Supplementary Methods

qPCR

Total RNA was extracted from cells using the Quick-RNA Miniprep Plus kit (Zymo Research) according to the manufacturer's protocol. 100 ng RNA was used per reaction for one-step SYBR Green RT-qPCR (Hifair Advanced One-Step RT-qPCR Kit, Yeasen) on a Roche LightCycler 96 instrument. Primer sets included two internal receptor-targeting primers per receptor, a receptor–bGH junction primer pair (transgene-specific), and housekeeping genes Gnb1 and Fkbp1a for CHO-K1 cells (GAPDH and RPL32 for SH-SY5Y cells). See table below. Cycling conditions were 50°C for 6 min (reverse transcription), 95°C for 5 min, followed by 40 cycles of 90°C for 15 s and 60°C for 30 s with fluorescence acquisition. Relative expression was quantified using the ΔΔCt method normalized to the geometric mean of the housekeeping genes.

Target	Primer Pair	Direction	Sequence (5' to 3')	Cell Line(s)
GALR1	bGH junction	Forward	AAGTCGAATAGACACCCCAC	CHO-K1, SH-SY5Y
GALR1	bGH junction	Reverse	TAGAAGGCACAGTCGAGG	CHO-K1, SH-SY5Y
GALR1	Internal 1	Forward	TGGTGGTTGTGGTGTTTGGA	CHO-K1, SH-SY5Y
GALR1	Internal 1	Reverse	AGTGGGCGGTGATTCTGAAG	CHO-K1, SH-SY5Y
GALR2	bGH junction	Forward	CAGATATCCAGCACAGTGGC	CHO-K1
GALR2	bGH junction	Reverse	TAGAAGGCACAGTCGAGG	CHO-K1
GALR2	Internal 1	Forward	GCCACTTATGCGCTTCGC	CHO-K1
GALR2	Internal 1	Reverse	TAAACGATGGGGTTGACGCA	CHO-K1
GALR2	Internal 2	Forward	CATCCTCGAGCCCTGTCCTG	CHO-K1
GALR2	Internal 2	Reverse	GCCACATCAACCGTCAGGATG	CHO-K1
GALR3	bGH junction	Forward	CCCGAGGACCGGAATAACT	CHO-K1
GALR3	bGH junction	Reverse	TAGAAGGCACAGTCGAGG	CHO-K1
GALR3	Internal 1	Forward	GCTCATCCTGTGCTTCTGGTA	CHO-K1
GALR3	Internal 1	Reverse	GTAGGCCAGGCAGTGTGAGG	CHO-K1
GALR3	Internal 2	Forward	TCTTTGGGGCCCTCGTCTG	CHO-K1
GALR3	Internal 2	Reverse	GTAAAGCTGCTGGCGTACAT	CHO-K1
Gnb1	Housekeeping	Forward	CCATATGTTTTCTTTCCAATGGC	CHO-K1
Gnb1	Housekeeping	Reverse	AAGTCGTCGTACCCAGCAAG	CHO-K1
Fkbp1a	Housekeeping	Forward	CTCTCGGGACAGAAACAAGC	CHO-K1
Fkbp1a	Housekeeping	Reverse	GACCTACACTCATCTGGGCTAC	CHO-K1
GAPDH	Housekeeping	Forward	GAGGGTGGTGCCAAGAAAGT	SH-SY5Y
GAPDH	Housekeeping	Reverse	TGGCTTGGGTCGTAGGCATCA	SH-SY5Y

RPL32	Housekeeping	Forward	CACCAGTCAGACCGATATGTCAAAA	SH-SY5Y
RPL32	Housekeeping	Reverse	TGTTGTCAATGCCTCTGGGTTT	SH-SY5Y

cAMP assay

For testing, growth media was aspirated and cells were incubated with 100 µL per well of compound-containing assay buffer for 15 minutes at room temperature. Following incubation, assay buffer was removed and cells were lysed by addition of 100 µL/well 0.1 M HCl for 20 minutes. Intracellular cAMP in the lysate was quantified using a commercially available cAMP ELISA kit (Cayman Chemical, 581002) following the manufacturer's protocol. Specifically, cell lysate was diluted 1:5 in ELISA buffer and transferred to a 96-well ELISA plate, and cAMP tracer and antiserum were added per kit protocol. A cAMP standard curve and appropriate buffer-only controls were included on each plate. After overnight incubation, plates were washed and developed with Ellman's reagent for 30 minutes, then absorbance was measured at 410 nm using a Biotek Synergy HTX plate reader. Raw absorbance values were converted to cAMP concentrations by back-calculating from the standard curve fitted to the cAMP standards (logit-linear fit), per kit protocol. Computed cAMP concentrations were normalized to the per-plate buffer-only controls as appropriate.

Calcium flux assay

For testing, growth media was aspirated and cells were washed 2 times with 150 µL/well PBS to remove serum-containing media. Next, 50 µL of dye loading solution containing 3 µM Fluo-4 AM (Ion Biosciences, 1041F), 2.5 mM probenecid, and 1× pluronic F-127 (a detergent that helps disperse the calcium dye; Ion Biosciences, 7601A) was added to the cells, which were incubated for 1 hour at 37°C. After this, the dye loading solution was removed and replaced with 50 µL of compound-containing buffer. The plate was immediately read on a Biotek Synergy HTX plate reader using the following settings: kinetic read at 5 second intervals over 5 minutes, excitation at 485/20 nm and emission at 528/20 nm, read from bottom of the plate. In order to minimize the sampling interval of the kinetic read, the cell plate was processed one column at a time.

To quantify the normalized fluorescence response for each sample, a bi-exponential rise-decay curve was fit to the raw fluorescence traces for each sample replicate:

$$F(t) = b + a \cdot (e^{-t/\tau 2} - e^{-t/\tau 1})$$

- t = time in seconds
- F(t) = fluorescence at time t (in relative fluorescence units, RFUs)
- b = baseline fluorescence
- a = scaling factor
- τ_1 = time constant for the rising phase of the calcium transient
- τ_2 = time constant for the decay phase

The curve was fitted using the Levenberg–Marquardt algorithm as implemented in the scipy.optimize.curve_fit function. The normalized fluorescence response for each sample was computed from the fitted curve as:

$$\Delta F/F0 = (Fpeak - F0)/F0$$

- F_{peak} = maximum of the fitted curve (computed by setting $dF(t)/dt = 0$ and solving for t, then evaluating $F(t_{peak})$)
- F_0 = mean fluorescence across all time points of the control (buffer-only) condition

Acetylcholine release assay

On testing day, assay buffer (HBSS with 10 mM HEPES and 0.1% BSA; Gibco) was prepared and supplemented with 100 μM neostigmine bromide (Ambeed, A198984) to block endogenous acetylcholinesterase activity in the cells. For the Ca^{2+}-free controls, HBSS was substituted with calcium-free HBSS (Gibco) and additionally supplemented with 1 mM EGTA (Thermo Scientific, A16086.09). Galanin (Arctom Scientific, HY-P1127) and PAC-832 (custom synthesis) solutions were prepared via serial dilution into assay buffer in the same manner as the cAMP assay. Separate high K^+ (50 mM) solutions were prepared for each compound solution by addition of 1 M KCl.

For testing, growth media was aspirated and cells were washed with 100 μL/well PBS to remove serum-containing media. Cells were pre-incubated with 100 μL/well compound-containing buffer for 10 minutes. The buffer was aspirated and replaced with compound-matched 100 μL/well high K^+ buffer. The cells were incubated for 10 minutes, then the high K^+ buffer was aspirated and pooled into microcentrifuge tubes (4 replicates per tube). The tubes were vacuum-concentrated on a Savant SVC100H SpeedVac for 6 hours in order to reduce the volume by 75% (necessary to reach the minimum 15 pg/mL ACh detection threshold of the ELISA kit used to quantify ACh).

Next, the ACh concentration was measured in the concentrated treatment buffer using a commercially available ACh ELISA kit (Elabscience, E-EL-0081) following the manufacturer's protocol. Specifically, the concentrated treatment buffer was transferred to a 96-well ELISA plate, then biotinylated detection antibody followed by HRP-streptavidin conjugate were added per kit protocol. An ACh standard curve and appropriate buffer-only controls were included on each plate. Plates were washed and developed with TMB substrate, then absorbance was measured at 450 nm using a Biotek Synergy HTX plate reader. Raw absorbance values were converted to ACh concentrations by back-calculating from the standard curve fitted to the ACh standards (four-parameter logistic fit). Computed ACh concentrations were normalized to the per-plate buffer-only controls as appropriate.

Concentration response curves

All concentration-response data were fit to a four-parameter logistic equation:

$$y = a + (d - a) / (1 + 10(c - \log[\text{drug}]) \cdot b)$$

- a = bottom asymptote
- b = Hill slope
- c = $\log(EC_{50})$
- d = top asymptote

Parameters were estimated using the Levenberg–Marquardt algorithm as implemented in the scipy.optimize.curve_fit function. Potency metrics IC/EC_{50} and I_{max}/E_{max} were derived directly from the fitted parameters. 95% confidence intervals for IC/EC_{50} were computed from the asymptotic covariance matrix of the fitted parameters returned by the optimizer.

The statistical significance of the curve fit was computed using an extra sum-of-squares F test:

$$F = ((SS_{null} - SS_{4PL}) / (dfnull - df4PL)) / (SS4PL / df4PL)$$

where SS_{null} and SS_{4PL} are the residual sums of squares for the null (flat-line) and 4PL models, respectively, $df_{null} = n - 1$, and $df_{4PL} = n - 4$.

In vivo compound formulations

Scopolamine hydrobromide trihydrate (A2B Chem, AI54488) and donepezil hydrochloride (A2B Chem, AA33596) were formulated in 10% DMSO in 0.9% saline. PAC-832 (custom synthesis) was formulated in 10% DMSO, 20% 2-Hydroxypropyl-β-cyclodextrin (w/v), and 0.5% Tween-80 in 0.9% saline, with isethionic acid added at 1.1 equivalents to form the corresponding salt. All solutions were sterile-filtered through a 0.22 μm filter prior to injection.

HPLC sample preparation and chromatography

Brain homogenates were prepared by combining whole brain tissue with water at a 1:4 (w/v) ratio and homogenizing using a glass Dounce homogenizer. 250 μL of homogenate was analyzed per replicate. 250 μL carbonate buffer (pH 10) was added to the brain homogenate, followed by a heptane wash to remove nonpolar lipids. PAC-832 was extracted from the aqueous phase by two sequential liquid-liquid extractions with ethyl acetate (2 × 600 μL). The combined ethyl acetate was evaporated to dryness using a vacuum concentrator, then reconstituted in 50 μL initial mobile phase (40:60 acetonitrile:water containing 0.1% formic acid). For plasma samples, 50 μL of plasma was analyzed per replicate. Plasma samples were processed analogously as the brain samples, though with scaled-down volumes to account for smaller starting volume.

Chromatographic analysis was performed on a Waters 2695 HPLC system equipped with a Waters 2996 Photodiode Array UV detector and a PerkinElmer Harmony C18 column (250 × 4.6 mm, 5 μm). Mobile phase consisted of acetonitrile with 0.1% formic acid (A) and water with 0.1% formic acid (B). A linear gradient was run from 40:60 A:B to 90:10 A:B over 10 minutes at a flow rate of 1 mL/min and an injection volume of 10 μL. Peak areas were quantified using Empower 3 (Waters).

Calibration standards were prepared by spiking blank plasma or brain homogenate with PAC-832 to final concentrations of 0.3, 1, 3, 10, and 30 µg/mL (plasma) or µg/g (brain) and processed identically to study samples. Calibration curves were fit using weighted linear regression with $1/x^2$ weights. The practical quantification limit under these conditions was ~1 µg/mL.

Equilibrium dialysis

Plasma protein binding and brain homogenate binding was determined by equilibrium dialysis using Pierce RED Device Inserts (8 kDa MWCO; Thermo Scientific). Blank plasma and brain homogenate (1:4 brain:water) were was spiked with PAC-832 to a nominal concentration of 400 µM. After dialysis, equal aliquots were collected from donor and receiver chambers. Samples were processed for HPLC quantification following the same protocol as above.

For plasma, the unbound fraction was calculated as $f_{u,p} = C_{buffer} / C_{plasma}$, where C_{buffer} is the concentration measured in the receiver chamber and C_{plasma} is the concentration measured in the donor chamber. For brain homogenate, the unbound fraction in intact brain was estimated using the standard dilution correction: $f_{u,brain} = 1 / [D \times ((1 / f_{u,hom}) - 1) + 1]$, where $D = 5$.

Supplementary Figures

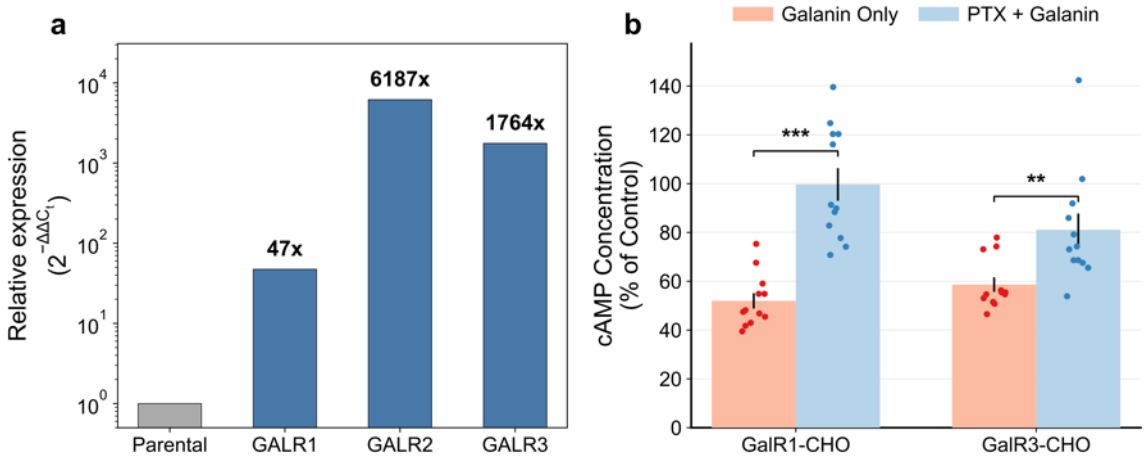

Fig. S1. CHO-K1 model validation. (**a**) Relative expression levels of galanin receptors in stable CHO lines compared to parental line. (**b**) Effects of pertussis toxin on cAMP levels in stable CHO lines (n=12 per group). *p < 0.05, **p < 0.01, ***p < 0.001 (Welch's t-test).

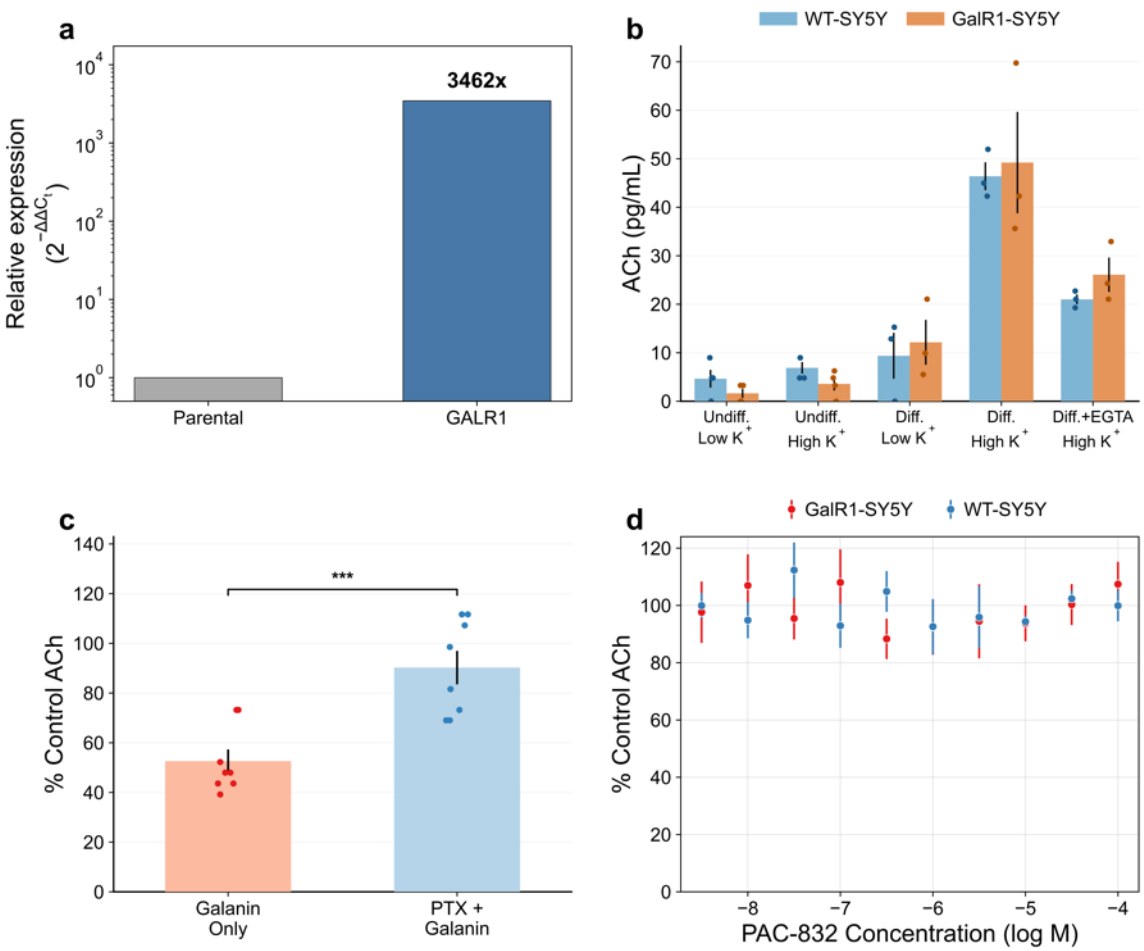

Fig. S2. SH-SY5Y model validation. (**a**) qPCR validation of GalR1 overexpression. (**b**) K^+-induced ACh release in WT-SY5Y and GalR1-SY5Y cells (n=3 per group). Error bars = SEM. (**c**) PTX control for galanin-induced ACh suppression (n=8 per group). *p < 0.05, **p < 0.01, ***p < 0.001 (Welch's t-test). (**d**) PAC-832 alone on ACh release (n=4 per group).

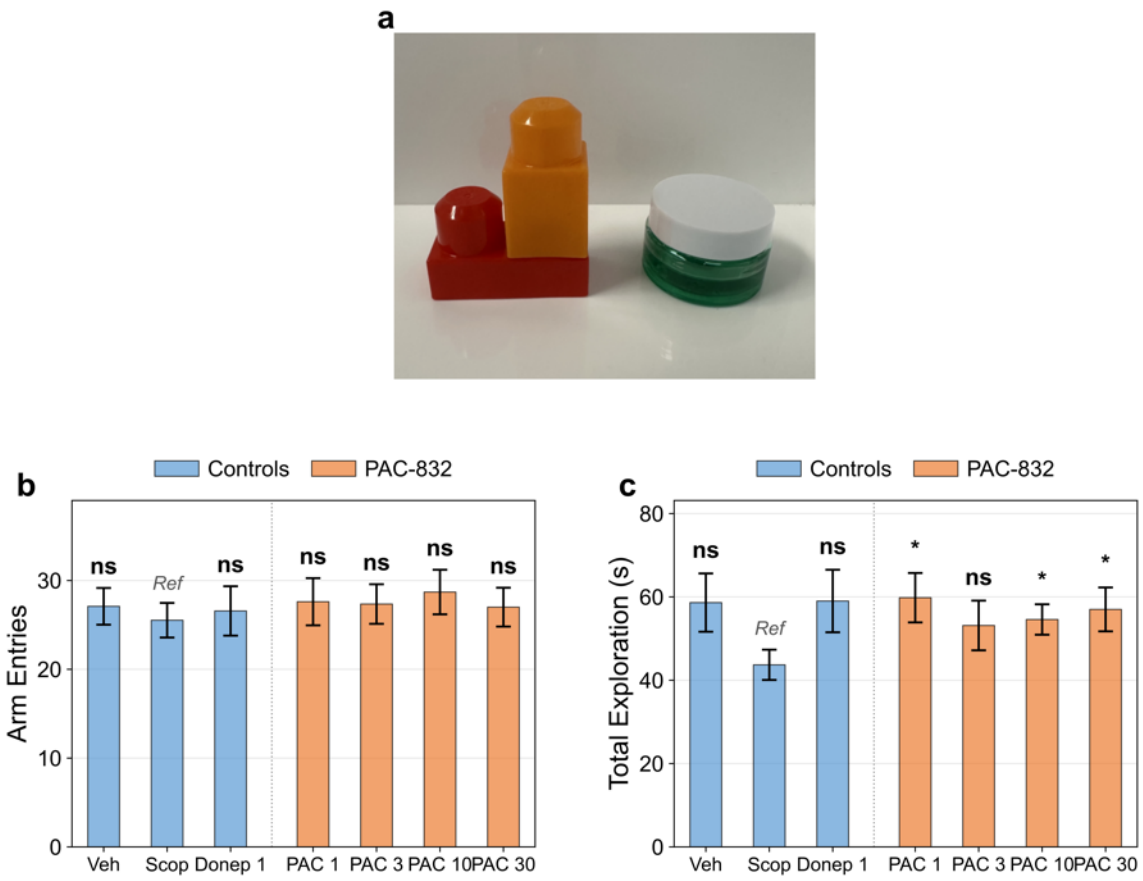

Fig. S3. Behavioral controls. (**a**) Objects used for novel object recognition test. (**b**) Total arm entries in the Y-maze spontaneous alternation test (n=25 per group). (**c**) Total object exploration time in the novel object recognition test (n=25 per group). *p < 0.05, **p < 0.01, ***p < 0.001 (Welch's t-test vs. scopolamine).