SUPPORTING INFORMATION

Synaptic adhesion protein ELFN1 is a selective allosteric modulator of group III metabotropic glutamate receptors *in trans*

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Supplementary Materials and Methods

cDNA Constructs

mGluR constructs were obtained from Missouri S&T cDNA Resource Center (GRM2 Cat#GRM2000000, GRM6 Cat#GRM6000000, GRM7 Cat#GRM7000001, GRM8 Cat#GRM8000000) and Bloomsburg University cDNA Resource Center (GRM4 Cat#GRM4000000). mGluR5B was a gift from Dr. Stephen M Strittmatter (Departments of Neurology and of Neuroscience, Yale University School of Medicine, New Haven, CT 06510, USA). ELFN1-myc and ELFN1 Ecto-Fc have been previously described (Cao et al., 2015). ELFN1 EctoΔCT-Fc, EctoΔFN3-Fc, EctoΔNT-Fc, and EctoΔSP-pHLsec-Fc were designed by PCR amplifying products into a modified secreted mammalian expression vector termed pHL-FcHis (1) (a gift from E. Yvonne Jones, Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK.). This vector has secretion signal sequence, site for insert, 3C protease cleavage site followed by the human IgGγ1 hinge and Fc regions, and a KHis6 tag. ELFN1 EctoΔCT-Fc and EctoΔFN3-Fc were cloned with native secretion signal peptide and EctoΔNT-Fc and EctoΔSP-pHLsec-Fc were cloned in frame with vector pHLsec secretion signal sequence. EctoΔSP-Caspr2 was generated by PCR amplifying the predicted extracellular domains of ELFN1 (aa26-418), and subcloning the product into p3CPro (a gift from Dr. Davide Comoletti, Robert Wood Johnson Medical School and Dept. of Neuroscience & Cell Biology, The Child Health Institute of NJ, New Brunswick, NJ, USA) between and in frame with the Caspr2 signal peptide and human Fc. ELFN1ΔFN3-myc and Caspr2-ELFN1-myc were designed with the same modifications as ecto-domain constructs while including intact transmembrane and carboxyl terminal regions in pcDNA3.1

expression vector. All constructs were confirmed by DNA sequencing. -22F cAMP pGloSensor construct was attained from Promega. Nluc-EPAC-VV and G protein coupling constructs were described previously (2).

Cell Culture

HEK 293T/17 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, minimum essential medium nonessential amino acids (Life Technologies), 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator containing 5% CO_2. For each experiment, cells were seeded in 6-cm dishes without penicillin and streptomycin and transfected the following day at ~70% confluency. Cells were transiently transfected with the appropriate expression constructs using Lipofectamine® LTX with Plus™ Reagent (specific details provided in each experimental section). The empty vector pcDNA3.1 was used to normalize the amount of DNA in each transfection.

Co-immunoprecipitation

For each experiment, three populations of HEK293 cells were transiently transfected with 1 μg of the following: (1) mGluR-expressing cells, (2) pcDNA3.1-expressing (control) cells, and (3) ELFN1-myc-expressing cells. Cells were lysed using 1% Triton-X lysis buffer and combined as described in Figure 1. Lysate combinations were incubated with Protein G Beads for ~1 hour at 4ºC and washed 3 times with centrifugation and fresh lysis buffer. Proteins were eluted using β-mercaptoethanol-containing sample buffer and SDS-PAGE was performed followed by Western

blotting for inputs and immunoprecipitated proteins. Each co-immunoprecipitation experiment was performed a minimum of 3 times per receptor.

Protein G pull-down

For each experiment, three populations of HEK293 cells were transiently transfected: (1) mGluR-expressing cells, (2) Fc-expressing (control) cells, and (3) Fc-tagged ELFN1 ecto domain-expressing cells. Fc and Fc-tagged ELFN1 constructs were transfected at varying levels to normalize level of expression. The following day, media was removed and replaced with OPTI-MEM to capture secreted constructs for up to 72 hours. Media was collected for secreted constructs and mGluR-expressing cells were lysed using 1% Triton-X lysis buffer and combined as described in Figure 1 & 2. Media/lysate combinations were incubated alone for ~1 hour at 4ºC. Protein G Beads were added for an additional hour and then washed 3 times with centrifugation and fresh lysis buffer. Proteins were eluted using β-mercaptoethanol-containing sample buffer and SDS-PAGE was performed followed by Western blotting for inputs and immunoprecipitated proteins. Protein G pull-down experiments recapitulating co-immunoprecipitation data were performed once. Protein G pull-down experiments using various mutant ecto-domains were performed a minimum of 3 times per mutant construct.

Bioinformatics

Multiple sequence alignment was performed with 165 sequences corresponding to ELFN1 across species identified by a similarity search using the blastp program against the Refseq_protein database (protein sequences from NCBI Reference Sequence project). Parameters used included at least 90% coverage and 55% homology compared to the ELFN1 mouse sequence

(NP_780731.1). Amino acids that were identical in all 165 sequences were highlighted (orange) to recognize the most conserved regions of ELFN1 protein.

Ecto-domain purification and N-terminal sequencing

Expression and purification of constructs were performed as previously described (3); however, Fc-tagged recombinant protein was transiently transfected in HEK293S cells (in suspension) to maximize yield. The secreted protein in the media was cleared by centrifugation to remove cells. Secreted protein from clarified supernatant was affinity purified by protein G column. The sample was prepared from purified protein for N-terminal sequencing as per protocol of two separate sequencing facilities: Tufts Core Facility, Physiology Dept., Stearns Bldg. Rm. 808, 136 Harrison Ave, Boston, MA 02111 (617) 636-2422 and The Protein Facility of Iowa State University. The first 5 amino acids were identified by Edman degradation using 5 rounds of cycle.

Trans-cellular GPCR complex signaling platform.

Cells were separated into two general categories: (1) cells expressing GPCR and biosensor construct(s), and (2) cells expressing empty pcDNA3.1 vector (Control) or ELFN1-myc (ELFN1) without any biosensor. 0.42µg of GPCR was transfected compared to 5 µg of vector/ELFN1; however, 3µg of mGluR4 was used in -22F pGloSensor experiments to effectively suppress FSK-mediated cAMP accumulation. ~24 hours after transfection, cells were lifted with PBS and centrifuged at 500 x g for 5 minutes at room temperature. PBS was removed and replaced with Tyrode's solution (137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 0.2mM Na_2HPO_4, 12mM $NaHCO_3$, 5.5mM D-glucose), with exception of EPAC cAMP sensor

experiments that used PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose. Pelleted cells were dissociated via pipetting and centrifuged at 500 x g for 5 minutes at room temperature. Buffer was removed and replaced with fresh corresponding buffer. GPCR/biosensor cells were plated at ~100 000 cells/well in white 96 well plates and control/ELFN1 cells were co-cultured with these cells at 4:1 for ~2 hours. Experiments were performed in suspension with control/ELFN1 constructs and cells outnumbering GPCR constructs and cells to maximize GPCR saturation. Importantly, each biological replicate represents measurements derived from the same homogenous cell population run in parallel and are identical with exception to experimental Control or ELFN1 co-culture conditions.

Measurements of cAMP dynamics

-22F pGloSensor Format. Cells were prepared using the transcellular GPCR complex signaling platform with 2.52µg of Promega -22F cAMP pGloSensor in mGluR-expressing cells. Cells were incubated in Promega GLO reagent during ~2-hour co-culture period with control/ELFN1-cells. Cells were pre-treated with various concentrations of L-glutamic acid (L-Glu) or L-2-amino-4-phosphonobutyric acid (L-AP4) and baseline luminescence was read on a BMG LabTech PHERAstar FSX. After 5 minutes, cells were treated with 1µM of forskolin (FSK) and readings were continued for up to 20 minutes. mGluR activation was calculated as the decrease in FSK-mediated luminescence amplitude. For constitutive activity readings (Supplementary Figure 1), the slope of FSK-mediated increase in luminescence in the absence of agonist was used to normalize for extrinsic factors between experimental days, where a decrease in slope was attributed to increased constitutive mGluR activity.

BRET-based EPAC format. Cells were prepared using the transcellular GPCR complex signaling platform with 0.84μg of Nluc-EPAC-VV in mGluR6-expressing cells. Cells were incubated for ~2-hour with either control or ELFN1-cells prior to stimulation. For desensitization experiments, $G\alpha_q$/PKC-dependent group III mGluR desensitization was facilitated with 1 μM adenosine-5'-triphosphate (ATP) pre-treatment to pre-activate endogenous $G\alpha_q$-coupled purinergic receptors (4, 5). Cells were treated with 300 μM L-Glu 5, 30, or 60 minutes and luminescence and fluorescence were read on a BMG LabTech PHERAstar FSX to attain a baseline bioluminescence resonance energy transfer (BRET) ratio (535nm/460nm). Cells were then treated with 1μM of forskolin and readings were continued for up to 20 minutes. mGluR activation was calculated as the inhibition of forskolin-mediated decrease in BRET ratio. For desensitization experiments, mGluR activation was normalized to 5 minutes L-Glu treatment to compare desensitization rates.

Biotinylation Experiments for GPCR membrane expression

Cells were prepared using the same transcellular GPCR complex signaling platform however no biosensor was present. Cells were incubated for ~2-hour in 60mm dishes with either control or ELFN1-cells. Cells were put on ice for 15 minutes and surface proteins were labelled with 1mg/mL Sulfo-NHS-SS-biotin for 1 hour at 4ºC. Cells were then washed and the biotinylation of surface proteins reaction was quenched with cold 100mM glycine for 30 minutes. Cells were washed and then lysed using 1% Triton-X lysis buffer. Insoluble material was pelleted and lysate supernatant was incubated with Streptavidin Sepharose beads for 1 hour at 4ºC. Samples were centrifuged and supernatant was removed, followed by 2 further centrifuge washes. Biotinylated membrane proteins were eluted from the beads using β-mercaptoethanol-containing sample

buffer and SDS-PAGE and Western blotting was performed with indicated antibodies. mGluR6 band intensities were quantified using ImageJ 1.50i.

Real-time kinetic BRET assays for G protein activation

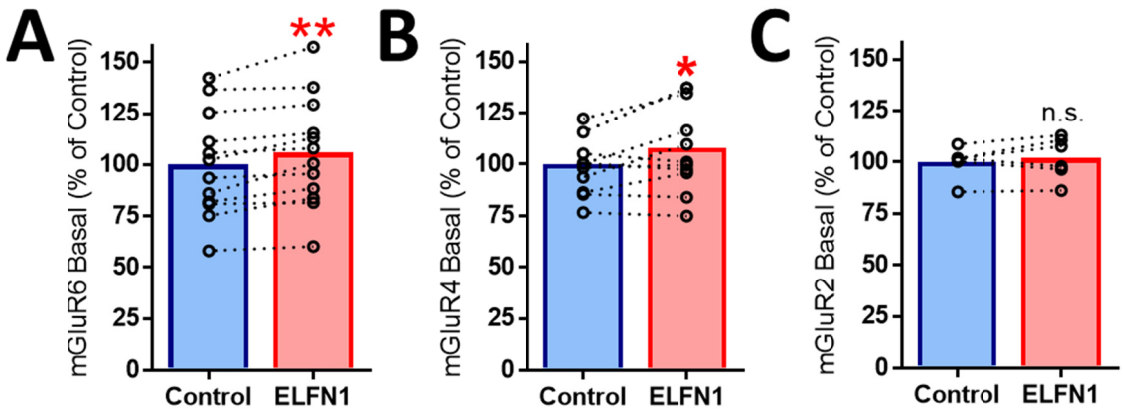
Cells were prepared using the transcellular GPCR complex signaling platform with 0.84µg of $G\alpha_{i/o}$, 0.42µg of Venus 156-239-$G\beta_1$, 0.42µg of Venus 1-155-$G\gamma_2$, and 0.42µg of masGRK3ct-Nluc (2) in mGluR-expressing cells. Cells were incubated for ~2-hour with either control or ELFN1-cells prior to stimulation. Cells were injected with Promega Nano-Glo® Luciferase Assay Reagent to 0.067% and luminescence and fluorescence were read on a BMG LabTech PHERAstar FSX to attain baseline BRET ratio (535nm/460nm). Upon stabilization of baseline BRET ratios, cells were injected with 300µM L-Glu and continually read every 60ms for up to 20s. mGluR6-$G\alpha_o$ experiments utilized 100µM L-Glu to prevent assay saturation. mGluR activation was calculated as the change in BRET ratio (ΔBRET) following agonist treatment. Rate of activation ($1/\tau$) was calculated using ClampFit 10.3 software to fit exponential curves and calculate τ. Integrated activation constant was calculated by E_{max}/τ.

Statistics

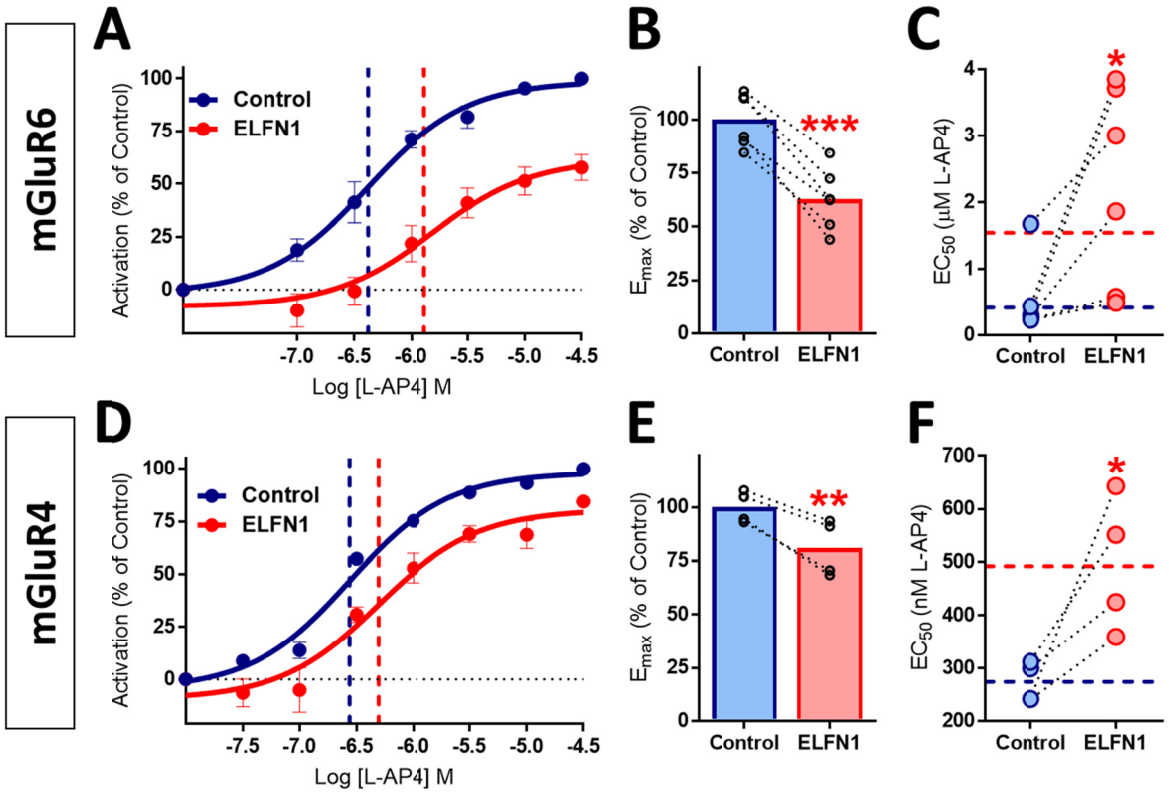
Statistics were performed using GraphPad Prism 7.02 software. Because of the identical cell populations in each biological replicate and the parallel experimental design, all statistical tests utilized two-tailed, paired t-tests (related samples t tests), with exception of Supplemental Figure 3 which utilized a repeated-measures (matched) one-way ANOVA with Tukey's multiple comparison test and Figure 4F which utilized a repeated-measures (matched) two-way ANOVA with Sidak's multiple comparisons tests. Statistical significance on bar graphs and dot plots was

expressed as * $P<0.05$, ** $P<0.01$, and *** $P<0.001$. Not significant (n.s.) was indicated where appropriate. Data were expressed as percentage of the mean of all controls with all data points provided representing n and dotted lines representing identical cell populations run in parallel.

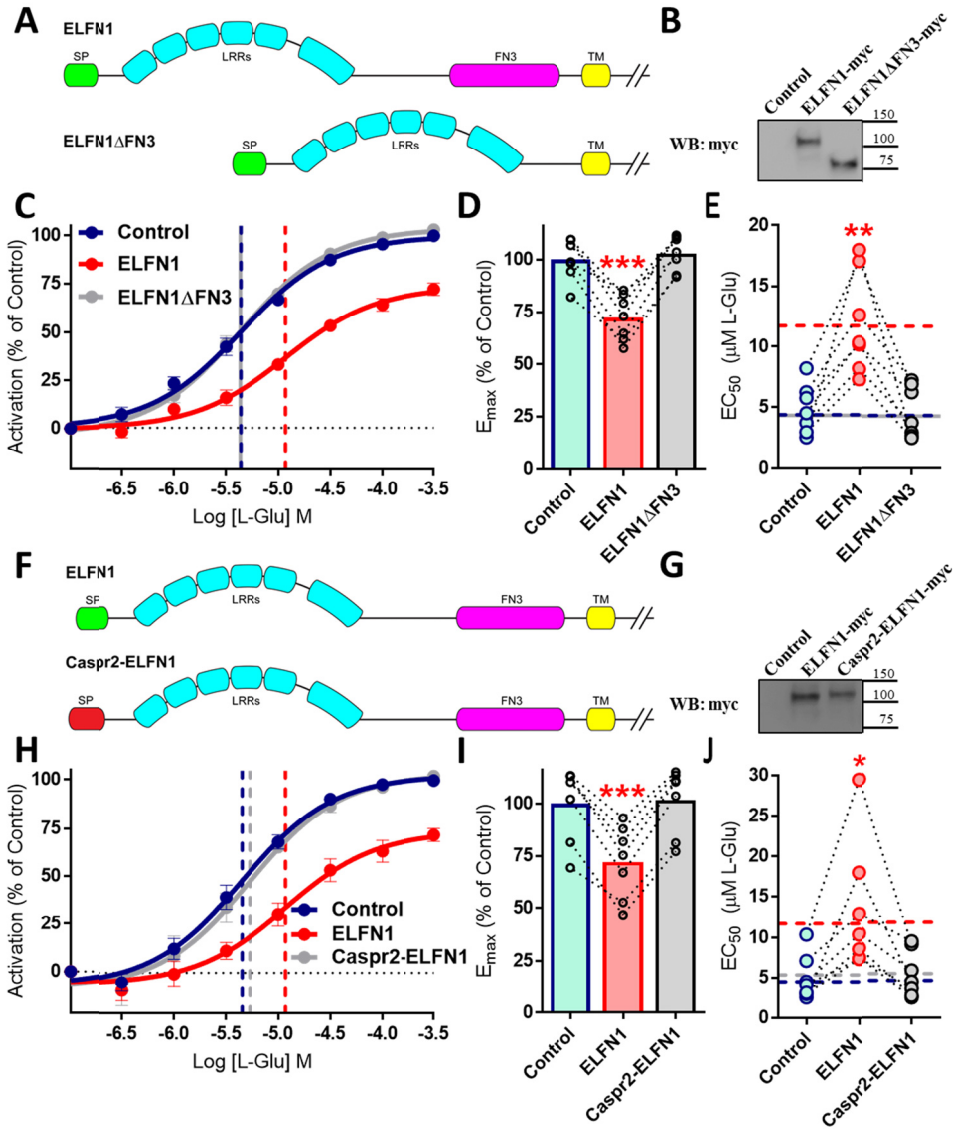
Supplementary Figures



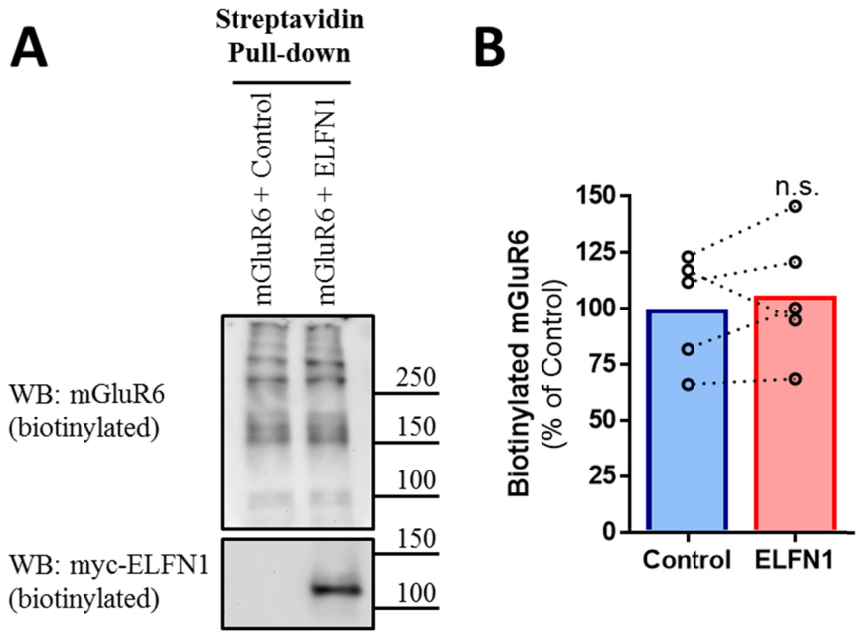
Supplementary Figure 1. ELFN1 interactions promote constitutive activity of Group III mGluRs. Trans-cellular GPCR signaling assay utilizing -22F cAMP pGloSensor in mGluR-expressing cells exposed to Control or ELFN1-expressing cells that lack biosensor (see Figure 3). **(A)** In the absence of agonist, ELFN1 exposure results in increased mGluR6 basal activity, defined by the inhibition of forskolin response. **(B)** ELFN1 similarly increased mGluR4 basal activity, **(C)** but had no significant effect on mGluR2 basal activity.



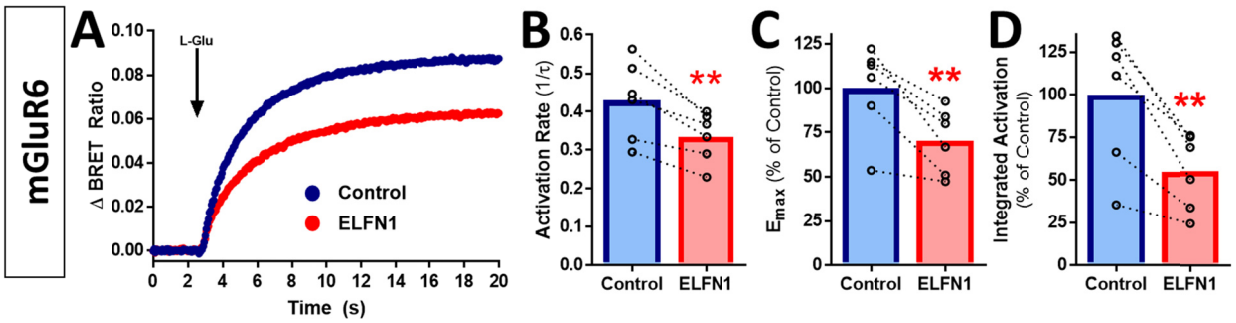
Supplementary Figure 2. ELFN1 modulates pharmacological properties of group III mGluRs *in trans*. Trans-cellular GPCR signaling assay utilizing -22F cAMP pGloSensor in mGluR-expressing cells exposed to Control or ELFN1-expressing cells that lack biosensor (see Figure 3). **(A)** Dose-response curves for mGluR6- and biosensor-expressing cell population, differing only by exposure to either Control or ELFN1-expressing cells. Co-culturing with ELFN1 lead to **(B)** decreased maximal efficacy (E_{max}) and **(C)** increased half maximal effective concentration (EC_{50}) for group III mGluR-specific agonist L-AP4. **(D)** mGluR4- and biosensor-expressing cells exposed to ELFN1-expressing cells similarly demonstrated **(E)** decreased E_{max} and **(F)** increased EC_{50} for L-AP4.



Supplementary Figure 3. ELFN1 modulation of group III mGluRs require FN3 region and intact distal amino terminal. (A) Schematic representation of transmembrane ELFN1ΔFN3 construct designed from non-binding ecto-domain construct in Figure 2. **(B)** Confirmation of ELFN1ΔFN3 protein expression via Western blot. **(C)** Dose-response curves for mGluR6- and biosensor-expressing cell population, differing only by exposure to either Control, ELFN1-, or ELFN1ΔFN3-expressing cells (See Figure 3). Co-culturing with ELFN1, but not ELFN1ΔFN3, lead to **(D)** decreased maximal efficacy (E_{max}) and **(E)** increased half maximal effective concentration (EC_{50}) for L-glutamic acid. **(F)** Schematic representation of transmembrane Caspr2-ELFN1 construct designed from non-binding ecto-domain construct in Figure 2. **(G)** Confirmation of Caspr2-ELFN1 protein expression via Western blot. **(H)** Dose-response curves for mGluR6- and biosensor-expressing cell population, differing only by exposure to either Control, ELFN1-, or Caspr2-ELFN1-expressing cells. Co-culturing with ELFN1, but not Caspr2-ELFN1, lead to **(I)** decreased maximal efficacy (E_{max}) and **(J)** increased half maximal effective concentration (EC_{50}) for L-glutamic acid.



Supplementary Figure 4. Trans-cellular effect of ELFN1 on group III mGluR pharmacology is not a consequence of reduced membrane expression. (A) Representative immunoblot of biotinylated mGluR6 and ELFN1 representing membrane expression on opposing cells. **(B)** Densitometric quantification of biotinylated mGluR6 bands representing no significant change in mGluR6 membrane expression.



Supplementary Figure 5. Trans-cellular interactions with ELFN1 directly alter G protein-coupling efficiency of Group III mGluRs. Trans-cellular GPCR signaling assay utilizing real-time kinetic $G\alpha_i$ coupling biosensors in mGluR-expressing cells co-cultured with Control or ELFN1-expressing cells (see Figure 5). **(A)** Effect of ELFN1-expressing cell co-culture on average mGluR6 activation of $G\alpha_i$, where increased BRET ratio corresponds to increased $G\alpha_i$ activation. Exposure to ELFN1 **(C)** decreased activation rate and **(D)** efficacy of mGluR6-mediated $G\alpha_i$ activation via L-glutamic acid, **(E)** thereby reducing integrated mGluR6-mediated $G\alpha_i$ activation value.

Supplementary References

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