruby3-iATPSnFR$^{1.0}$	Mammalian cells	modified pDisplay	102551
cyto-LSSmOrange-iATP	Mammalian cells	modified pDisplay	
GfaABC$_1$D-pm-iATPSnFR$^{1.0}$	Mouse astrocytes	pZac	102552
GfaABC$_1$D-pm-iATPSnFR$^{1.1}$	Mouse astrocytes	pZac	
GfaABC$_1$D-cyto-iATPSnFR$^{1.0}$	Mouse astrocytes	pZac	102553
GfaABC$_1$D-cyto-iATPSnFR$^{1.0}$-mScarlet	Mouse astrocytes	pZac	
GfaABC$_1$D-cyto-Ruby3-iATPSnFR$^{1.0}$	Mouse astrocytes	pZac	102554
GfaABC$_1$D-cyto-LSSmOrange-iATPSnFR$^{1.0}$	Mouse astrocytes	pZac	
GfaABC$_1$D-cyto-iATPSnFR$^{1.0}$-P2A-mScarlet	Mouse astrocytes	pZac	
Synapsin-pm-iATPSnFR$^{1.0}$	Mouse neurons	pSynapsin	102555
Synapsin-pm-iATPSnFR$^{1.1}$	Mouse neurons	pSynapsin	
Synapsin-cyto-iATPSnFR$^{1.0}$	Mouse neurons	pSynapsin	102556
Synapsin-cyto-iATPSnFR$^{1.0}$-mScarlet	Mouse neurons	pSynapsin	
Synapsin-cyto-Ruby3-iATPSnFR$^{1.0}$	Mouse neurons	pSynapsin	102557
Synapsin-cyto-LSSmOrange-iATPSnFR$^{1.0}$	Mouse neurons	pSynapsin	
Synapsin-cyto-iATPSnFR$^{1.0}$-P2A-mScarlet	Mouse neurons	pSynapsin	

Supplementary Table 1. Details of the new plasmids generated in this study along with the Addgene IDs.

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