Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supplementary Information

High-throughput synthesis of stable isotope-labeled transmembrane proteins for targeted transmembrane proteomics using a wheat germ cell-free protein synthesis system

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1. Experimental Procedure

1.1 Concurrent synthesis of SI-labeled TMPs using WG-CFS

We selected 263 cDNA clones encoding TMPs of interest from the Functional Annotation of Mouse (FANTOM) full-length cDNA library (DNAFORM, Yokohama, Japan). Split-primer PCR was performed using specific primers to produce DNA templates for *in vitro* transcription, as described previously.[24] A peptide tag sequence (MGPGGRAIIIRAAQAGTVR) was designed to measure the absolute amount of synthesized TMPs using the tryptic fragment AIIIR; the specific nucleotide sequence of the tag peptide was fused to each targeted TMP cDNA at the 5′ end. First, PCR was performed using a 10 nM primer set consisting of the gene-specific sense primer (Table S1) and an antisense primer (5′-GTCAGACCCCGTAGAAAAGA-3′) corresponding to a common sequence on the plasmid DNA from the FANTOM library. Subsequently, the template DNA for *in vitro* transcription was amplified by PCR using a primer mix consisting of 100 nM SPu primer (5′-GCGTAGCATTTAGGTGACACT-3′), 1 nM deSP6E01-PT primer (5′-GGTGACACTATAGAACTCACCTATCCCCAACACCTAATAACATTCAATCACTC TTTCCACTAACCACCTATCTACATCACCAACCACCCACCACCACCAATG-3′), and 100 nM antisense primer. The PCR product was then used as the template for protein synthesis.

A bilayer reaction method and GenDecoder1000 robotic synthesizer (CellFree Sciences, Matsuyama, Japan) were used to perform *in vitro* transcription and translation automatically.[16,24] For SI-labeling, [$^{13}C_6$,$^{15}N_2$]-Lys and [$^{13}C_6$,$^{15}N_4$]-Arg (99% isotopic purity, Cambridge Isotope Laboratories, Andover, MA, USA) were used (final concentration of 0.3 mM each). Liposomes were prepared from soybean asolectin (Sigma-Aldrich, St. Louis, MO, USA), as described previously.[18] Briefly, 10 g of asolectin powder was dissolved in 30 mL of chloroform, and then mixed with 180 mL of ice-cold acetone. After stirring for 2 h at room temperature, the suspension was stored at 4°C overnight. The supernatant was then discarded, and the remaining solvent was evaporated under a stream of N_2 gas. The lyophilized lipid powder was rehydrated with SUB-AMIX® SGC (CellFree Sciences) to a final concentration of 100 mg/mL, and stored at −80°C until use. For the synthesis of TMPs, liposome-supplemented SUB-AMIX® SGC was added to the translation layer (10 mg/mL final concentration of asolectin). The bilayer reaction was then performed at 16°C for 24 h. Synthesized TMP/liposome complexes were precipitated by centrifugation at 15,000 × *g* and 4°C for 30 min. The pellets were washed with ice-cold

phosphate-buffered saline (PBS) three times, re-precipitated, and stored at −80°C until analysis. TMPs without SI labels were synthesized as references for MS analysis.

1.2 Enzymatic digestion of gel-separated recombinant proteins

Synthesized TMPs were solubilized at room temperature in 50 μL of SDS lysis buffer containing 50 mM dithiothreitol. They were then separated by SDS-PAGE, and stained using Bio-Safe CBB (Bio-Rad, Hercules, CA, USA). Bands corresponding to target proteins were excised from the gel, and each gel piece was destained using 50% (v/v) acetonitrile in100 mM ammonium bicarbonate. After washing with 100 mM ammonium bicarbonate, proteins in gel pieces were reduced by soaking in 10 μL of 40 mM DTT for 1.5 h at 37°C, and then alkylated by incubation in 10 μL of 250 mM acrylamide for 30 min at room temperature. Individual gel pieces were dehydrated with 1 mL of acetonitrile, and then rehydrated with 2 μL of 0.1 μg/μL sequencing-grade modified trypsin or chymotrypsin (Promega, Madison, WI, USA), followed by incubation in 50 μL of 50 mM ammonium bicarbonate solution at 37°C overnight. Peptides were then extracted from the gel pieces three times with 50 μL of 50% (v/v) acetonitrile/5% (v/v) trifluoroacetic acid (TFA). Peptide samples were concentrated using a centrifugal vacuum concentrator, and then reconstituted with 0.2% (v/v) TFA solution for MS analysis.

1.3 Tissue sample preparation for MS analysis

We prepared three individual sets of brain samples from C57BL/6 male mice (12 weeks old, n = 3). Animal experiments were approved by and performed in accordance with the guidelines for the care and use of laboratory animals established by the Animal Experiments Committee of Ehime University. Mice were anesthetized using pentobarbital, and perfused with saline. Brains were then removed and cut into 2-mm slices. Six brain regions (olfactory bulb, prefrontal cortex, striatum, hippocampus, midbrain, and cerebellum) were excised under a stereomicroscope, frozen immediately on dry ice, and stored at −80°C until use. Each brain tissue (10–20 mg) was homogenized in 400 μL of ice-cold PBS containing complete mini-protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) using a glass homogenizer. Tissue homogenates (120-μg total protein) were mixed with an internal standard mixture containing SI-labeled recombinant neurotransmitter receptors, and then sonicated at 4°C for 3 min. After centrifugation at 15,000 × *g* for 30 min at 4°C, the pellets were re-dissolved in 400 μL of 0.1 M Na_2CO_3 and

1 mM EDTA, pH 11.3, and incubated on ice for 30 min. After centrifugation at 15,000 × *g* for 30 min at 4°C, pellets were suspended with 40 μL of lysis buffer (0.15 M Tris-HCl pH 6.8, 4% SDS, and 50 mM dithiothreitol) and incubated at room temperature for 4 h. The samples were then centrifuged at 15,000 × *g* for 30 min at 24°C, and the supernatants were transferred to separate low-binding tubes (Eppendorf, Hamburg, Germany) and stored at −80°C until analysis. Protein concentrations were determined using Qubit® Protein Assay Kits (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

1.4 Tube-gel digestion of extracted proteins

Extracted proteins were digested using the tube-gel digestion protocol with minor modifications.[25] Briefly, 7 μL of protein solution (5 μg total protein), 5 μL of 30%/0.8% (w/v) acrylamide/bis-acrylamide solution, 1 μL of 1.5% ammonium persulfate, and 0.5 μl of 100% tetramethylethylenediamine were mixed in a 0.2-mL PCR tube. After polymerization, gel pieces were transferred to 1.5-mL plastic tubes and washed twice with 1 mL of wash solution A (50% [v/v] methanol, 5% [v/v] acetic acid) for 30 min, and twice with 1 mL of wash solution B (50% [v/v] acetonitrile in 50 mM ammonium bicarbonate) for 30 min. The gels were dehydrated with 100% acetonitrile, and then air-dried at room temperature for 30 min. In-gel digestion using trypsin or chymotrypsin was performed in 50 mM ammonium bicarbonate solution for 15 h at 37°C. Digested peptides were extracted three times using 200 μL of 50% (v/v) acetonitrile and 5% (v/v) TFA. After concentration and purification with a self-made C18 STAGE tip, peptide samples in 0.2% (v/v) TFA solution were analyzed using MS.

1.5 Mass spectrometry and data analysis

Tandem MS (MS/MS) analysis was performed on a QTRAP 5500 hybrid triple quadrupole/linear ion trap MS equipped with a Nanospray ion source (AB SCIEX, Framingham, MA, USA). The chromatographic separation of peptides was performed using a Prominence Nano-flow Liquid Chromatography System (Shimadzu Biotech, Kyoto, Japan). For online desalting, peptides were injected onto a C18 trap column (Chemicals Evaluation and Research Institute, Tokyo, Japan) and washed with 0.1% TFA for 10 min. Concentrated peptides were separated at a constant flow rate of 300 nL/min in a fused-silica capillary column packed with C18 resin (15 cm × 75 μm ID; Nikkyo Technos, Tokyo, Japan). For peptide separation, 0.1% formic acid (A) and 80% acetonitrile with 0.1%

formic acid (B) were used as the mobile phases. A gradient (5–100% mobile phase B) was applied for 50 min, followed by a 10 min wash using 100% mobile phase B. The column was then equilibrated for 20 min with 5% mobile phase B. Separated peptides were transferred to a QTRAP 5500 connected to the Prominence system for the acquisition of MS/MS spectra using information-dependent acquisition (IDA) workflows. Survey scans were performed in enhanced mass spectrum mode, and precursor ions were selected in accordance with the IDA criteria. Enhanced product ion scanning mode was used to determine the optimal collision energy to obtain high quality MS/MS spectra of the precursor ions. Ion spray voltage was maintained at 2,300 V, and the temperature of the interface heater was held at 150°C. MS/MS data analysis and protein identification were performed using Analyst version 1.5 and ProteinPilot version 4.0 software (both from AB SCIEX). Database searches were performed against an in-house database containing 263 mouse TMP sequences using the following parameters: modifications, biological modifications; instrument type, 5500QTRAP ESI; and search type, thorough mode. MRMPilot software (AB SCIEX) was used to select at least three transitions per target proteotypic peptide for SRM analysis using QTRAP 5500 coupled to the Prominence nano LC system.

1.6 Targeted quantitation of TMPs

The synthesized amount of the SI-labeled protein used as internal standard was detected by using the peptide tag (AIIIR) released from the N-terminus of the SI-labeled protein by trypsin digestion. At the end of the translation reaction, the synthesized protein solution was dispensed into 40-μL aliquots. After the separated SI-labeled protein solution was digested with trypsin using the tube-gel digestion protocol, the non-SI-labeled synthetic peptide AIIIR (10 pmol) was added to the resulting digested peptide mixture. The AIIIR peptide present in the purified digested peptide sample was detected by SRM analysis using the QTRAP 5500 coupled to the Prominence LC system, and the synthesized protein concentration in the samples was calculated from the ratio of the heavy (m/z 595.42 > 185.13) and light (m/z 585.41 > 175.12) peptide peak areas. The area under each peak was measured using MultiQuant version 2.0 software (AB SCIEX). The quantitation of each endogenous protein was performed by comparing the area of an endogenous peptide to the corresponding SI-labeled peptide.

2. Supplementary Results

2.1 In vitro synthesis of full-length proteins and effective SI-incorporation using WG-CFS.

We evaluated the efficiency of SI-incorporation into synthesized proteins using WG-CFS. The cDNAs encoding beta-glucuronidase fused with glutathione S-transferase (GST-GUS) and green fluorescent protein (GFP) were PCR-amplified, and then subcloned into pEU-E01-MCS (CellFree Sciences). Two hundred and fifty microliters of a transcription mixture containing 25 μg of plasmid DNA, 50 μL of 5× TB (CellFree Sciences), 2.5 mM of each nucleoside triphosphate (CellFree Sciences), 250 U of SP6 RNA polymerase (Promega), and 250 U of RNasin (Promega) was incubated for 6 h at 37°C. The *in vitro* translation of SI-labeled GFP and GUS were performed using bilayer reactions in 96-well plates. The translation layer was prepared by mixing 10 μL of the transcription mixture, 10 μL of WEPRO® (CellFree Sciences), 0.08 μL of 20 mg/mL creatine kinase, 16 μL of a heavy labeled amino acid solution containing 1.5 mM [$^{13}C_6$,$^{15}N_2$]-Lys (99% isotopic purity, Cambridge Isotope Laboratories), 1.5 mM of [$^{13}C_6$,$^{15}N_4$]-Arg (99% isotopic purity, Cambridge Isotope Laboratories), and 4 μL of 5×SUB-AMIX® SGC (CellFree Sciences) not including Lys and Arg. SUB-AMIX® SGC (200 μL) was loaded over the translation mixture, and the bilayer reaction was performed at 15°C for 20 h. The synthesis of SI-labeled proteins was carried out using different amino acid concentrations, as shown in Fig. S1A. After the *in vitro* translation of GFP and GST-GUS, the protein extracts were separated and visualized by SDS-PAGE followed by CBB staining (Fig. S1B). In-gel digestion with then performed using trypsin. To assess the efficiency of SI-incorporation, multiple reaction monitoring cubed (MRM^3) analyses were performed on a QTRAP 5500 coupled to a Eksigent nanoLC-Ultra system via a cHiPLC-nanoflex module (AB-SCIEX) (Fig. S1C). Peptides were separated on a nano cHiPLC C18-reversed phase column (Chrome XP C18CL, 200 μm ID × 15 cm) and eluted at a constant flow rate of 1200 nL/min. A linear gradient (2–30% mobile phase B) was applied for 5 min, followed by a 2 min wash with 90% mobile phase B; the column was then equilibrated for 2 min with 2% mobile phase B. The MS parameters used for MRM^3 analyses are shown in Table S7. The light-to-heavy ratio of a targeted peptide was used to determine the SI-incorporation efficiency of isotopically heavy [$^{13}C_6$,$^{15}N_2$]-Lys into the GFP tryptic peptide FICTTGK, or the GST-GUS tryptic peptide IEAIPQIDK (Fig. S1D). In both peptides, the addition of 0.3 mM [$^{13}C_6$,$^{15}N_2$]-Lys to both layers and 1.8 mM [$^{13}C_6$,$^{15}N_2$]-Lys to the bottom layer (final concentration, 0.3 mM) achieved ~98% labeling efficiency. An efficiency >99% was

achieved by increasing the concentration of SI-labeled Lys in bottom layer to 6 mM (final concentration, 1 mM) (Fig. S1D); however, further increases in [$^{13}C_6$,$^{15}N_2$]-Lys to 9 or 12 mM had little effect on incorporation or yield. Similar results were obtained with the incorporation of [$^{13}C_6$,$^{15}N_4$]-Arg (97–99 %; Fig. S1D).

References

24 T. Sawasaki, Y. Hasegawa, M. Tsuchimochi, N. Kamura, T. Ogasawara, T. Kuroita, and Y. Endo, *FEBS Lett.*, 2002, **514**, 102-105.

25 X. Lu and H. Zhu, *Mol. Cell. Proteomics*, 2005, **4**, 1948-1958.

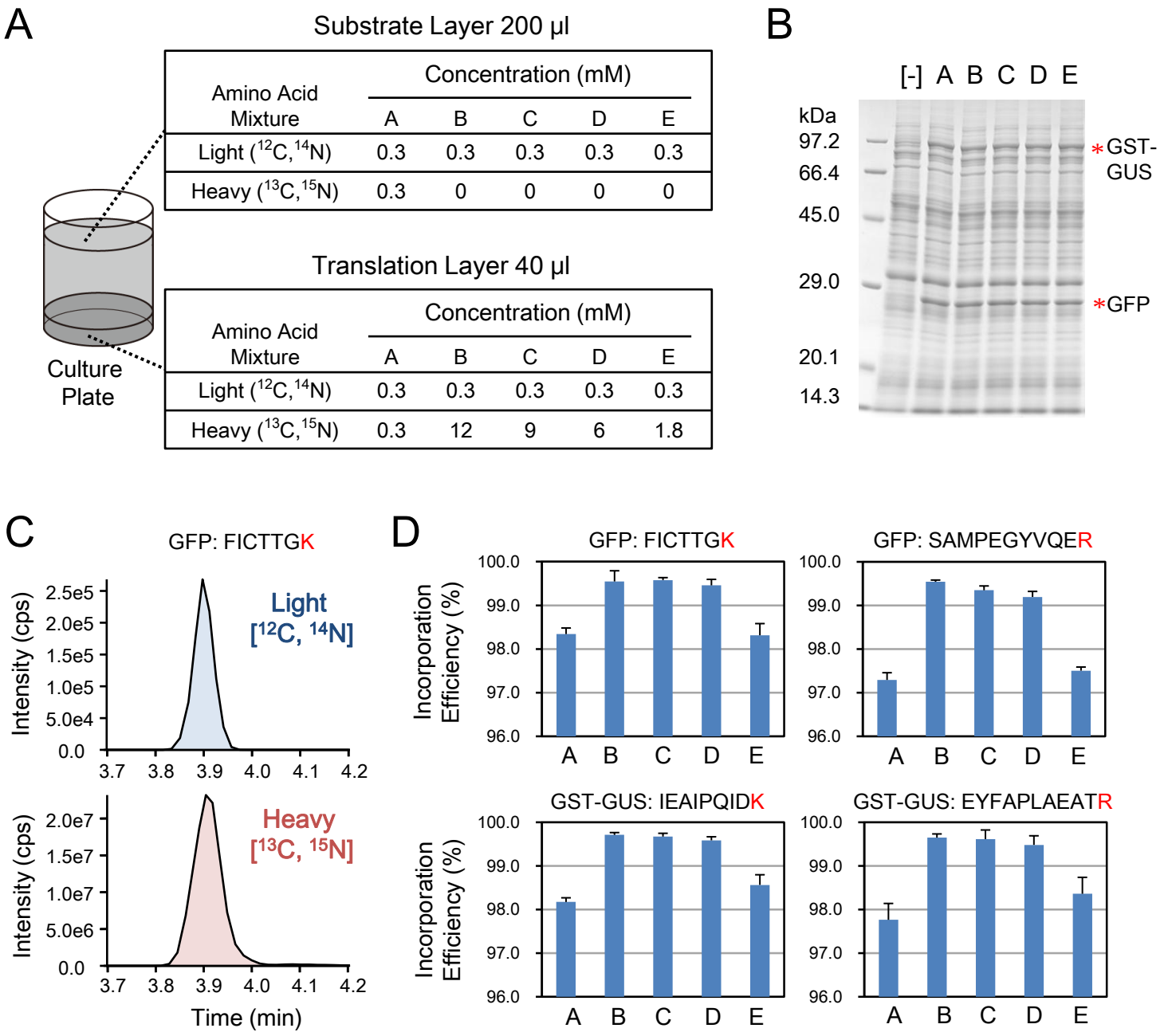


Fig. S1 Highly effective incorporation of stable isotope (SI)-labeled amino acids in a bilayer cell-free system. (A) *In vitro* synthesis of SI-labeled glutathione *S*-transferase (GST-GUS) and green fluorescent protein (GFP) using a wheat germ cell-free system (WG-CFS). In the lower layer, the concentration of the light (^{12}C,^{14}N) amino acid mixture was maintained at 0.3 mM, whereas the concentration of the heavy (^{13}C,^{15}N) amino acid mixture ranged from 0.3–12 mM. Light (^{12}C,^{14}N): 18 types of non-SI-labeled amino acids, excluding Lys and Arg. Heavy (^{13}C,^{15}N): SI-labeled Lys and Arg. (B) Representative SDS-PAGE image of SI-labeled GST-GUS and GFP (asterisks) visualized using Coomassie brilliant blue. The proteins were subjected to in-gel digestion with trypsin for MS analysis. [−], negative control. (C) MRM^3 ion chromatogram of the GFP tryptic peptide FICTTGK. The ratio of the isotopically light (m/z 420.7 > 580.3 > 305.1) and heavy (m/z 424.7 > 588.3 > 313.1) peptide peak areas was used to estimate the efficiency of SI-incorporation. (D) Efficiency of the incorporation of SI-labeled amino acids into GFP and GST-GUS tryptic peptides. The efficiency was high at all SI concentrations tested in (A). Even at 0.3 mM, >98% of SI-labeled amino acids were incorporated into the proteins (n = 3).

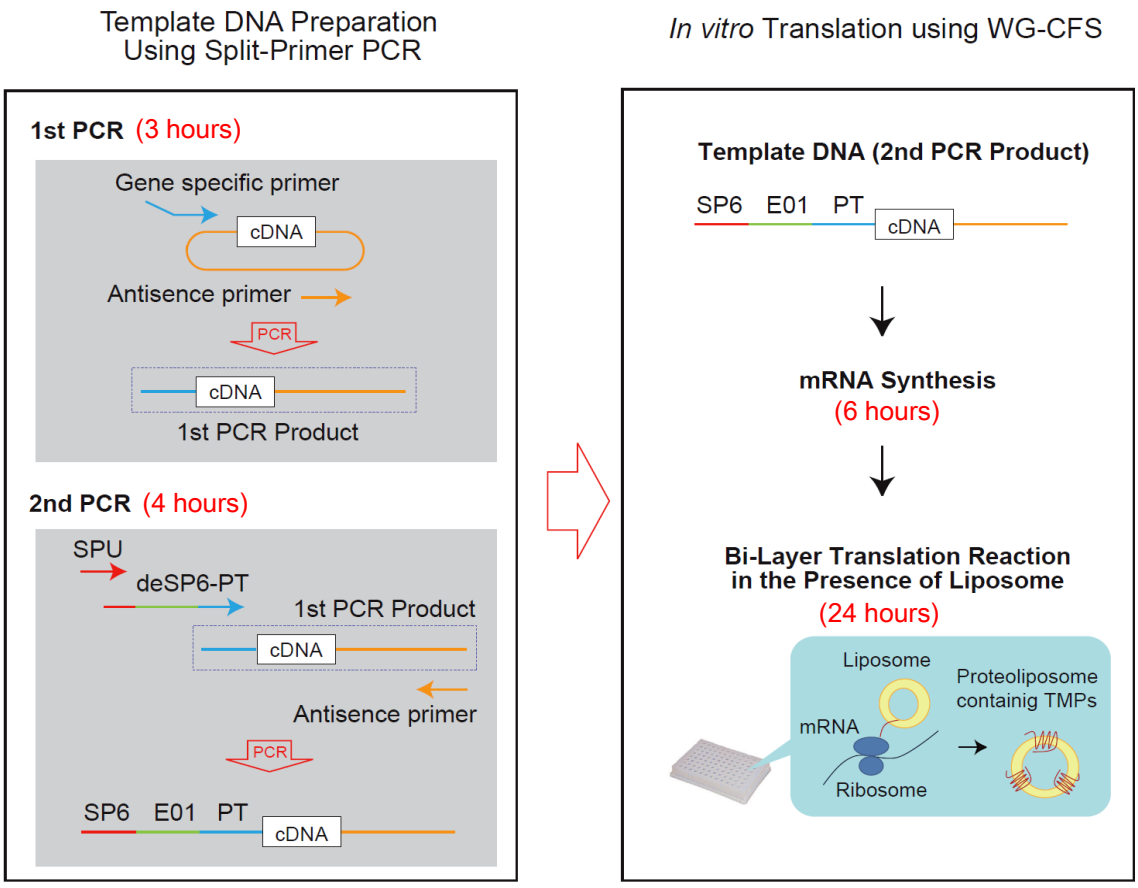


Fig. S2 A workflow diagram for the bilayer cell-free synthesis of transmembrane proteins (TMPs). PCR amplification of mouse cDNA encoding for a target TMP was performed in two steps using gene- and vector-specific primers. PCR products were used as the templates for the cell-free synthesis of mouse TMPs in liposome-supplemented wheat germ cell-free system (WG-CFS). To maximize the throughput of protein synthesis, a protein synthesizer was used to automate the transcription and translation processes. PT, a peptide tag sequence for the quantitation of synthesized TMPs.

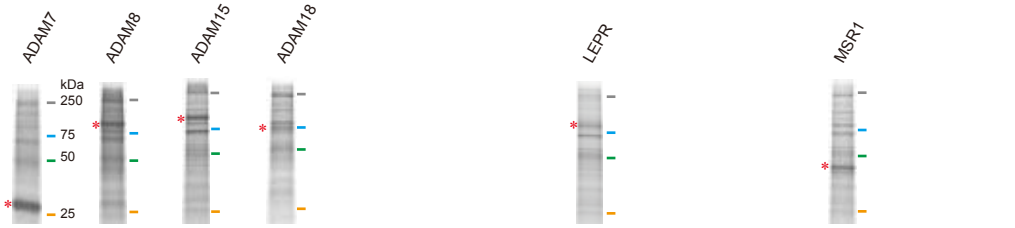
Fig. S3 SDS-PAGE images of mouse transmembrane proteins (TMPs).

Single-pass membrane protein

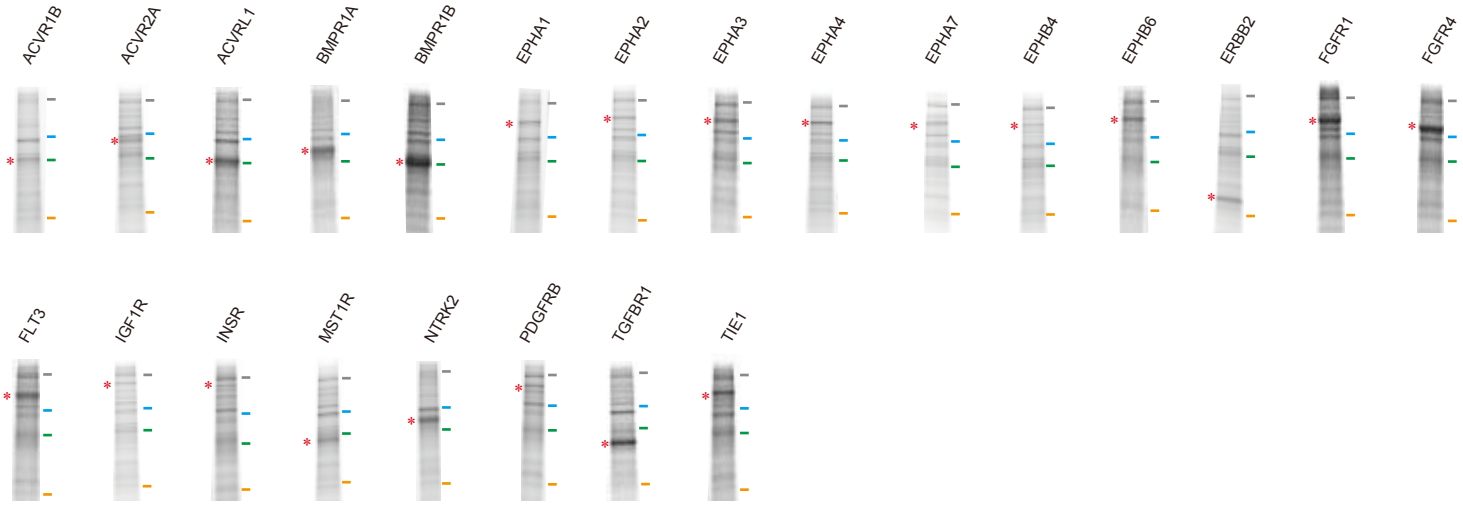
ADAM (a disintegrin and metallopeptidase)

Leptin receptor

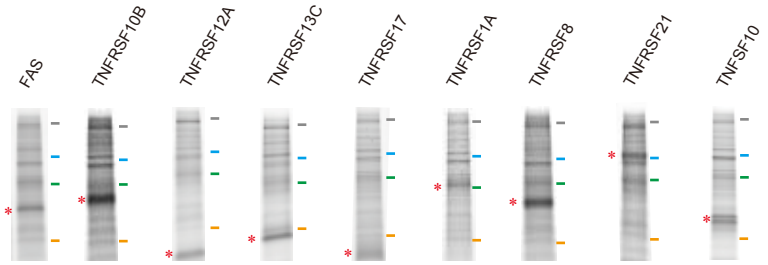
Macrophage scavenger receptor



Receptor tyrosine kinases

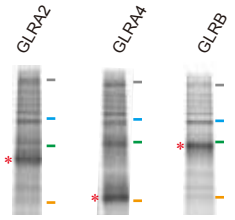


Tumor necrosis factor (ligand) and receptor

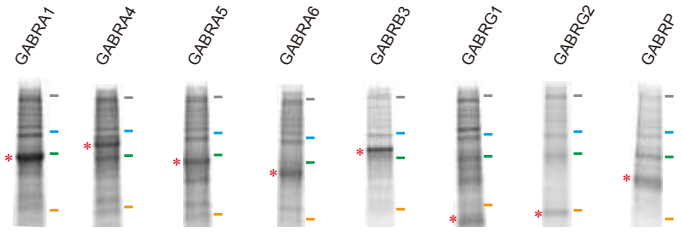


Multi-pass membrane protein: Ionotropic neurotransmitter receptor

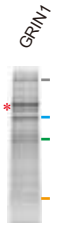
Neurotransmitter receptors
(Glycine receptor)



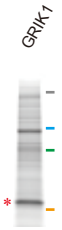
Neurotransmitter receptors
(GABAA receptor)



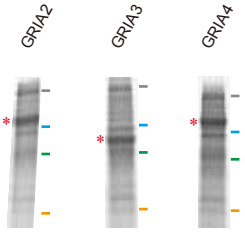
Neurotransmitter receptors
(NMDA receptor)



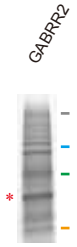
Neurotransmitter receptors
(Kainate receptor)



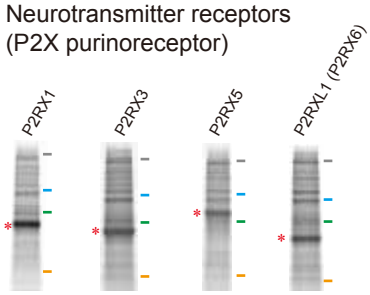
Neurotransmitter receptors
(AMPA receptor)



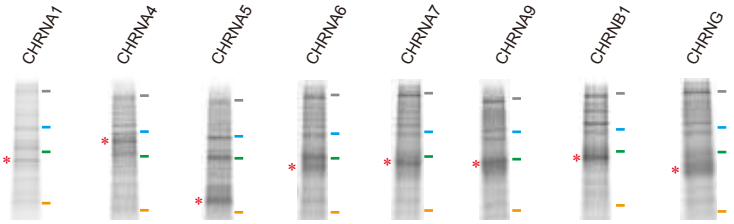
Neurotransmitter receptors
(GABAC receptor)



Neurotransmitter receptors
(P2X purinoreceptor)

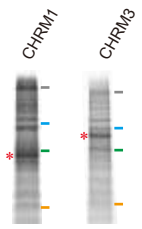


Neurotransmitter receptors
(Nicotinic acetylcholine receptor)

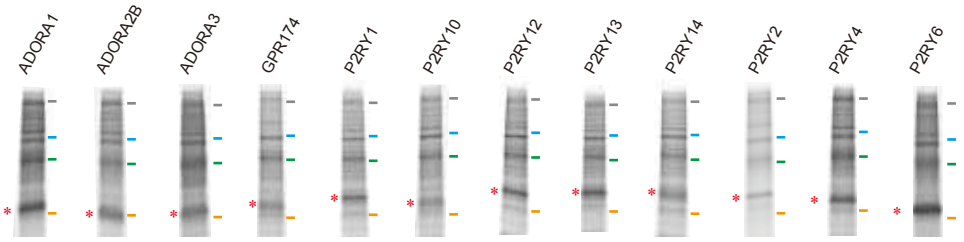


Multi-pass membrane protein: G protein-coupled receptors (GPCRs)

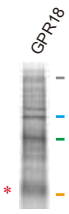
GPCR 1 family
Acetylcholine receptors



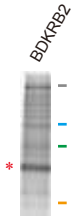
GPCR 1 family
Adenosine and adenine nucleotide receptors



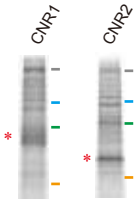
GPCR 1 family
Arachidonyl glycine receptors



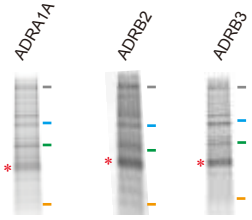
GPCR 1 family
Bradykinin receptors



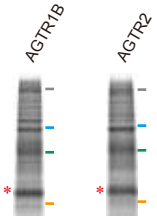
GPCR 1 family
Cannabinoids receptors



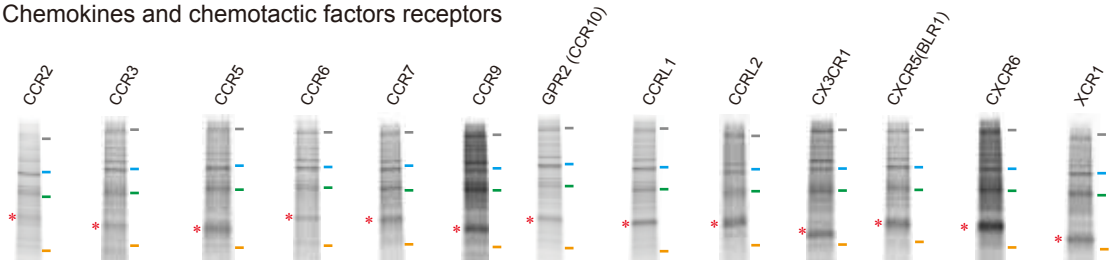
GPCR 1 family
Adrenergic receptors



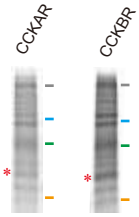
GPCR 1 family
Angiotensin receptors



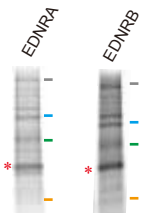
GPCR 1 family
Chemokines and chemotactic factors receptors



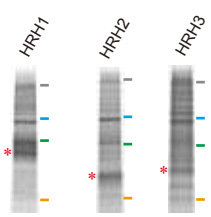
GPCR 1 family
Cholecystokinin receptors



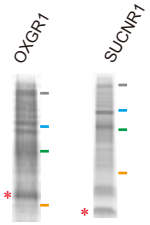
GPCR 1 family
Endothelin receptors



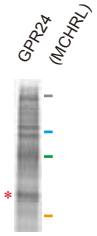
GPCR 1 family
Histamine receptors



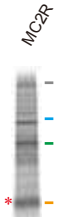
GPCR 1 family
Krebs cycle intermediates receptors



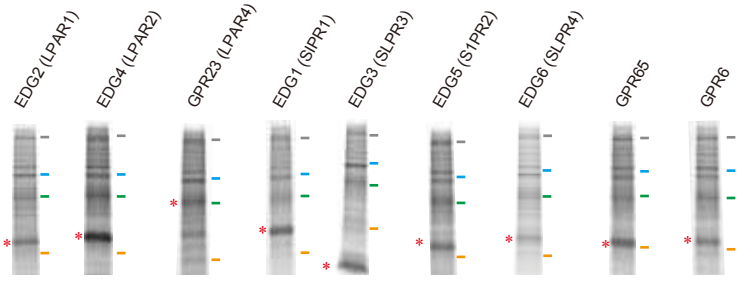
GPCR 1 family
Melanin-concentrating hormone receptors



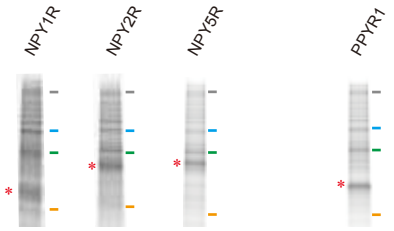
GPCR 1 family
Melanocortins receptors



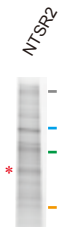
GPCR 1 family Lysolipids receptors



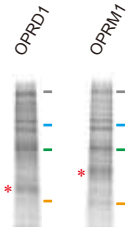
GPCR 1 family
Neuropeptide Y receptors



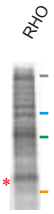
GPCR 1 family
Neurotensin receptors



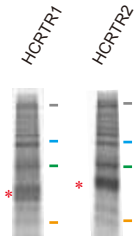
GPCR 1 family
Opioid peptides receptors



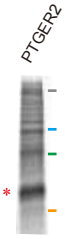
GPCR 1 family
Opsins



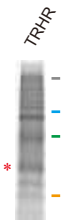
GPCR 1 family
Orexins receptors



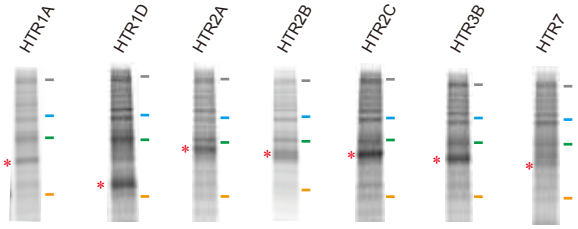
GPCR 1 family
Prostanoids receptors



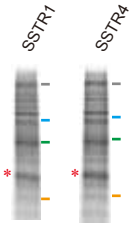
GPCR 1 family
Releasing hormones receptors



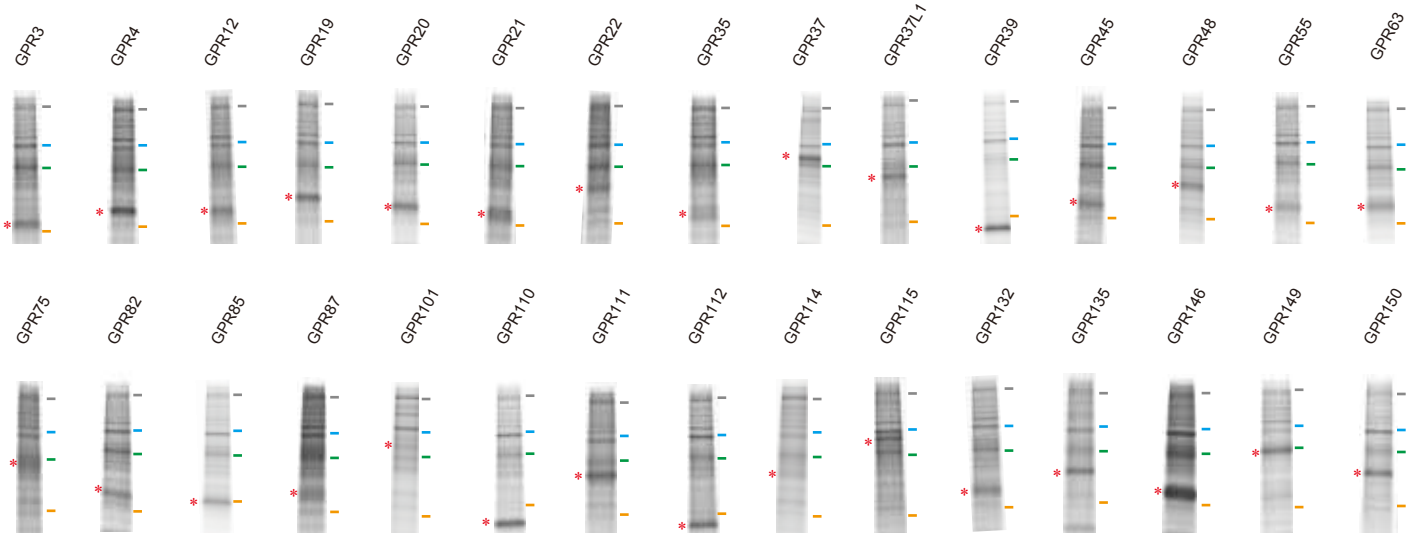
GPCR 1 family
Serotonin receptors

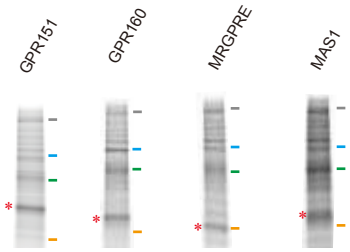


GPCR 1 family
Somatostatin and urotensin receptors

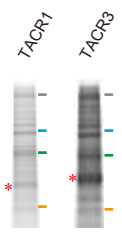


GPCR 1 family
Orphan receptors

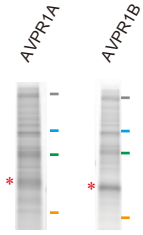




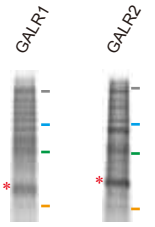
GPCR 1 family
Tachykinin receptors



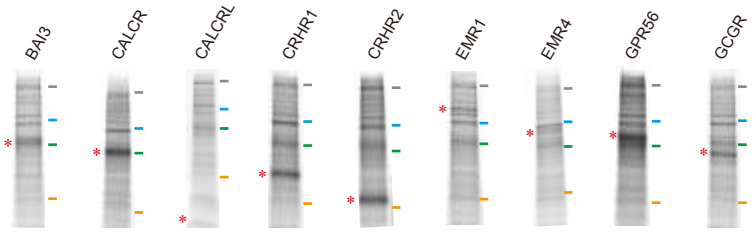
GPCR 1 family
Vasopressin / oxytocin receptors



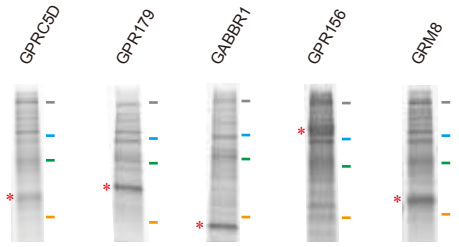
GPCR 1 family
Other receptors



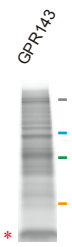
GPCR 2 family



GPCR 3 family

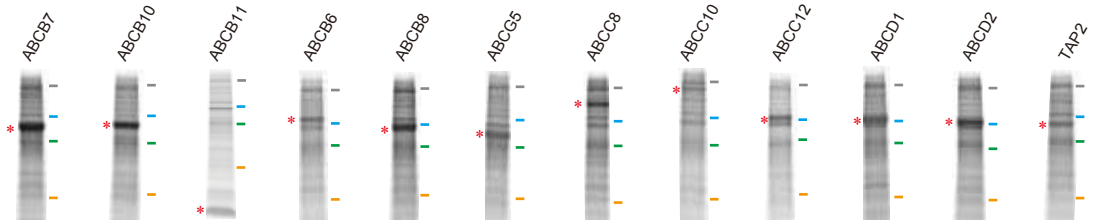


GPCR family OA

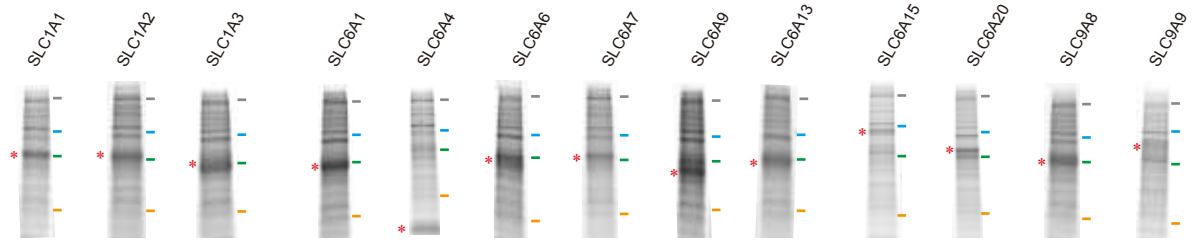


Multi-pass membrane protein: Other receptors

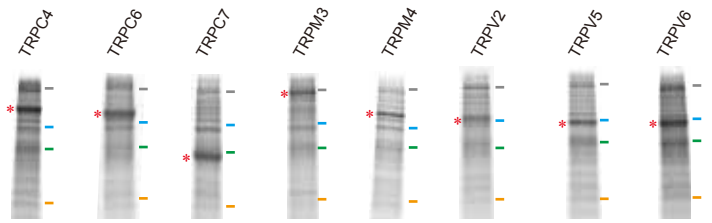
ATP-binding cassette



Solute carrier family



TRP channel



Adiponectin receptor

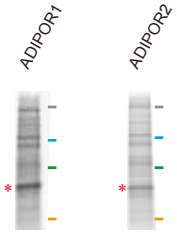


Fig. S4 Western blot analysis of mouse GRIA3 in six brain regions. Equal amounts of protein were loaded for SDS-PAGE (7.5% polyacrylamide gel) of each sample with 40 μg/lane. Gel-separated proteins were transferred to PVDF membrane prior to western blotting. The PVDF membrane was blocked for 1 h with TBST containing 5% nonfat skim milk, and incubated at 4°C overnight with rabbit anti-GRIA3 antibody (Frontier Institute, Ishikari, Japan) at a dilution of 1:3,000. The membrane was then washed three times with TBST and incubated with HRP -conjugated secondary anti-rabibit IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) in 1% nonfat skim milk in TBST. The results were visualized by ImageQuant LAS 4000 (GE Healthcare UK Ltd, Buckinghamshire, England). FCTX, frontal cortex; CBL, cerebellum; HP, hippocampus; MB, midbrain; OB, olfactory bulb; STR, striatum.

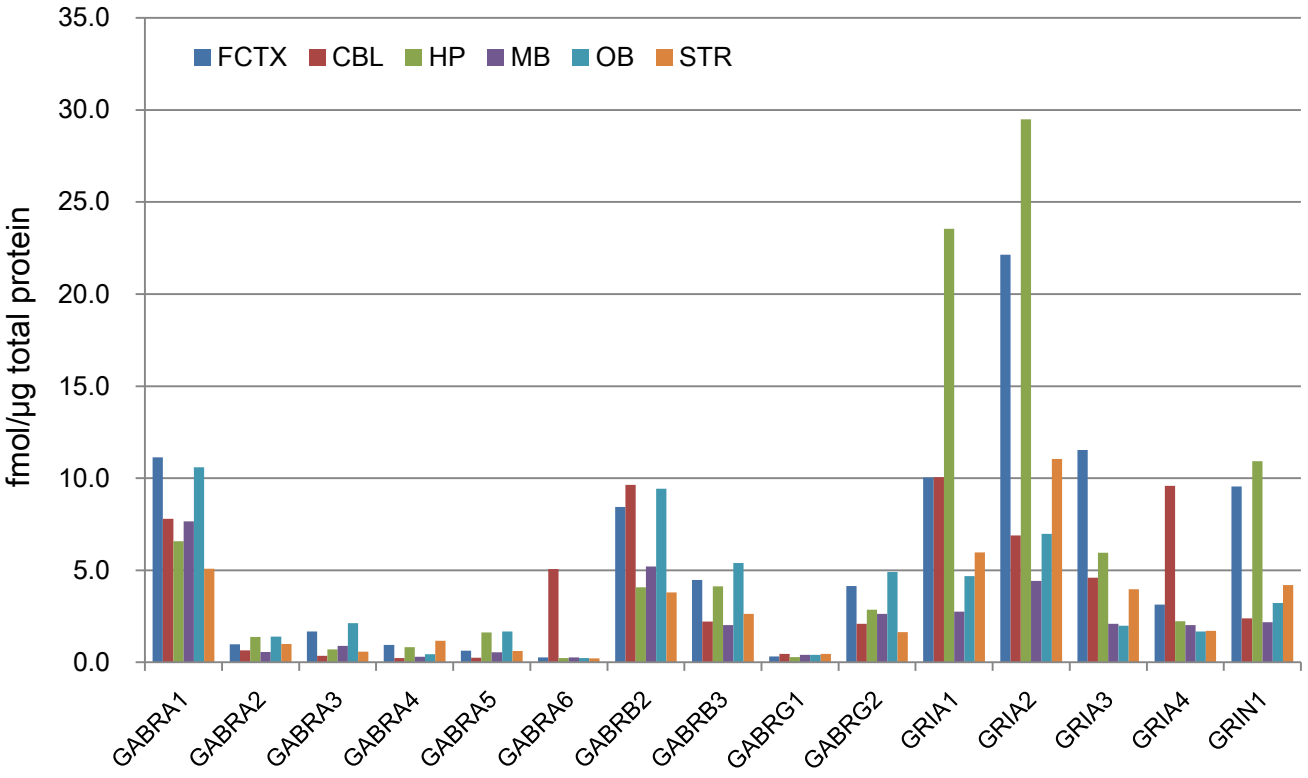


Fig. S5 Expression profiles of endogenous neurotransmitter receptors in six brain regions. Fifteen neurotransmitter receptors (5 glutamate and 10 gamma-aminobutyric acid [GABA] receptors) were quantified successfully. FCTX, frontal cortex; CBL, cerebellum; HP, hippocampus; MB, midbrain; OB, olfactory bulb; STR, striatum.