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Supplementary Information for

GHB analogs confer neuroprotection through specific interaction with the CaMKIIα hub domain

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Supplementary Information Text

1. Experimental design

A number of techniques ranging from chemical biology, structural biology, biochemistry, *in vitro* and *in vivo* pharmacology were used in this work. Major parts of the *in vitro* studies were exploratory by nature. Prespecified formats with regards to assay design, and data analysis are described in the individual sections and number of biological and technical replicates and specific statistical tests in all relevant cases stated in the figure legends. For *in vivo* studies, power analysis and prespecified components are described in a separate section along with statistics.

2. Materials

2.1. Compounds and radioligands

GHB (γ-hydroxybutyrate) sodium salt, 5-HDC (5-hydroxydiclofenac) and KN-93 were purchased from Sigma-Aldrich (St. Louis, MO, USA). HOCPCA (3-hydroxycyclopent-1-enecarboxylic acid) sodium salt was synthesized as described previously (1) and is available in small amounts by request. The engineered photoligand, SBV-3, was synthesized as described. Purity of ligands were at least 95%. [^{3}H]NCS-382 (spec. activity 20 Ci/mmol, #ART-1114) and [^{3}H]GHB (20 Ci/mmol, #ART-0365) were purchased from Biotrend (Köln, Germany). [^{3}H]-HOCPCA (28.6 Ci/mmol) was prepared as previously described (1). Peptides used for control experiments (CaM binding element peptide (CBEP), LKKFNARRKLKGAILTTMLA; syntide-II, PLARTLSVAGLPGKK; CN21, KRPPKLGQIGRSKRVVIEDDR; TatCN21, GRKKRRQRRRKRPPKLGQIGRSKRVVIEDDR (tat-CN21)) were obtained from Genscript Biotech (Leiden, Netherlands). Peptides were acetylated at the C-terminus, amidated at the N-terminus, purified by HPLC, and subjected to standard trifluoroacetic acid (TFA) removal (as TFA was found to interfere with ligand binding). CBEP corresponds to amino acids 290-309 of the regulatory segment of human CaMKIIα (2). Standard buffers were made using high-grade reagents and solvents purchased from Sigma-Aldrich unless otherwise noted.

2.2. Antibodies and Western blotting reagents

The following primary antibodies were used: streptavidin-HRP (#434323, RRID:AB_2619743; Invitrogen; validated in this study, Extended Data Fig. 2A), CaMKIIα (#NB100-1983, RRID:AB_10001339; mouse monoclonal IgG, clone 6g9, Novus Biologicals; validated in this study, Fig. S2N), CaMKIIβ (#12716, RRID:AB_2713889; mouse monoclonal IgG2b, clone CB-beta-1, Invitrogen; validated in this study, Fig. S7F), phospho-CaMKII (Thr286) (#AP12716b, RRID:AB_10820669; rabbit monoclonal IgG, clone D21E4, Cell Signaling Technology; validated in this study, Fig. S6F), GluN2B (#21920-1-AP, RRID:AB_11232223; rabbit polyclonal IgG, Proteintech; validated by the company (https://www.ptglab.com/products/GRIN2B-Antibody-21920-1-AP.htm#validation), MAP2 (#188004, RRID:AB_2138181; guinea pig polyclonal antiserum, Synaptic Systems; validated by the company (https://www.sysy.com/products/map2/facts-188004.php)), GAPDH (#NB300-221,

RRID:AB_10077627; mouse monoclonal IgG, clone 1D4, Novus Biologicals), Myc tag (#MA1-21316, RRID:AB_558473; mouse monoclonal IgG1, Invitrogen), Na^+/K^+-ATPase (#ab76020, RRID:AB_1310695; rabbit monoclonal, clone EP1845Y, Abcam). Secondary antibodies used were HRP-rabbit anti-mouse (#P0161, RRID:AB_2687969; polyclonal Ig fraction, Agilent), HRP-goat anti-rabbit (#P0448, RRID:AB_2617138; polyclonal, Agilent), Goat anti-mouse Alexa Fluor Plus 647 (#A32728, RRID:AB_2633277; polyclonal IgG, Invitrogen), polyclonal IgG, Jackson ImmunoResearch). Final antibody dilutions used, re-using of antibody solutions and their storage are stated below.

For Western blotting, a '1% protease/phosphatase inhibitor cocktail' was customarily used, consisting of 1% cOmplete™ protease inhibitor cocktail (Roche Diagnostics), 1% phosphatase inhibitor cocktail 2 (#P5726, Sigma-Aldrich) and 1% phosphatase inhibitor cocktail 3 (#P0044, Sigma-Aldrich). For protein determination, either the Bradford assay (#5000006, Bio-Rad Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Copenhagen, Denmark) or the Pierce™ BCA Protein Assay Kit (#23227, Thermo Fisher Scientific, West Palm Beach, FL, USA) were used according to the manufacturer's instructions, as specified.

2.3. Plasmids and mutants

pCMV6-CaMKIIα-Myc-DDK, (#RR201121), pCMV6-CaMKIIγ-Myc-DDK (#RR207416), and pCMV6-CaMKIIδ-Myc-DDK (#RR209882) were from Addgene. pCAGG-CaMKIIβ-pPGK-tdTOMATO was described previously (3). The CaMKIIα hub deletion mutant lacked amino acids 338-stop codon. All constructs used were rat.

2.4. Cell lines

The human embryonic kidney (HEK) 293T cell line was purchased from ATCC (293T-ATCC; #CRL-3216; authenticated to be mycoplasma-free).

3. Methods

3.1. Chemical synthesis

3.1.1. Synthesis of SBV3 precursor 5-(4-((3-Azido-5-(azidomethyl)benzyl)oxy)phenyl)dihydrofuran-2(3H)-one

SBV3-43 was synthesized from precursors 3-azido-5-(azidomethyl)benzyl methanesulfonate and 5-(4-hydroxyphenyl)dihydrofuran-2(3*H*)-one (4). 3-azido-5-(azidomethyl)benzyl methanesulfonate (681 mg, 2.41 mmol) and 5-(4-hydroxyphenyl)dihydrofuran-2(3*H*)-one (430 mg, 2.41 mmol) were dissolved in DMF (20 mL) before K_2CO_3 (500 mg, 3.62 mmol) was added and the resulting solution was stirred at 70 °C overnight. The thick, dark solution was poured into HCl (aq.) (0.1 M, 75 mL) and the aqueous phase was extracted with EtOAc (3 x 75 mL), washed with water (2 x 50 mL) and brine (20 mL), dried ($MgSO_4$) and the solvent was removed *in vacuo*. Column chromatography (2:1 Heptane/EtOAc) yielded SBV3 precursor as a yellow oil (502 mg, 1.38 mmol, 57%). ^1H NMR ($CDCl_3$)): δ 2.14–2.22 (m, H), 2.56–2.68 (m, 3H), 4.35 (s,2H), 5.04 (s, 2H), 5.43–5.46 (m, 1H), 6.91–6.94 (m,1H), 6.97 (d, J = 8.8, 2H),

7.06–7.08 (m, 1H), 7.14–7.16 (m, 1H), 7.27 (d, J = 8.4 Hz, 2H). ^{13}C NMR ($CDCl_3$)): δ 29.27, 30.83, 54.11, 69.15, 81.39, 115.06, 117.54, 118.06, 123.22, 127.19, 131.88, 137.96, 139.62, 158.56, 177.20. LC-MS: MH^+: 365.1.

3.1.2. Synthesis of SBV3

Lithium 4-(4-((3-azido-5-(azidomethyl)benzyl)oxy)phenyl)-4-hydroxybutanoate

5-(4-((3-Azido-5-(azidomethyl)benzyl)oxy)phenyl)dihydrofuran-2(3*H*)-one (50 mg, 0.137 mmol) was dissolved in THF (100 µL) and LiOH (aq) (206 µL, 2N, 0.41 mmol) was added. The orange solution was stirred for 2 h at room temperature. Water was added (10 mL) and the aqueous phase washed with diethyl ether (2 x 3 mL). The aqueous phase was evaporated to give the product as a yellow solid (75 mg, 100%). ^{1}H NMR (D_2O, dioxane): δ 1.81–1.96 (m, 2H), 2.00–2.11 (m, 2H), 4.22–4.25 (m, 2H), 4.49–4.52 (m, 1H), 4.90–4.95 (m, 2H), 6.85–6.92 (m, 3H), 6.96–6.99 (m, 1H), 7.06–7.10 (m, 1H). ^{13}C NMR (D_2O, dioxane): δ 34.12, 34.52, 53.66, 69.34, 73.28, 115.16, 117.79, 118.32, 123.96, 127.68, 136.84, 138.08, 139.43, 140.82, 157.22, 167.36, 171.15, 182.80. LC-MS $(M\text{-}N_2\text{-}OH)H^+$: 337.20.

3.2. Standard radioligand binding assays (brain homogenates)

3.2.1. Rat brain cortical homogenate preparation

Cortical P2 synaptosomally-enriched membranes (referred to as 'homogenate') were prepared from healthy adult male (250–300 g) Sprague Dawley rats obtained from commercial breeders (Janvier) (RRID: RGD_7246927), as described earlier (5). Typically, 20-23 rats were decapitated and tissue pooled for homogenate preparations. Care was taken to collect brains immediately after decapitation. The final pellet (5 x volume/weight) was washed 3-4 times in binding buffer (50 mM KH_2PO_4 buffer, pH 6.0) by a centrifugation-resuspension procedure using a SS-34 fixed angle rotor in a high-speed Sorvall centrifuge (48,000 x *g*) and aliquoted and stored at -20 °C. On the day of the assay, homogenates were quickly thawed by shaking in binding buffer, pelleted and resuspended in binding buffer to approx. 5 mg/mL. Protein concentrations were determined using the Bradford method. Homogenates were kept on ice until use.

3.2.2. Mouse brain crude homogenate preparation

Crude homogenates of cortical tissues from adult male *Camk2a* -/-, *Camk2b* -/- and corresponding +/+ litter mates were individually prepared to generate biological replicates. In brief, brain tissues were quickly dissected after decapitation and stored in liquid nitrogen. Tissues were thawed in ice-cold binding buffer (50 mM KH_2PO_4 buffer, pH 6.0) (5x volume/weight) followed by homogenization using 2 x 1 mm zirconium beads in a Bullet Blender (NextAdvance, NY, USA) for 20 sec at max speed. Homogenates were centrifuged at 11,000 x *g* for 2 min at 4 °C, supernatant removed, and the pellet resuspended in ice-cold binding buffer. Washing was repeated twice, and protein concentration of homogenates was determined using the Bradford method, aliquoted and stored at -20 °C until further use.

3.2.3. Equilibrium binding assays

Inhibitory affinities (K_I values) of 5-HDC and SBV3 were determined using well-established brain homogenate competition assays employing either [^{3}H]NCS-382 (16 nM) or [^{3}H]HOCPCA (5 nM) exactly as previously described (1, 5). Due to a limited amount of compound, SBV3 was only determined in the [^{3}H]NCS-382 assay. In brief, homogenate amounting to 25–40 µg total protein per 96-well was mixed with radioligand and test compound in a total volume of 200 µL. Non-specific binding (NSB) was determined with 1 mM GHB. After 1 h incubation at 0-4 °C, reactions were terminated by rapid filtration through GF/C unifilters (PerkinElmer, Waltham, MA, USA) using a cell harvester (Packard), washed quickly three times with ice-cold binding buffer, dried, and added scintillation liquid MicroScint-0 (PerkinElmer) before counting plates for radioactivity (3 min per well) in a TopCount NXT Microplate Scintillation counter (PerkinElmer). To permit K_I calculations, K_D values as well as actual radioligand concentrations from each experiment were used. Whereas the K_D value for [^{3}H]NCS-382 (430 nM) has been reported previously (5), the K_D value for [^{3}H]HOCPCA (259 nM) was determined by a saturation experiment using radioligand concentrations ranging from 10-15,000 nM and isotope dilutions at concentrations above 500 nM, using 5 mM GHB for NSB.

3.2.4. Data analysis

All homogenate binding assays were performed in technical triplicates using pooled membranes. Data are visualized as pooled data of at least three independent experiments (means ± SEM). Competitive inhibition curves were analyzed using the '*One site–Fit logIC$_{50}$*' function.

Specific binding was corrected for NSB and normalized to the total signal. IC_{50} values were converted to K_I values using the Cheng-Prusoff equation:

$$K_I = \frac{IC_{50}}{1 + [RL]/K_D}$$

where [RL] is the added concentration of radioligand and K_D is the dissociation constant determined from saturation experiments.

Saturation data were fitted to a one-site model by non-linear regression to determine K_D and B_{max}:

$$Specific\ bound = \frac{B_{max}}{1 + K_D/[L]}$$

Specific bound was corrected for NSB determined from each radioligand concentration. For concentrations based on isotope dilution, NSB was extrapolated. In some instances, points from isotope dilution were negative or close-to-zero corrected values in which case they were excluded from the analysis. This amounted to max two data points per curve leaving at least three points in the saturated range. Protein determinations were made by the Bradford method.

3.3. Photoaffinity labeling & affinity purification (target identification)

3.3.1. Photoaffinity labeling (PAL) to hippocampal homogenate

Rat hippocampal homogenate preparation and compound affinity determination was performed as previously described (5). Homogenate was incubated at a protein concentration of 0.125 mg/mL with 600 nM SBV3 for 60 min at 4 °C in the dark (concentration corresponds to 10x the IC_{50} value obtained in the [^{3}H]NCS-382 radioligand binding assay). For competition experiments, 5-HDC was added during this incubation step in increasing concentration. The homogenate was then transferred onto non-tissue culture-treated polystyrene plates and irradiated for 4 min at room temperature using a UVP Benchtop transilluminator (Thermo Fisher) set to high intensity (302 nm, 8 W, M-20 V) for photo-crosslinking to the aromatic azide group of SBV3. Excess SBV3 was subsequently washed away using 1x PBS and centrifugation. For Staudinger-Bertozzi ligation to the alkylic azide group, the membranes were resuspended to a protein concentration of 0.5 mg/mL in 1x PBS and solubilized with 0.1% SDS and 1 mM EDTA for 15 min at 37 °C. EZ-Link™ Phosphine-PEG_3-Biotin (#88901, Thermo Fisher) was added to a final concentration of 200 µM and the reaction was allowed to proceed for 60 min at 37 °C under shaking. Prior to streptavidin affinity enrichment, excess EZ-Link™ Phosphine-PEG_3-Biotin was removed using PD MiniTrap G25 spin columns (#GE28-9180-07, GE Healthcare Biosciences, Pittsburgh, PA, USA).

3.3.2. Streptavidin affinity enrichment

Biotinylated proteins were enriched using Pierce™ High Capacity Streptavidin Agarose (#20361, Thermo Fisher). The solubilized membranes were diluted to a final concentration of 0.01% SDS and incubated with the resin under rotation for 30 min at room temperature. Enrichment was followed by a rigorous washing procedure (3 x 1 min with 10 column volumes (CV) PBS, 0.01% Tween-20 and 3 x 10 min with 10 CV PBS, 0.01% Tween-20). Biotinylated proteins were eluted by boiling in 1x NuPAGE™ LDS sample buffer (#NP0007, Thermo Fisher) supplemented with 100 µM DL-dithiothreitol (DTT) at 100 °C for 10 min under vigorous shaking. Eluates were loaded onto NuPAGE™ 4-12% Bis-Tris gels (Thermo Fisher) and run for 50 min at 175 V. Gels were stained with GelCode™ Blue Stain (#24590, Thermo Fisher) according to the manufacturer's instructions. Gel sections between the 70 kDa and 25 kDa marker (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (#26616, Thermo Fisher) were cut out and diced into 1x1 mm cubes. After destaining using a 1:1 mixture of 5 mM ammonium bicarbonate pH 8.5 (ABC) and 100% acetonitrile for 10 min at room temperature, gel pieces were washed three times with ABC and 100% acetonitrile each. Proteins were reduced using 10 mM DTT for 10 min at 40 °C, followed by alkylation using 50 mM iodoacetamide (Sigma-Aldrich) for 20 min at room temperature in the dark. Samples were in-gel digested using 70 ng/band endoproteinase Lys-C (Sigma-Aldrich) overnight at 37 °C and 175 ng/band trypsin (Sigma-Aldrich) for 8 h at 37 °C. After acidification to a concentration of 1% formic acid, peptide extracts were loaded onto in-house packed C_{18} STAGE Tips (#14-386, 3M™ Empore) and eluted into a 96-well microtiter plate with 2 x 20 µL 40% acetonitrile, 0.5% acetic acid in water, followed by removal of organic solvents in a vacuum centrifuge and reconstitution of peptides in 2% acetonitrile, 0.5% acetic acid, 0.1% TFA in H_2O.

3.3.3. Target identification by LC-MS/MS data analysis

Raw LC-MS/MS data was processed using the MaxQuant software (v. 1.5.5.1) and further data analysis was performed using Perseus (v. 1.5.6.0, Max-Planck Institute of Biochemistry, Department of Proteomics and Signal Transduction, Munich, Germany), Microsoft Office Excel and GraphPad Prism (v. 7). The MaxQuant search against the rat and mouse UniProt databases (downloaded 13.03.2017), led to the identification of 1184 protein hits. In addition, the default contaminant protein database was included and any hits to this were excluded from further analysis. Further, proteins only identified by site were excluded. Carbamidomethylation of cysteine was specified as a fixed modification; phosphorylation of serine, threonine and tyrosine residues, oxidation of methionine, pyro-glutamate formation from glutamine and protein N-terminal acetylation were set as variable modifications. Proteins were quantified using the label free quantification (LFQ) algorithm (6). The LFQ intensity of proteins selectively labeled with SBV3 should decrease in a concentration-dependent manner with increasing concentrations of the competing ligand 5-HDC. Unspecific binding or endogenously biotinylated proteins should not show this concentration-dependent behavior. Proteins only identified by site or from the reverse database were excluded. Data were then exported after initial filtering in Perseus to GraphPad Prism, and non-linear regression was performed for all proteins using the '*One site–Fit* $logIC_{50}$' function. Best-fit values for Top (upper plateau of the concentration-response curve) were plotted against the coefficient of determination (R^2) values to identify proteins with competitive concentration-dependence behavior (*refer to Table S1* and *Data S1*).

3.3.4. Validation of PAL by anti-biotin Western blotting

To verify the photoaffinity labeling of GHB high-affinity binding sites by SBV3 and competition by 5-HDC, anti-biotin Western blotting using HRP-streptavidin was performed. Samples were prepared by adding 4x NuPAGE™ sample buffer (Thermo Fisher) and 100 mM DTT. Samples were denatured for 10 min at 40 °C, followed by centrifugation for 5 min at 11,000 x *g*. Samples were loaded onto Mini-PROTEAN® TGX™ gels (Bio-Rad) and run for 40 min at 200V in 1x Tris/glycine/SDS (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) running buffer. Proteins were transferred to a PVDF membrane using the Trans-Blot® Turbo™ System (#1704156, Bio-Rad) and blocked with 3% BSA in 1x tris-buffered saline (TBS) with 0.1% Tween-20 detergent (TBS-T) for 1 h at room temperature. The membrane was then incubated with 0.5 mg/L streptavidin-HRP in 1% BSA in 1x TBS-T for 30 min at room temperature, followed by three quick washes with milliQ H_2O and three washes with 1x TBS-T for 20 min at room temperature. The membrane was developed using Pierce ECL western blotting substrate (#32106, Thermo Fisher) for 4 min in the dark. Chemiluminescence was read using a FluorChem HD2 (Alpha Innotech, San Leandro, CA, USA).

3.3.5. PAL on knockout tissue homogenates

To further verify the observed selectivity for CaMKIIα, SBV3 as tested using crude homogenates from *Camk2a* and *2b* -/- and +/+ tissues exactly as described for PAL to hippocampal homogenates followed by anti-biotin Western blotting.

3.4. *In vitro* autoradiography

In vitro autoradiography with tritiated GHB radioligands was performed on slices from adult male *Camk2a* -/- and *Camk2b* -/- mice using wildtype (WT; +/+) littermates as controls (further detailed under *in vivo* section). The procedure was exactly as previously described using phosphorimaging (7), using either 1 nM [^{3}H]HOCPCA 7 nM [^{3}H]NCS-382 incubated for 30 min at room temperature or 30 nM [^{3}H]GHB incubated for 30 min at 4 °C. For determination of non-specific binding, either 1 mM GHB or HOCPCA (1 mM) were used. Section anatomy was validated using cresyl violet staining as described (7). Experiments were carried out on individual mice (*n* = 3-4) in four technical replicates.

3.5. CaMKIIα expression in HEK cells and radioligand binding assays

3.5.1. Cell culturing and transfection of CaMKII in HEK293T cells

HEK293T cells were maintained in DMEM GlutaMAX medium (#61965026, Gibco) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (#15140122, Invitrogen), in a humidified 5% CO_2 atmosphere at 37 °C. Transfections with various CaMKII constructs were performed using PEI (Polysciences Inc., Warrington, PA, USA). The day before transfection, cells were seeded in 15 cm culture dishes at a density of 4.5 x 10^6. On the day of transfection, 16 µg plasmid DNA was diluted in 2 mL serum-free medium and 48 µL 1 mg/mL PEI added. After 15 min of incubation at room temperature, the DNA/PEI mixture was added to the cells. Transfected cells were used for Western blotting or [^{3}H]HOCPCA radioligand binding experiments.

3.5.2. Western blotting

Membranes were probed with two primary antibodies targeting the myc-tag or CaMKIIβ (both diluted 1:1,000 and stored at -20 °C) and Na^+/K^+-ATPase (diluted 1:10,000 and stored at -20 °C) for 1 h at room temperature followed by 3 x 5 min washes with TBS-T. Subsequently, membranes incubated with species-specific secondary antibody, anti-mouse-HRP and anti-rabbit-HRP (1:2,000, 1 h at room temperature, stored at 4 °C) followed by 3 x 10 min washes with TBS-T. All antibodies were prepared in 1% (w/v) BSA in TBS-T and re-used up to four times. Membranes were probed with 1:1 mixture of ECL Detection Reagent (GE Healthcare) for up to 4 min before capturing the luminescence signal using a FluorChem HD2 (Alpha Innotech).

3.5.3. [3H]HOCPCA binding assay to whole cell homogenates

Whole cell homogenates were prepared 48 h post-transfection by washing the cells with ice-cold PBS and harvesting by scraping. Cells were collected and centrifuged for 10 min at 1,500 x *g*. Cell pellets were resuspended in ice-cold binding buffer (50 mM KH_2PO_4 buffer, pH 6.0), and homogenized using 2 x 1 mm zirconium beads in a Bullet Blender (NextAdvance, NY, USA) for 20 sec at max speed. Protein concentration was determined using the Bradford method. Aliquots were stored at -20 °C until the day of assay.

Binding experiments were performed in glass tubes ((#10682424, Corning) using a 48-well setup optimized to a protocol including 100-200 µg protein per well, 40 nM [^{3}H]HOCPCA for competition binding and test compound in 400 µL total volume of

binding buffer (50 mM KH_2PO_4 buffer, pH 6.0). Either WT or mutant CaMKII whole-cell homogenates were used for the competition binding. NSB was determined with 30 mM GHB. Saturation experiments employed [^{3}H]HOCPCA in concentrations 10-10,000 nM (isotope dilution above 500 nM). Equilibrium binding was achieved by incubation for 60 min at 0-4 °C. To permit filtration, proteins were precipitated by addition of ice-cold acetone (4x of assay volume) followed by vortexing and incubation at -20 °C for 60 min. Protein-ligand bound complexes were collected by rapid filtration through GF/C unifilters (Whatman Schleicher and Schuell, Keene, NH) using a Brandell M48-T cell harvester (Alpha Biotech) and rapid washing with binding buffer. Radioactivity counts (DPM) was measured in a Packard Tricarb 2100 liquid scintillation counter using 3 mL of OptiFluor scintillation liquid (PerkinElmer) and counting for 3 min per sample. All experiments were replicated in at least three individual experiments with technical triplicates using at least three different batches of cell homogenate. IC_{50} values were converted to K_I values by means of the Cheng-Prusoff equation (8). K_D values were obtained from saturation experiments. For concentrations based on isotope dilution, NSB was extrapolated. In some instances, points from isotope dilution were negative or close-to-zero corrected values in which case they were excluded from the analysis. This amounted to 0-1 data points of the individual saturation experiments leaving at least three points in the saturated range. Protein determinations were made by the Bradford method.

3.6. Protein purification and crystallography

3.6.1. Expression and purification of the CaMKIIa hub domain

The human CaMKIIα hub domain (UniprotKB Q9UQM7, residues 345-475), WT or containing six mutations (Thr354Asn, Glu355Gln, Thr412Asn, Ile414Met, Ile464His, and Phe467Met, referred to as the 6x Hub), was expressed and purified similar as to previously described (9, 10). Briefly, the hub domain with an N-terminal 6His-prescission protease expression tag was inserted into a pSKB2 vector with kanamycin resistance. BL21 (DE3) *E. coli* cells transformed with the vector were cultured in TB media supplemented with phosphates. Protein expression was induced at OD_{600} = 0.6-0.8 by addition of 1 mM IPTG. Expression proceeded for 15-18 h while shaking at 18 °C. All subsequent purification steps were performed at 4 °C and all columns were made by GE Healthcare unless otherwise noted.

Induced cells were pelleted, resuspended in buffer A (25 mM Tris, 150 mM KCl, 50 mM imidazole, 0.5 mM DTT, 10% (v/v) glycerol, pH 8.5 at 4 °C), and lysed using a cell disrupter. Soluble cell lysate was passed over a 5 mL Nickel IMAC column. The column was washed with buffer A, and immobilized protein was eluted with a mixture of 25% buffer A and 75% buffer B (25 mM Tris, 150 mM KCl, 1 M imidazole, 10% glycerol, pH 8.5 at 4 °C). A HiPrep 26/10 column was used to exchange the protein into buffer C (25 mM Tris, 150 mM KCl, 10 mM imidazole, 1 mM DTT, 10% glycerol, pH 8.5 at 4 °C). Prescission protease was added overnight to remove the 6His tag. The protein was then concentrated using Amicon Ultra filters with a molecular weight cutoff of 50 kDa. A final purification step was performed using a Superose-6 gel filtration column equilibrated with the final protein storage buffer (25 mM Tris, 150 mM KCl, 10% (v/v) glycerol, 2 mM DTT, 1 mM TCEP, pH 8.0 at 4°C). Fraction purity was assessed by SDS-PAGE.

Sufficiently pure fractions were pooled, concentrated, and flash-frozen in liquid nitrogen for storage at -80 °C.

3.6.2. Co-crystallization of 5-HDC with the 6x Hub
Crystals were grown via sitting drop vapor diffusion at 20 °C. Reservoir solution contained 20% w/v PEG3350, 200 mM potassium acetate, pH 8.1. Protein stock concentration was 17 mg/mL. 5-HDC was dissolved in DMSO to 100 mM and 5 µL was added to 50 mL of protein solution for a final 5-HDC concentration of approximately 10 mM, 10x the protein molarity. The protein and 5-HDC were incubated at 4 °C for 2 h prior to dispensing. 200 nL drops were dispensed by adding 100 nL of protein stock to 100 nL of reservoir solution. Drops were equilibrated against 45 µL of reservoir solution. Crystals were harvested and frozen in cryoprotectant (20% w/v PEG3350, 20% v/v glycerol, 100 mM HEPES pH 7.0) 7 days after trays were set. For X-ray diffraction data see Table S4.

3.6.3. X-ray structure determination
X-ray diffraction data was collected at the Advanced Light Source, Beamline 8.3.1, at wavelength 1.115830 Å and temperature 100 K. Data were processed using XDS (11) and scaled and merged with Aimless (12) in the CCP4 suite (13). The space group was C2221 with cell dimensions a = 101.43, b = 182.96, c = 106.47, $\alpha = \beta = \gamma = 90°$. Phases were determined by molecular replacement using Phenix Phaser (14). A previously determined structure of the protein in an unliganded state (PDB entry 6OF8) was used as the search model (9). Phenix Refine (14) was used for structure refinement and Coot (15) was used for model building. The cif dictionary file for 5-HDC was generated using Phenix Elbow (16). Software used in this project was curated by SBGrid (17). Electron density in the shape of the compound was seen at the hypothesized binding sites of 6 of the 7 subunits in the crystallographic asymmetric unit prior to any structure refinement. Partial occupancy of the ligand at the binding site of the seventh subunit (chain A, the lone 'Trp in' subunit) was observed but not modeled (See electron density maps in *Fig. S5*; data collection and refinement of X-ray data in *Table S4*).

3.7. Computational modeling of Trp403 movement

All computational work was performed using the Maestro Schrödinger package (Schrödinger Release: 2018-1). The co-crystal structure complex of CaMKIIα-5-HDC was prepared using the Protein Preparation Wizard (18) including hydrogen optimization at pH 6.4 of the ionizable polar groups using PROPKA (19). The PDB entry 5IG3 (2) was used to illustrate the 'Trp-in' observation. The Trp403 movement comparison was observed via a Protein Superimposition of the backbone atoms of both our reported ligand bound structure reported herein and that of the PDB entry 5IG3 (RMSD=1.091 Å).

3.8. Biophysical & biochemical assays

3.8.1. Surface plasmon resonance (SPR) biosensor analysis
SPR measurements were performed at 25 °C using a Pioneer FE instrument (Molecular Devices, FortéBio). 6x Hub human protein, WT hub or full-length human recombinant

CaMKIIα (#02-109, Carna Biosciences) were immobilized on to a biosensor surfaces by amine coupling using a 20 mM NaAc pH 5 immobilization buffer. HBS-P (10 mM Hepes, 150 mM NaCl, 0.005% Tween-20, 1 mM DTT) pH 7.4 or pH 6 running buffer were used for all experiments with CaMKIIα hub, while for experiments with full-length CaMKIIα, HBS-P buffer pH 7.4 supplemented with $CaCl_2$ (Ca^{2+}) (500 μM) was used. The ligands were injected in 2-fold serial dilution over sensor chip surface with immobilized proteins. Control peptide CBEP and calmodulin (CaM) were used as positive controls to evaluate activity of the SPR assay with CaMKIIα hub and full-length protein, respectively. Between CaM injections, the biosensor chip surface was regenerated by injections of HBS-P buffer supplemented with 100 μM EDTA. The data were analyzed using Qdat Data Analysis Tool version 2.6.3.0 (Molecular Devices, FortéBio). The sensorgrams were corrected for buffer bulk effects and unspecific binding of the samples to the chip matrix by blank and reference surface subtraction (flow cell channel activated by injection of EDC/NHS and inactivated by injection of ethanolamine). The dissociation constants (K_D) were estimated by plotting responses at equilibrium (R_{eq}) against the injected concentration and curve fitted to a Langmuir (1:1) binding isotherm.

3.8.2. Intrinsic tryptophan fluorescence (Trp flip) assay

Human hub recombinant purified proteins and compounds (HOCPCA and 5-HDC) were diluted in assay buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM DTT) and mixed in a microplate to obtain a protein concentration of 5 μM 6x Hub and 3.4 μM WT hub. For absorbance and background fluorescence measurements, compounds were mixed with buffer for each compound concentration. Neither HOCPCA nor 5-HDC showed fluorescent properties. All measurements were performed in black half-area 96-well format low-binding OptiPlates (#6052260, PerkinElmer) for fluorescence and half-area UV-Star microplates (#675801, Greiner Bio-One) for absorbance. All measurements were recorded at 25 °C on a Safire^2 plate reader (Tecan). Emission was recorded in the wavelength range of 300-450 nm with 1 nm increments and an excitation wavelength of 290 nm with 5 nm band widths. Fluorescence intensities at 340 nm were used for data analysis. To check for inner filter correction, the absorbance was measured in the range of 270-400 nm. Neither compound showed absorbance or inner filter effect.
The fluorescence intensities were normalized according to:

$$F = \frac{(F_{obs} - F_b) - F_{min}}{F_{max} - F_{min}}$$

F_{obs} is the observed fluorescence intensity and F_b is the background fluorescence for compound in buffer alone. F_{max} is the fluorescence intensity of hub alone without compound, and F_{min} is the fluorescence intensity when plateau is reached at high compound concentrations in the presence of hub. Since HOCPCA did not reach a plateau at high compound concentrations, F_{min} was set to the fluorescence intensity of buffer for all compounds tested. Fluorescence intensities usually spanned from 10,000-55,000 for 5-HDC and 36,000-59,000 for HOCPCA, while the fluorescence intensity for buffer was around 1000. Non-linear regression was used for curve-fitting using the equation for '*log(inhibitor) vs. response with variable slope*' to determine IC_{50} values (GraphPad

Prism, v. 8). All curves are pooled data (means ± SEM) each performed in technical triplicates using at least two different batches and several different aliquots of protein. For 6x Hub (n = 8) and for WT hub (n = 2-5). Supporting computational modeling is described in section 3.7.

3.8.3. ADP-Glo assay

Syntide-II substrate phosphorylation was assayed using the ADP-Glo Assay kit (#V9101, Promega) using CaMKIIα full-length protein with a N-terminal GST-tag (#02-109, Carna Biosciences) or CaMKIIα full-length protein with a C-terminal 6xHis-tag (#PR4586C, Thermo Fisher). All experiments were performed in 384-well white polypropylene plates (#784075, Greiner) with a working volume of 25 µL. Kinase detection reagent was prepared according to manufacturer's protocol. Linearity of the assay was assessed using various concentrations of ADP to ATP mixture in the ADP-Glo kinase reaction buffer (40 mM Tris (pH 7.5), 0.5 mM $CaCl_2$, 20 mM $MgCl_2$, 0.1 mg/mL BSA, 30 µg/mL calmodulin (#P1431, Sigma-Aldrich), 50 µM DTT) as described in the manufacturer's protocol. For inhibitor studies, the total assay volume was 20 µL, the volume of the kinase reaction was 5 µL (1 µL 5x inhibitor + 2 µL 2.5x CaMKIIα (= 3 ng final) + 2 µL 2.5x substrate mix (= 25 µM ATP and 50 µM syntide-II final concentrations). Kinase reactions were incubated 60 min at 37 °C, after this 5 µL ADP-Glo reagent was added to each well and incubated for 40 min at room temperature. Finally, 10 µL kinase detection reagent was added to each well and after 30 min of incubation at room temperature, luminescence was recorded on an EnSpire® Multimode Plate Reader (PerkinElmer). Kinase reactions without enzyme or substrate were used as controls. Curve fitting and data analysis was done using GraphPad (v. 8), and IC_{50} values were determined using the equation for '*log(inhibitor) vs. response with variable slope'*. All curves are pooled data (means ± SEM) of at least three independent experiments each performed in technical triplicates.

3.8.4. Differential scanning fluorimetry (DSF)

Thermal melting points (T_m) of the CaMKIIα WT hub with and without the presence of GHB, HOCPCA or 5-HDC were assessed by DSF measured on a Mx3005P qPCR System (Agilent Technologies, Waldbronn, Germany). Samples were prepared in qPCR 96-well plates (25 µL/well) with a final concentration of 0.1 mg/mL CaMKIIα and 8x SYPRO® Orange Protein Gel Stain (#S6650; Life Technologies) in MES buffer (20 mM MES, 150 mM NaCl, 1 mM DTT; pH 6). Compounds were tested in 3-fold dilution series. Fluorescence was monitored using excitation at 492 nm and emission at 610 nm from 25–114 °C in 90 cycles with a 1 °C temperature increase per min. Data was processed in GraphPad Prism (v. 8). T_m values were calculated by fitting the sigmoidal curves of normalized fluorescence intensity versus temperature to
the Boltzmann equation. The difference in T_m (ΔT_m) of each compound concentration compared to CaMKIIα WT hub was plotted as concentration-response curves and maximum ΔT_m was obtained via non-linear regression using the ''*One site–Fit logIC$_{50}$*' function. Experiments were performed in at least three independent experiments using singlicates.

3.9. *In vitro* assays cultured neurons

3.9.1. Preparation and culturing of cortical neurons

Primary cortical neurons were prepared from E16-E18 embryos from time-mated female C57BL6/*J*Rj female mice (Janvier Laboratories, Le Genest-Saint-Isle, France) or from genetically modified *Camk2a* -/- or +/+ mice as described). Tissue dissociation and neuronal isolation (approximately 6-9 embryos per female mouse) was performed using validated antibodies with MACS® technology according to the manufacturer's description (#130-094-802 and #130-115-389, Miltenyi Biotec). When more than $6x10^7$ cells were obtained from tissue dissociation, two separation columns were used in neuronal isolation. As a result, approximately $2x10^7 – 3x10^7$ cells were frequently obtained. Primary hippocampal neuronal cultures were prepared according to the procedure described in Goslin and Banker (1991) (20). Briefly, hippocampi were isolated from brains of E16.5 embryos and collected altogether in 10 ml of Neurobasal™-A medium (NB) (#10888-022, Invitrogen) on ice. After two washings with NB, the neurons were dissociated with Gibco™ trypsin/EDTA solution (#25300054, Thermo Fisher). Isolated cortical and dissociated hippocampal neurons were resuspended in NB supplemented with 2% B-27™ Supplement (#17504044, Invitrogen), 1% GlutaMAX™ Supplement (#35050038, Invitrogen) and 1% penicillin-streptomycin (Invitrogen), and plated in poly-D-lysine-coated 96-well, 12-well or 24-well dishes (~60,000, 100,000 or 400,000 cells/well). Cultures were maintained at 37 °C and 5% CO_2 and fed every 48-62 h, where half of the conditioned media were replaced with fresh media.

3.9.2. Determination of mRNA levels by qPCR

To determine the expression level of *Camk2a* and *Camk2b* during culturing time days-in-vitro (DIV) 4-14, mRNA levels were verified by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Cortical neuronal cultures plated in 24-well dishes were harvested in RLT plus Lysis buffer (Qiagen) and stored at -80 °C prior to RNA extraction. Each sample was homogenized with zirconium oxide beads (2 x 2 mm) using a Bullet Blender and total RNA was extracted with RNeasy Plus Mini kit (#74136, Qiagen) and PureLink® DNase (#12185010, Invitrogen) according to the manufacturer's instructions with minor changes. Total RNA amounts were quantified using NanoDrop 2000 (Thermo Fisher). 500 ng of RNA was reverse-transcribed using qScript® cDNA Supermix (Quanta Bio) on an Eppendorf Mastercycler Personal PCR machine. The cDNA was diluted to approximately 100 ng/µL and stored at -20 °C until RT-qPCR was performed. The PCR samples were prepared on ice by combining 5 µL cDNA, 10 µL Power SYBR green Master Mix (2x) (Applied Biosystems) and 0.15 µL of forward and reverse primers (*Camk2a* (F) 5'-GCTCTTCGAGGAATTGGGCAA-3' (R) 5'CCTCTGAGATGCTGTCATGTAGT-3', *Camk2b* (F) 5'GCACACCAGGCTACCTGTC-3' (R) 5'-GGACGGGAAGTCATAGGCA-3', succinate dehydrogenase A (*SDHA*) (21) (housekeeping gene) (F) 5'-GGAACACTCCAAAAACAGACCT-3' (R) 5'-CCACCACTGGGTATTGAGTAGAA-3'; TAG Copenhagen) with distilled water to a final volume of 20 µL. Further, the RT-qPCR was initiated by 10 min heating to 95 °C followed by 40 PCR cycles of 15 s at 95 °C and 60 s at 60 °C on a Stratagene Mx3005P (Agilent Technologies). The cycle

threshold (Ct) of the fluorescent PCR product was determined by default in MxPro qPCR Software (Agilent Technologies), and used to calculate the relative fold gene expression:

$$\Delta CT = 2^{(Ct_{housekeeping} - Ct_{target})}$$

Data are given as means ± SEM from three independent cultures (*n* = 3) each performed in technical triplicates.

3.9.3. Determination of CaMKIIα protein levels by Western blotting

Protein expression levels (DIV 4-20) were examined by Western blot. Neurons (400,000 cells/well in 24-well dish) were harvested in 150 µL ice-cold radioimmunoprecipitation (RIPA) buffer supplied with a 1% protease/phosphatase inhibitor cocktail. Protein concentrations were determined with the Pierce BCA method, and neuronal samples were stored at -80 °C until Western blot analysis. Western blot samples were prepared by adding 4x Fluorescent Compatible Sample Buffer (Thermo Fisher), 100 mM DTT and harvested neuron samples to a final concentration of 1 mg/mL. Samples were heated for 10 min at 37 °C, followed by sonication and centrifugation for 2 min at 4 °C and 11,000 x *g*. 10 µg sample were loaded on 4-20% Mini-PROTEAN® TGX™ gels (Bio-Rad) with iBright™ Prestained Protein Ladder (#LC5615, Invitrogen). SDS-PAGE was performed at 200 V for 40 min with Tris/glycine/SDS running buffer. Proteins were transferred to a low-fluorescence polyvinylidene difluoride (PVDF) membrane (Thermo Fisher) using the Trans-Blot® Turbo™ transfer system (Bio-Rad). Membranes were blocked for 30 min at room temperature under constant agitation with 1x Blocker™ FL Fluorescent Blocking Buffer (#37565, Thermo Fisher). Membranes were probed with primary antibody targeting CaMKIIα (1:1,000, incubation overnight at 4 °C, stored at -20 °C), CaMKIIα-pThr286 (1:1,000, incubation overnight at 4 °C, stored at -20 °C) or GAPDH (1:10,000, incubation 1 h at room temperature, stored at 4 °C) followed by 3 x 5 min washes with TBS-T. Subsequently, membranes were incubated with species-specific secondary antibody (1:2,000, 1 h at room temperature, stored at 4 °C) followed by 3 x 10 min washes with TBS-T. All antibodies were prepared in 1% (w/v) BSA in TBS-T and re-used up to five times. Images were detected with the iBright FL1500 imaging system (Invitrogen) and quantified in Image Studio (Lite version 5.2). CaMKIIα-pThr286 signals were normalized to total CaMKIIα. Data are from one culture carried using three different wells (means ± SD).

3.9.4. [^{3}H]HOCPCA binding

Neurons were grown in 6-cm dishes (DIV 10) and harvested in ice-cold binding buffer (50 mM K_2PO_4, pH 6.0) and a crude homogenate prepared. Briefly, cells were collected in Eppendorf tubes, subjected to three centrifugation (11,000 x *g*)-resuspension steps and Bullet Blender homogenization. Membranes were diluted to give 10-20 µg final protein per well and competition binding carried out using [^{3}H]HOCPCA (40 nM) and GHB (8 mM) for NSB as previously described (1). Data are from three different cultures carried out in technical replicates (means ± SEM).

3.9.5. pThr286 autophosphorylation
Cortical neuronal cultures (DIV 16-20) plated in 24-well dishes were incubated for 1 h (37 °C, 5% CO_2) with the desired compounds diluted in assay buffer (HBSS supplied with 20 mM HEPES, pH 7.4). Buffer was used for measurement of basal and Ca^{2+} as stimulation control, typically 5 mM. Subsequently, neurons were harvested in 150 μL ice-cold RIPA buffer supplied with a 1% protease/phosphatase inhibitor cocktail. Protein concentrations were determined using Pierce BCA method, and neuronal samples were stored at -80 °C until Western blot analysis targeting CaMKIIα and CaMKIIα-pThr286 as described in the section 'Determination of CaMKIIα protein levels by Western blotting'. Statistical analysis was performed with GraphPad Prism (v. 8). If groups had equal variances, One-way ANOVA was used, followed by Dunnett's post-hoc test comparing Ca^{2+} stimulation with treatments. If no equal variance was obtained, statistical analysis was done with Brown-Forsythe and Welch ANOVA, followed by Dunnett's T3 multiple comparisons test to compare basal and treatment. Exact number of replications/batches of cultures are stated in the legend to Fig. 3.

3.9.6. Excitotoxic stimulation
Cortical neuronal cultures (DIV 16-18) were exposed to a pathological stimulus of 1-200 μM L-glutamate (Glu) plus 20 μM glyine (Gly) for 1 h (37 °C, 5% CO_2). HBSS supplemented with HEPES (pH 7.4) was used as assay buffer. The neuronal media was replaced with 50 μL assay buffer and an equal volume of assay buffer supplemented with 2x Glu/Gly was added. Subsequently, the cultures were washed with assay buffer and replaced with fresh NB medium. When compounds were added during the excitotoxic insult, half of the assay buffer was replaced with buffer containing 1x Glu/Gly and 2x compound. With application of compounds following excitotoxic insult, half of the fresh NB medium was replaced with NB medium containing 2x compound. All compound incubations were 1 h at 37 °C and 5% CO_2. HOCPCA was used in the indicated concentrations. Tat-CN21 (10 μM) was used as a positive control. Finally, the neurons were washed with assay buffer and incubated 20-24 h at 37 °C and 5% CO_2 in NB until further assay analysis.

Hippocampal neuronal cultures (DIV 16-17) were stimulated with 400 μM Glu in NB (supplemented with B-27, GlutaMAX and penicillin-streptomycin) for 5 minutes. After stimulation, the neurons were placed back in their original medium and incubated for 1 h at 37°C and 5% CO_2. HOCPCA (1 mM) and H_2O (control) were added 1 hour after stimulation, followed by 20-24 h incubation at 37 °C and 5% CO_2 until further analysis. Tat-CN21 (10 μM) was added for 20 min before Glu stimulation.

3.9.7. Cell viability lactate dehydrogenase (LDH) assay
Following 20-24 h Glu/Gly stimulation (for optimization see *Fig. S7*), neuronal cell death was assessed using the CyQuant™ lactate dehydrogenase (LDH) Cytotoxic Assay kit (#C20301, Invitrogen) according to the manufacturer's instructions using 96-well flat-bottom ELISA plates (#655101, Greiner). Triplicates or quadruplicates were performed with each condition. Control wells containing only media were subtracted before further calculation of percentage LDH release compared to maximum cell death. One-way ANOVA was used for statistical analysis in GraphPad Prism (v. 8), followed by

Dunnett's post-hoc test comparing control (Glu/Gly alone) with treatment. Exact number of replications/batches of cultures used are stated in the legend to Fig. 3.

3.9.8. Live/death staining

Hippocampal neurons were stained 20-24 h after Glu stimulation with calcein AM (1:2000, #425201, Biolegend, stored at -20°C) and ethidium homodimer-1 (1:2000, E1169, Invitrogen, stored at -20°C) in DPBS. The cultures were incubated for 20 min at 37°C and 5% CO_2 and imaged with a confocal microscope (Zeiss LSM700). Three images were taken per well and analysed with Fiji ImageJ (National Institutes of Health, USA) to determine the live/dead ratio per well. One-way ANOVA was used for statistical analysis in GraphPad Prism (v. 8), followed by Dunnett's post-hoc test comparing Glu with treatment and control.

3.9.9. Co-localization studies in hippocampal neurons

Primary hippocampal neurons (DIV 14-19) plated in 12-well dishes were used for immunocytochemistry. Media was removed and replaced with Tyrode's solution (5 mM) with tetrodotoxin (TTX, 1 μM, #1078), NBQX (50 μM, #0373, both from Tocris Biosciences, Bristol, UK), and L-AP5 (10 μM, #A5282, Sigma-Aldrich) to block the basal activity of the neurons. After 10 min in H_2O, tat-CN21(10 μM) according to (22), or HOCPCA (2 mM) were added to the cultures for 20 min, followed by 2 min stimulation with Glu (400 μM). The neurons were fixated with 4% PFA/4% sucrose for 10 min at room temperature and washed 3 times 5 minutes in PBS. The coverslips with the fixated neurons were incubated in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30 mM phosphate buffer (PB), pH 7.4) with the primary antibodies: rabbit polyclonal GluN2B (1:100, stored at -20°C), mouse CaMKIIα (1:100) and guinea pig MAP2 (1:500) (both stored at 4°C) overnight in the dark at 4 °C. All antibodies were used only once. After incubation, coverslips were washed 3 times in PBS at room temperature, followed by a 1 h incubation at room temperature in GDB buffer with the secondary antibodies donkey anti-rabbit Alexa488 (1:100), donkey anti-mouse Cy3 (1:100), donkey anti-guinea pig Alexa647 (1:100, all stored at -20°C). Finally, the coverslips were washed 3 times 5 min in DPBS, rinsed briefly in MilliQ, mounted with Mowiol® 4-88 (#81381, Sigma-Aldrich) and dried overnight at 4 °C. An LSM700 Zeiss confocal microscope was used to make airyscan images of the neurons. Five to seven images were taken per coverslip. Colocalization of GluN2B and CaMKIIα in the secondary dendrites was determined using the Confined Displacement Algorithm (CDA) plugin in Fiji ImageJ. Data was normalized to the control group. For statistical analysis, GraphPad Prism (v. 8) was used. To show a significant effect of the treatments, one-way ANOVA was used, followed by Dunnett's post-hoc test comparing control with treatment. Exact number of replications/batches of cultures used are stated in the legend to Fig. 4.

3.10. *In vivo* & *ex vivo* mouse studies

3.10.1. Animals and mouse breeding

Specific details pertaining to age, weight and sex of animals used are given in the relevant sections. All breeding protocols and animal experiments were approved by the respective University animal ethics committees following national and international guidelines.

Mice for *in vitro* studies (homogenate binding and autoradiography) were *Camk2a* (*Camk2a*^{tm3Sva}, MGI:2389262) or *Camk2b* knockouts (-/-) or corresponding littermates (+/+) backcrossed in the C57BL/6*J* background. Generation of *Camk2b* knockout mouse lines has been described elsewhere (23). *Camk2* lines were bred as heterozygotes to generate +/+ and -/- littermates, except for neuronal cultures where homozygous breeding, between either +/+ or -/- mice was also used. Genotyping was performed by a technician blinded to the experiments.

3.10.2. Study design & power analysis

All *in vivo* stroke studies were approved by the University of Otago Animal Ethics Committee and are reported according to the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines. For behavioral experiments, six animals per group are required to achieve >80% power (86% calculated), considering the following parameters: α=0.05; with an effect size=1.5. For histological experiments, 5 animals per group are required to achieve >80% power (91% calculated), considering the following parameters: α=0.05; effect size 1; 3 concentrations; 2 groups, and correlation between measures=0.5. Parameters were determined from our prior work, in which we have demonstrated significant behavioural effects (24), and on the assumption that variance was about 25%. It should be noted that more conservative effect sizes were used for such experiments, as it is harder to assess recovery over time between groups than looking at the effects of drug treatments on stroke size. Mice were allocated randomly to treatment groups, and the experiments performed blinded.

3.10.3 Temperature recordings in mice

Ethical permission for the following procedures were granted by the Danish Animal Experiments Inspectorate (permission 2017-15-0201-01248), and all animal procedures were performed in compliance with Directive 2010/63/EU of the European Parliament and of the Council, and with Danish Law and Order regulating animal experiments (LBK no. 253, 08/03/2013 and BEK no. 88, 30/01/2013). Mice (C57BL/6*J*Rj, Janvier, 8 weeks, *n* = 8) were habituated with i.p. injections (0.9% saline) for 4 days prior to the experiment to minimize stress on the day of the experiment. Experiments were conducted in a quiet room (22-23 °C), in which mice were left undisturbed for at least 2 h prior to the experiment. The core body temperature was measured rectally by a thermometer (Harvard Apparatus, Edenbridge, UK) via a lubricated thermistor probe (1.6 mm diameter OD probe; Harvard Apparatus) at various time points after drug or vehicle administration. Mice were held at the base of the tail and measured until a stable temperature was obtained (approx. 15 sec). Data are means ± SD, and statistical analysis used is two-way ANOVA followed by Dunnett's post-hoc test with time and treatment as factors.

3.10.4. Ex vivo LTP recordings

Adult male mice (> 8 w old) were used (C57BL/6JOlaHsd from in-house breeding). After anesthesia with isoflurane (Nicholas Piramal) and decapitation, the brain was taken out quickly and submerged in ice-cold oxygenated (95%) and carbonated (5%) artificial CSF (ACSF; <4.0°) containing the following (in mM): 120 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose. Using a vibratome, 400-µm-thick sagittal slices were made. Hippocampal sections were dissected out afterward and maintained at room temperature for at least 1.5 h in an oxygenated and carbonated bath to recover before experiments were initiated. At the onset of experiments hippocampal slices were placed in a submerged recording chamber and perfused continuously at a rate of 2 mL/min with ACSF equilibrated with 95% O2, 5% CO_2 at 30 °C. Extracellular recording of field EPSPs (fEPSPs) and stimulation were done using bipolar platinum (Pt)/iridium (Ir) electrodes (Frederick Haer). Stimulus duration of 100 µs for all experiments was used. In CA3–CA1 measurements, the stimulating electrode and recording electrode were placed on the CA3– CA1 Schaffer collateral afferents and apical dendrites of CA1 pyramidal cells (both 150–200 µm from stratum pyramidale), respectively. Upon placement of the electrodes, slices were given 20–30 min to rest before continuing measurements. HOCPCA (100 µM) was washed in 20 min before and continued to be washed in during the experiment. All paired-pulse facilitation (PPF) experiments were stimulated at one-third of slice maximum. Varying intervals were used in PPF: 10, 25, 50, 100, 200, and 400 ms. CaMKII-dependent LTP was evoked using a 100 Hz induction protocol (1 train of 1 s at 100 Hz, stimulated at one-third of slice maximum). During LTP slices were stimulated once per minute. Potentiation was measured as the normalized increase of the mean fEPSP slope for the duration of the baseline. Only stable recordings were included. This judgment was made blinded. Average LTP was defined as the mean last 10 min of the normalized fEPSP slope. Number of mice per experiment was at least $n = 20$ as specified in the figure legend and are given as means ± SEM. For statistical analysis Repeated Measures ANOVA was used.

3.10.5. Photothrombotic stroke

All procedures were performed in accordance with the guidelines on the care and use of laboratory animals set out by the University of Otago, Animal Research Committee and the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85–23, 1996). All mice for the stroke studies were obtained from the Biomedical Research Facility, University of Otago, New Zealand.

Focal stroke was induced by photothrombosis in young adult male C57BL/6*J* mice (3-4 months, 27-30 g) or aged female C57BL/6*J* mice (20-24 months, 32-40 g) as previously described(25). Under isoflurane anesthesia (4% induction, 2-2.5% maintenance in O_2) mice were placed in a stereotactic frame (9000RR-B-U, KOPF; CA, USA), and buprenorphine hydrochloride (0.1 mL of a 0.5 mg/kg solution, Temgesic®) was administered subcutaneously as pre-emptive post-surgical pain relief. Following sterilization of the skin using chlorhexidine (30% in 70% ethanol, Hibitane®), the skull was exposed through a midline incision, cleared of connective tissue and dried. A cold light source (KL1500 LCD, Zeiss, Auckland, New Zealand) attached to a 40x objective providing a 2-mm diameter illumination was positioned 1.5 mm lateral from bregma. Then, 0.2 mL of Rose Bengal (Sigma-Aldrich, Auckland, New Zealand; 10 mg/mL in

normal saline) was administered i.p. After 5 min, the brain was illuminated through the exposed intact skull for 15 minutes, while keeping body temperature at 37 °C using a heating pad. The color temperature intensity used for all experiments to induce a stroke was 3300 K. The skin was glued and animals left in a cage placed on a heating pad during the wake-up phase. Sham surgery was performed in the exact same way, except that saline was injected instead of Rose Bengal. Mice were housed in groups of two to five under standard conditions in individually ventilated cages (IVC: Tecniplast): 21 ± 2 °C and humidity of 50 ± 10%, on a reverse 12 h light/dark cycle (white lights off from 07:00-19:00) with ad libitum access to food and water. Further, the mice were monitored and weighed on a daily basis. All animals were randomly assigned to a treatment groups and all assessments were carried out by observers blinded to the treatment group. No deaths were reported during these studies. Five mice were excluded from analysis, two from the stroke vehicle groups and three from the stroke + treatment groups due to the lack of any visible stroke being detected. This is most likely due to experimenter error with the Rose Bengal most likely being injected into the bladder. Additional animals were set-up to replace these two mice.

The sodium salts of GHB and HOCPCA were dissolved in sterile dH_2O. Drugs were administered i.p. as 10 mg/mL and 10 µL of solution per gram mouse body weight. Injection of compound (i.p.) was performed 30 min, 3 h, 6 h or 12 h after induction of the photothrombotic stroke. The vehicle groups received a corresponding volume of saline (0.9%).

3.10.6. pThr286 autophosphorylation after photothrombotic stroke

Photothrombotic strokes were induced as described and brains collected. Mice were euthanized by cervical dislocation, followed by rapid extraction of the brain at 3, 6 and 12 h after stroke induction. Brains were snap-frozen and stored at -80 °C until further processing. On the day of the assay, brains were cut using a CM1860 cryostat (Leica) at -20 °C and the peri-infarct was collected using a tissue punch from the top quadrant of the left hemisphere. Tissue homogenization was achieved using a Bullet Blender in RIPA buffer supplemented with a 1% protease/phosphatase inhibitor cocktail. Protein concentrations were determined with the Pierce method. Samples were prepared for Western blot analysis by addition of 4x Fluorescent Compatible Sample Buffer (#LC2570, Thermo Fisher) and 100 mM DTT with a protein concentration of 2 µg/µL. Samples were heated for 5 min at 95 °C, sonicated and centrifuged 11,000 x *g* for 2 min at 4 °C. Western blotting and antibody incubation was performed exactly as described under 'Determination of CaMKIIα protein levels by Western blotting', however with 20 µg sample loaded. Experiments were carried out in 4-5 biological replicates using three technical replications. For statistics, One-way ANOVA, post-hoc Dunnett's test was used.

3.10.7. Histological assessment

For quantification of the infarcted area, animals were deeply anaesthetized (i.p. pentobarbital 100 mg/kg) and transcardially perfused with saline followed by 4% PFA at either 3- or 7-days post-stroke. Brains were dissected out and post-fixed in 4% PFA for post-fixing overnight prior to being transferred into 30% sucrose solution for cryopreservation and kept at 4° C until sectioning. Brains were cut on a sliding microtome

attached with a freezing stage (-21°C, Leica, Model 1300). A layer of sucrose solution (30%) was used to mount the brain onto the freezing stage and brains left to freeze (15-20 min) before 40 µm coronal sections were cut and collected into 24-well plates containing a cryoprotectant solution (0.1 M PBS, 30% sucrose, 1% polyvinyl-pyrrolidone, 30% ethylene glycol). Tissue was stored at -20 °C until required.

Every sixth section was washed in 0.05 M TBS solution for three consecutive 10 min washes. Sections were mounted on gelatin subbed slides, dried overnight, stained using 0.1% cresyl violet solution, passed through ascending concentration of alcohols, cleared in xylenes, and coverslipped using DPX mounting solution. Images of cresyl violet staining were then taken using an inverted montaging microscope (Model Ti2E Wideview, Nikon, Japan) set with a 2.5x objective lens. Images were then exported as TIFF files and opened on Fiji ImageJ to quantify infarct volume. Stroke volume was calculated as per the equation:

$$Infarct\ Volume\ (mm^3) = Area\ (mm^2)\ x\ Section\ Thickness\ x\ Section\ Interval$$

3.10.8. Behavioral assessment

Motor performance was determined using both the cylinder and grid-walking tasks as previously described (25). All animals were tested one week prior to surgery on both behavioral tasks to establish baseline performance levels, and again seven days post-stroke, at approximately the same time each day, at the end of their dark cycle. All behaviors were scored by observers who were blind to the treatment group of the animals in the study as previously described (24).

3.10.9. Grid-walking test

The grid-walking apparatus was manufactured using 12 mm square wire mesh with a grid area 32 cm / 20 cm / 50 cm (length / width / height). A mirror was placed beneath the apparatus to allow video footage in order to assess the animals' stepping errors (i.e. 'foot faults'). Each mouse was placed individually atop of the elevated wire grid and allowed to freely walk for a period of 5 min (measured in real time by stopwatch and confirmed afterwards by reviewing videotape footage). During this 5 min period, the total number of footfaults for each limb along with the total number of non-foot fault steps was counted and a ratio between footfaults and total steps taken calculated. Percent foot faults were calculated by: [#foot faults / (#foot faults + #non-foot fault steps) * 100]. To take into account differences in the degree of locomotion between animals and trials, a ratio between foot faults and total steps taken was used.

3.10.11. Cylinder task

The spontaneous forelimb task encourages the use of forelimbs for vertical wall exploration / press in a cylinder. When placed in a cylinder, the animal rears to a standing position, whilst supporting its weight with either one or both of its forelimbs on the side of the cylinder wall. A cylinder 15 cm in height with a diameter of 10 cm is used. Videotape footage of animals in the cylinder was evaluated quantitatively in order to determine forelimb preference during vertical exploratory movements. While the video footage was played in slow motion (1/5th real time speed), the time (sec) during each rear that each animal spent on either the right forelimb, the left forelimb, or on both forelimbs

were calculated. Only rears in which both forelimbs could be clearly seen were timed. From these three measures, the total amount of time spent on either limb independently as well as the time the animal spent rearing using both limbs was derived. The percentage of time spent on each limb was calculated and these data were used to derive a spontaneous forelimb asymmetry index (% ipsilateral use / % contralateral use). The 'contact time' method of examining the behavior was chosen over the 'contact placement' method, as it takes into account the slips that often occur during a bilateral wall press post-stroke.

3.10.12. Compound action potential (CAP) electrophysiological recordings from corpus callosum

Functional changes within the corpus callosum were measured by assessing compound action potential recordings (CAPs) based on Crawford *et al* (26). Briefly, 14-days post-sham or stroke surgery, mice were anaesthetized with isoflurane, decapitated, and the brain rapidly removed. Coronal slices (400 µm thick) were cut with a vibratome (Campden Instruments model #MA752) in ice-cold artificial cerebrospinal fluid (aCSF (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.25, $NaHCO_3$ 26, $MgSO_4$ 1.3, $CaCl_2$ 2, glucose 10; pH 7.4; gassed with Carbogen: 95% O_2/5% CO_2). Slices were then transferred to a holding chamber containing oxygenated aCSF at room temperature and were allowed to equilibrate under these conditions for at least 1 h prior to recording.

Brain slices were placed between nylon mesh nets in a commercial brain slice recording chamber (Kerr Scientific Instruments Ltd., Christchurch, NZ) that was maintained at room temperature, and constantly superfused at 1 mL/min with oxygenated aCSF. The slices were left undisturbed for 1 h before commencement of recordings. Electrical stimulation of the corpus callosum was performed using a bipolar microstimulating electrodes (50 µm diameter Teflon-coated tungsten wire), positioned over the corpus callosum in order to evoke CAPs. Biphasic electrical stimulation was applied as 0.1 msec pulses delivered at 0.1 Hz, at intensities ranging from 2 to 30 V. The evoked CAPs were recorded extracellularly at an approximate distance of 0.8-1.2 mm from the stimulation site using a crimped copper electrode inserted inside a glass microelectrode filled with aCSF. The recording electrode was attached to a KSI Bio Amplifier, with waveforms were filtered and amplified using a Powerlab 2/25 analog-digital converter (ADInstruments Pty., Sydney, Australia) and stored on a Macintosh computer hard drive for off-line analysis using LabChart 7 Pro data acquisition software (ADInstruments Pty.). Waveforms were analyzed to assess peak responses for N1 (myelinated axons) and N2 (unmyelinated axons) and expressed as CAP responses (mV).

3.10.13. Quantification of biotinylated dextran amine (BDA)-labeled axonal projections

Injection of BDA was carried out as we have previously described (24). On day 7 post-stroke, animals were anaesthetized and positioned in a stereotaxic frame (KOPF), and buprenorphine hydrochloride (0.1 mL of a 0.5 mg/kg solution, Temgesic®) was administered subcutaneously as pre-emptive post-surgical pain relief. Following sterilization of the skin using chlorhexidine (30% in 70% ethanol, Hibitane®), an incision was made to expose the skull and connective tissue cleared and the skull dried. A glass Hamilton syringe (5 mL; Hamilton Company, NV, USA) containing 10% BDA (10,000 MW; Invitrogen) was positioned above bregma (1.5 mm AP, 1.75 mm ML) to work out

where to drill. Using a small drill (Micro 8v Max, Dremel), a 1 mm diameter burr-hole was carefully made through the skull. The Hamilton syringe was then lined back up over the burr-hole and lowered slowly through the skull into the left premotor cortex (0.75 mm DV). Once positioned, 300 nL of BDA was infused at 0.125 μL/min. Following infusion, the needle was left for 2-5 minutes before being carefully retracted. The skin was then glued back together and the mouse gently removed from the facemask and stereotaxic frame and allowed to recover on a heating mat before being returned to the home cage.

For quantification of BDA-labeled axonal projections through the corpus callosum, animals were deeply anaesthetized (i.p. pentobarbital 100 mg/kg) and transcardially perfused with saline followed by 4% PFA 1 week after tracer injection (14-days post-stroke). Brains were dissected free, post-fixed and 40 μm thick coronal sections collected into 24-well plates containing cryoprotectant. Tissue was stored at -20 °C until required. To visualize BDA fiber staining, sections were incubated with avidin–biotin–peroxidase complex (Vectastain) followed by diaminobenzidine (DAB). Sections were mounted onto gelatin-coated glass slides, lightly air-dried, passed sequentially through alcohols (50%, 70%, 95% and 100%) before being passed through xylene and then cover-slipped using DPX mounting solution before being processed for densitometric analysis of axonal labels. Statistical analysis was performed using GraphPad Prism (v. 8). Data are displayed as mean ± SD plotted as box and whisker graphs and one-way ANOVA followed by Tukey's post-hoc test was used for statistical assessment. $P<0.05$ was considered statistically significant.

3.10.14. Statistical analysis

All statistical analyses relating to this section were performed using GraphPad Prism (v. 8). For *in vivo* studies, data are displayed as mean ± SD and plotted as box and whisker graphs, unless otherwise noted. For histological, electrophysiological and behavioral assessments post-stroke, one-way and two-way ANOVA followed by Dunnett's, Tukey's or Bonferroni's post-hoc test as well as two-tailed Student's *t*-test were used when as appropriate and specified accordingly in the figure legends. For histological assessment of groups with an $n = 5$/group, one-way ANOVA followed by Kruskal-Wallis test was performed. $P<0.05$ was considered statistically significant.

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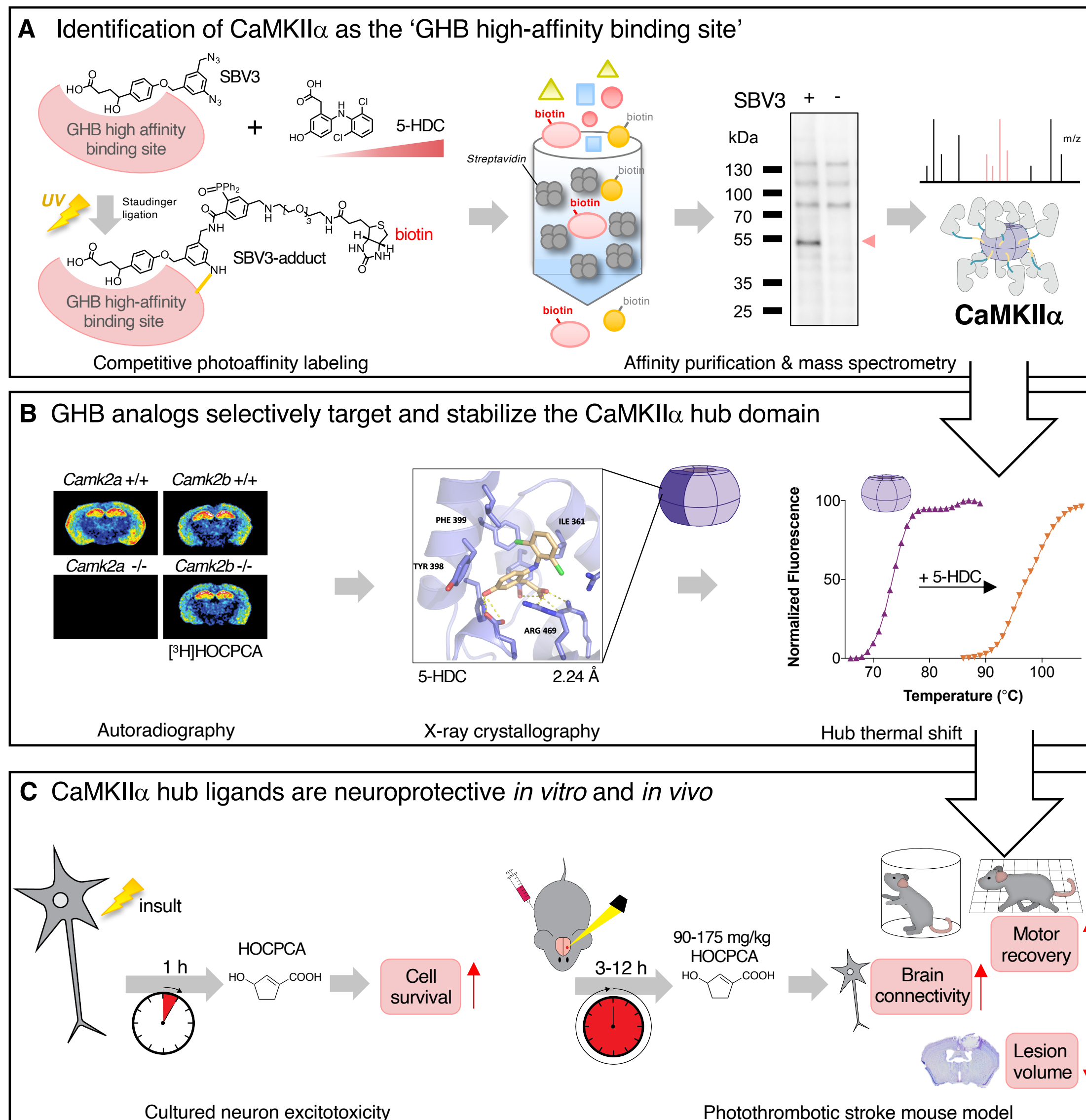

Fig. S1. Schematic summary of results

(A) CaMKIIα was identified as the 'GHB high-affinity binding site' with a workflow consisting of competitive photoaffinity labeling (PAL), affinity purification (AP) and quantitative chemical proteomics. Rat hippocampal homogenate was labeled with the photoligand SBV3, containing a GHB-binding moiety, an aryl azide group for photolabeling, and an alkyl azide group for subsequent ligation of a phosphine-PEG$_3$-biotin linker. PAL was competed with free 5-HDC. After streptavidin AP of biotinylated proteins, the enriched binding protein was subjected to SDS-PAGE, in-gel digestion and LC-MS/MS analysis. (B) The clear absence of [^{3}H]HOCPCA binding to *Camk2a* knockout (-/-) mouse brain tissue validates CaMKIIα as the GHB binding protein. A deep binding cavity in the hub domain was identified as the binding site for the ligand 5-HDC by co-crystallization. 5-HDC was shown to dramatically stabilize the hub protein in a thermal shift assay. (C) The GHB-related compound HOCPCA shows neuroprotective effects both *in vitro* in cultured neurons after Glu exposure and *in vivo* in mice when administered up to 12 h after a photothrombotic stroke and assessed 1-2 weeks later.

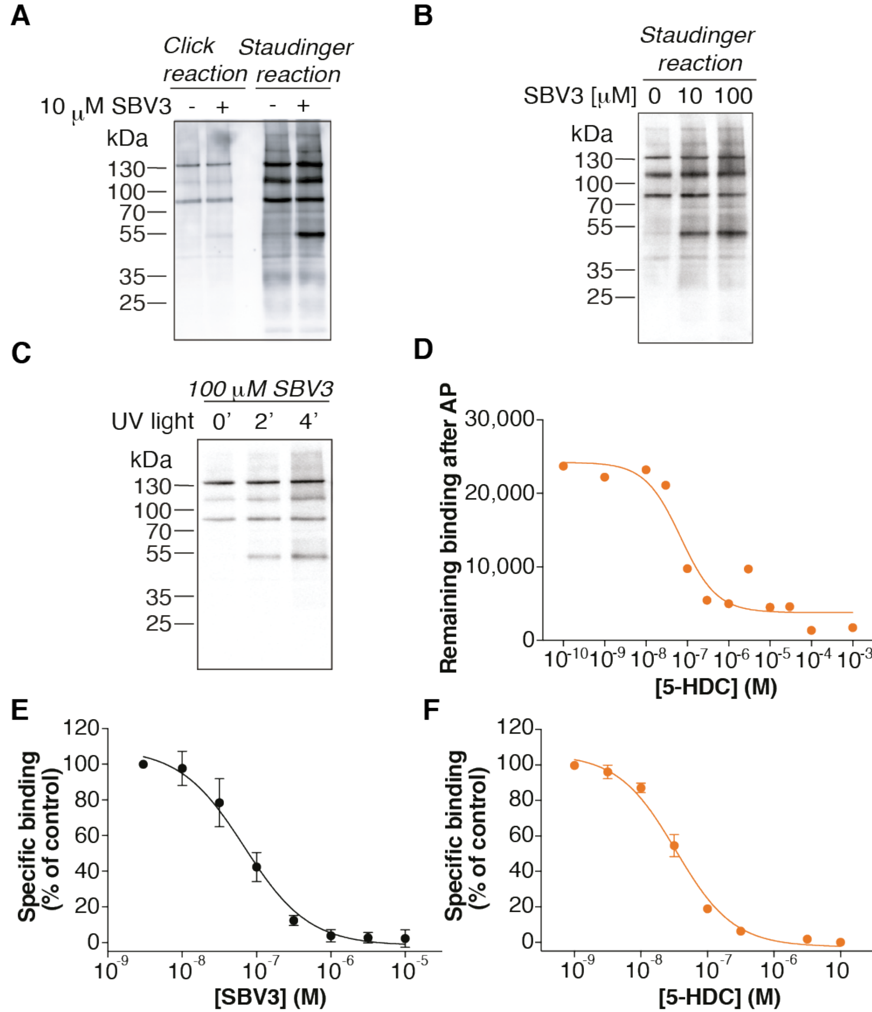

Fig. S2. Optimization of conditions for PAL reaction
(A-C) Optimization of photolabeling (shown with biotinylation) achieved by either Huisgen click reaction (*left*) or Staudinger-Bertozzi ligation (*right)* followed by anti-biotin Western blotting (concentration and time). The signature band at ~55 kDa is detectable only after photolinking with SBV3 (10 µM). (D) Concentration-dependent inhibition of the SBV3-induced PAL-AP reaction by 5-HDC. IC_{50} value obtained 69 nM (single experiment). Concentration-dependent inhibition of [^{3}H]NCS-382 binding to rat cortical homogenate by (E) the photoligand SBV3 and (F) the ligand 5-HDC used for inhibition of PAL. Results are given as means ± SEM of (n = 3-5). Average K_I (pK_I ± SEM) values were 66 nM (7.2 ± 0.104) and 35 nM (7.5 ± 0.05), respectively.

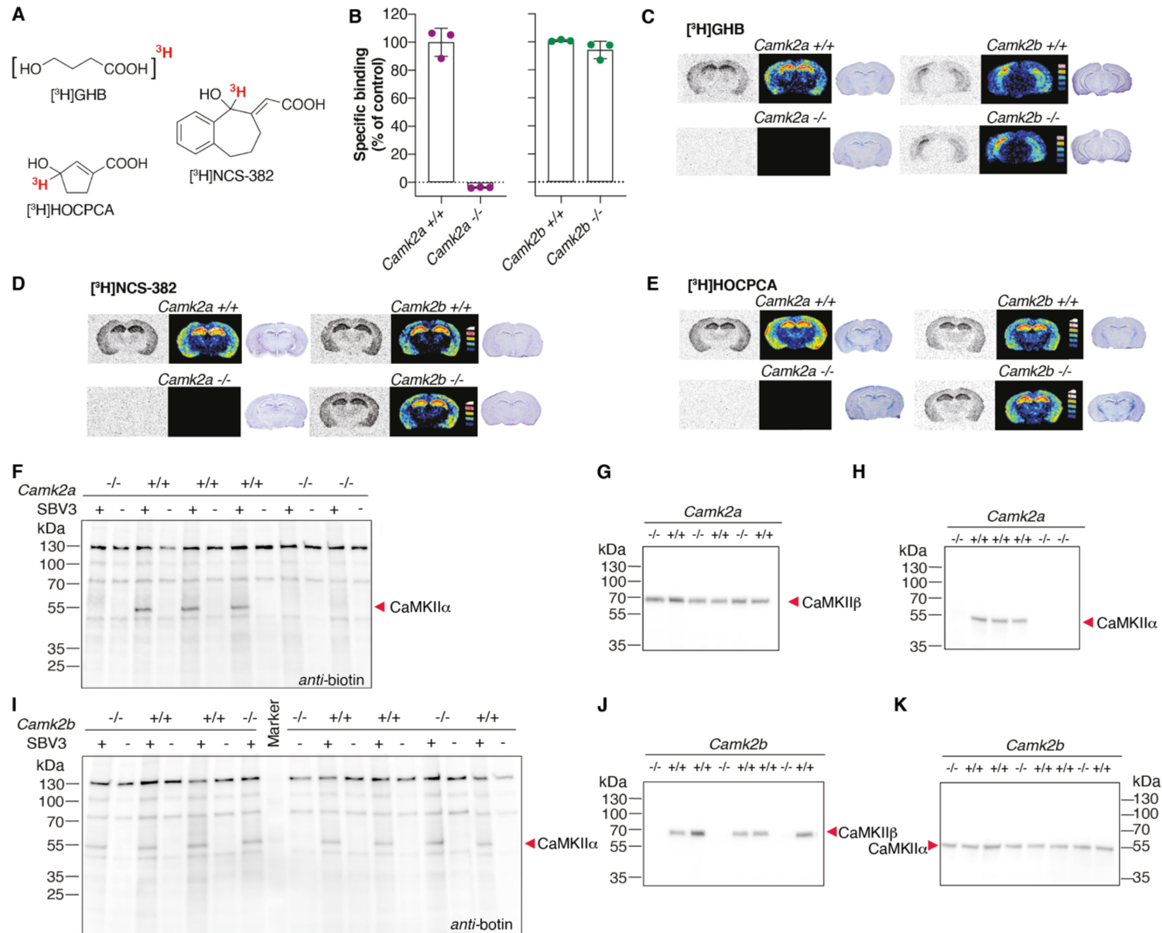

Fig. S3. Validation of the CaMKIIα binding site using labeled ligands
(A) Chemical structures of radioligands. (B) No [^{3}H]HOCPCA (5 nM) binding to cortical homogenate from *Camk2a* -/- cf. *Camk2a* +/+, *Camk2b* -/- and *Camk2a* +/+ samples (unpaired t-test with Welch's correction (*n* = 3). (C-E) *In vitro* autoradiography with [^{3}H]GHB (30 nM), [^{3}H]NCS-382 (7 nM), and [^{3}H]HOCPCA (1 nM) confirming absence of binding to *Camk2a* -/- tissue. Representative autoradiograms are supported by pseudo color images as well as cresyl violet staining of coronal sections (*n* = 3-4, 4 sections per animal). Scale bars with coloring information low-high (blue-to-white) are included (F-K) Validation of photolinked band by Western blots. (F) Anti-biotin WB showing the complete absence of the 55 kDa-PAL band in *Camk2a* -/- cf. +/+ samples. (G, H) Anti-CaMKIIα and CaMKIIβ Western blots of *Camk2a* -/- cf. +/+ brain samples, confirming the deletion of CaMKIIα only. (I) Anti-biotin Western blot showing an intact 55 kDa band in both *Camk2b* +/+ and -/- samples. (J-K) Anti-CaMKIIα and CaMKIIβ Western blots of *Camk2b* -/- cf. +/+ brain samples, confirming the deletion of CaMKIIβ only. Red arrows indicate relevant bands.

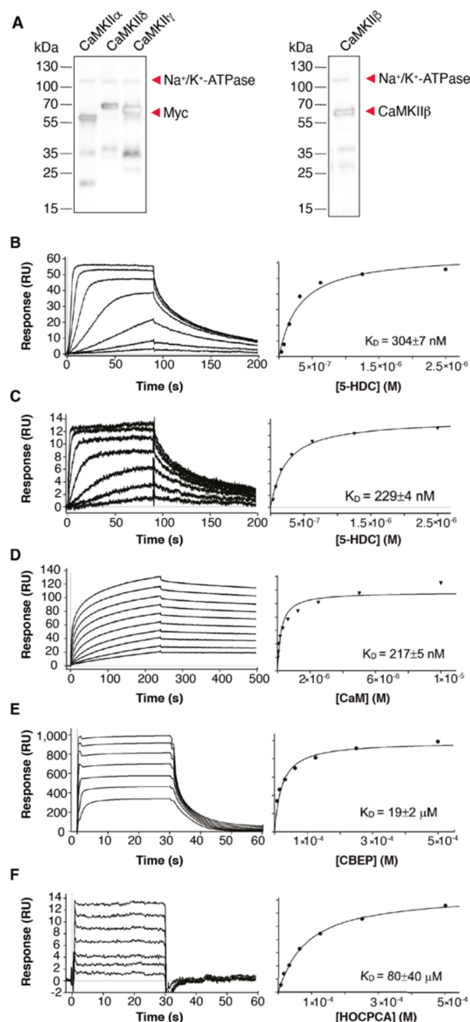

Fig. S4. Western blot validation of CaMKIIα/β/γ/δ expression in HEK whole cell homogenate and Surface Plasmon Resonance (SPR) binding

(A) Western blots showing bands at 55 kDa for CaMKIIα, 70 kDa for CaMKIIβ, 65 kDa for CaMKIIγ and 60 kDa for CaMKIIδ, corresponding to the expected sizes of the subtypes plus the c-terminal myc tag (not present in CaMKIIβ). Red arrows indicate the relevant bands Bands for Na^+/K^+-ATPase at 110 kDa confirm equal loading of samples. (B-F) SPR sensorgrams of ligands interacting with immobilized CaMKIIα. Compounds were injected in 2-fold serial dilutions over immobilized CaMKIIα (WT hub, 6X Hub or full-length, as specified). (B) Binding of 5-HDC is unchanged to CaMKIIα WT hub (cf. 6x Hub in Fig. 2B), and (C) full-length CaMKIIα. (D) CaM control binding to immobilized full-length CaMKIIα (pH 7.4). The binding of CaM to CaMKIIα full-length was fully regenerated between concentrations by injection of 1 mM EDTA. (E) Control peptide CBEP, and (F) binding of HOCPCA to the 6x Hub (pH 6 as for 5-HDC in Fig. 2B). SPR sensorgrams are blank injection and reference surface subtracted (*left* panels). Plots of equilibrium binding responses at the end of the analyte injections against analyte concentration (*right* panels). Steady state K_D ± SEM were derived from curves fitted to a 1:1 model based on at least seven concentration-response measurements (collected in *Table S3*).

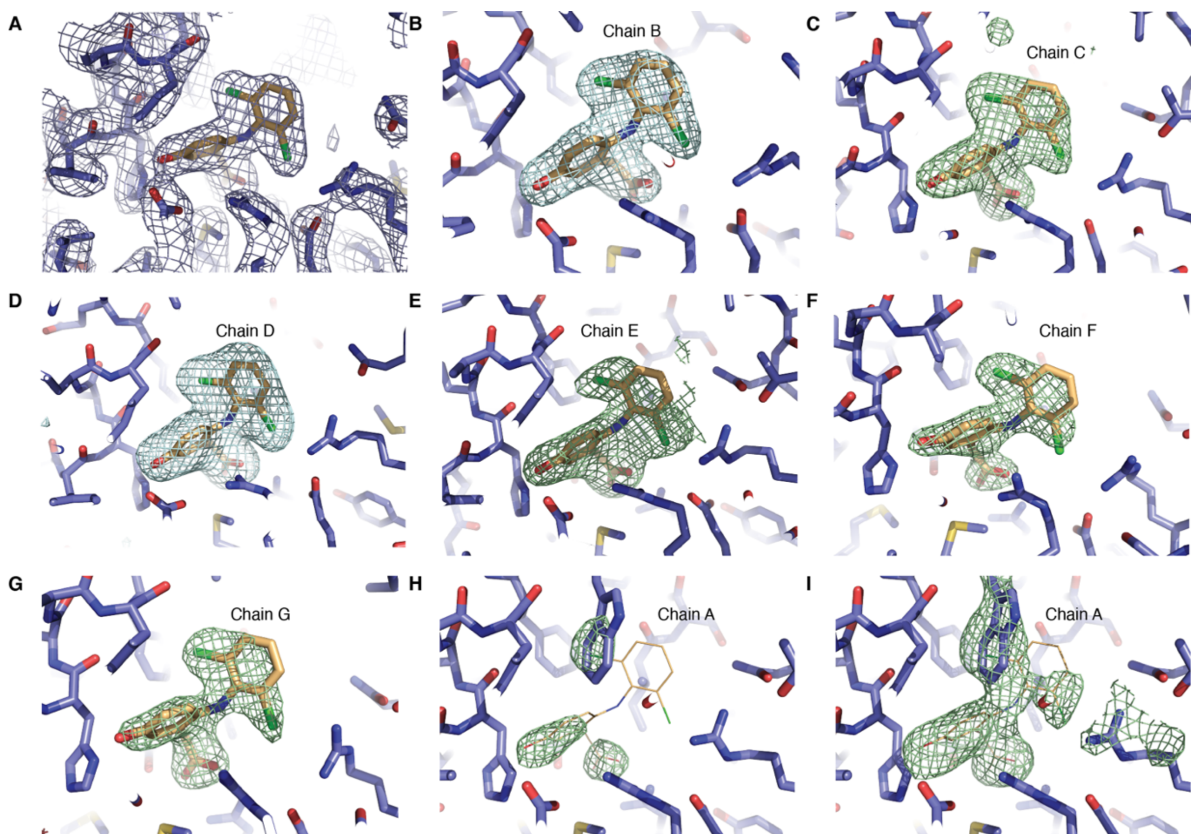

Fig. S5. Electron density maps of 5-HDC bound to CaMKIIα 6x Hub
(A) 2Fo-Fc density showing 5-HDC bound to a pocket of the hub monomer. (B-G) Polder omit maps (27) where the ligand in the chain indicated is omitted in the map calculation, along with the surrounding solvent. Maps are contoured at 4.5 sigma. (H), Polder omit map for the chain A pocket with tryptophan 403 (Trp403) modeled inwards. Trp403, nearby ordered waters, and the surrounding solvent were omitted from the map calculation. The expected binding pose of the ligand (upon Trp 403 displacement) is shown with a line model. This ligand is not modeled in the final structure. Map contoured at 4.5 sigma. (I) Same as in (H), but the map is contoured at 3 sigma. Weak density is observed for both bound-ligand and inward-pointing Trp 403, indicating a mixture of two states in the crystal.

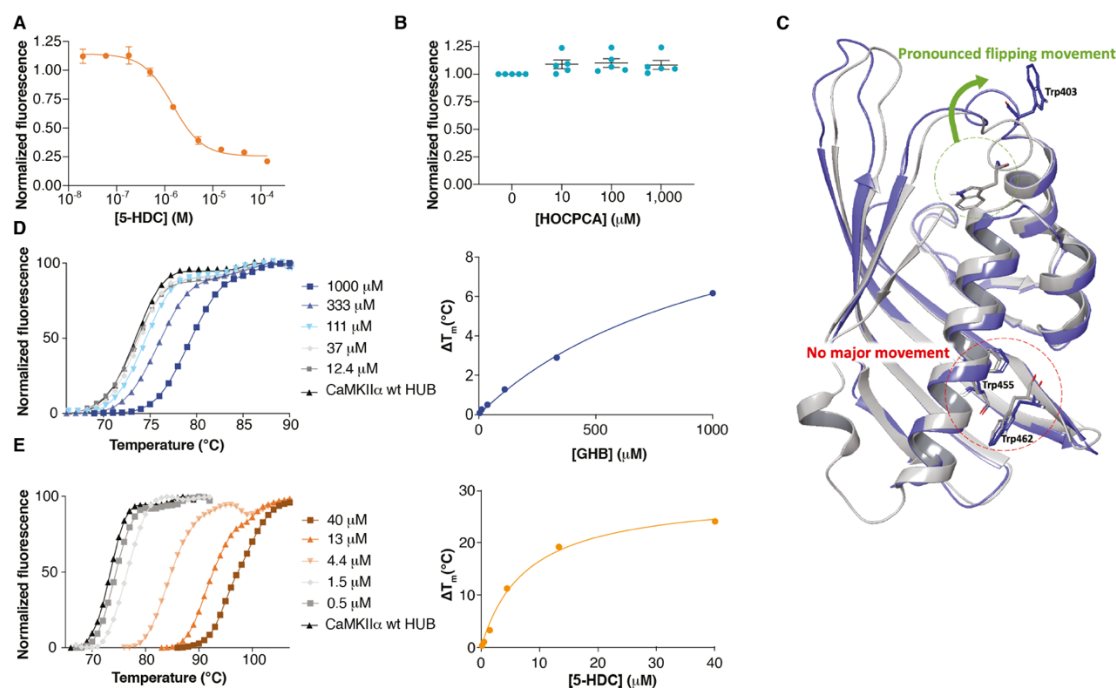

Fig. S6. Structural effects of GHB analogs on the hub domain

(A-B) Concentration-dependent quenching of Trp403 fluorescence. (A) 5-HDC gives a similar response in the WT hub protein (IC_{50} value of 1.47 μM) cf. 6x Hub (main Fig. 2G). (B) No effect of HOCPCA at increasing concentrations (no change in fluorescence observed). For 5-HDC, the graph is based on pooled data (normalized means ± SEM) of two technical replicates. For HOCPCA, each data point represents independent data as means of five technical replicates. (C) Protein superimposition comparing the relative positioning of the three hub domain Trp residues without (grey, PDB entry 5IG3) and with 5-HDC bound (purple). Upon ligand binding there is a pronounced flip of Trp403 but no movement of Trp455 and Trp462. (D-E) TSA melting curves from differential scanning fluorimetry of the CaMKIIα WT hub upon GHB or 5-HDC binding. Melting curves (*left* panels) and maximum ΔT_m of CaMKIIα hub by compounds, derived from plots of ΔT_m against analyte concentration fitted to a 1:1 model (*right* panels) of (D) GHB (12-1000 μM), (E) 5-HDC (0.5-40 μM). The maximum ΔT_m of CaMKIIα by GHB and 5-HDC were estimated to 13.0 °C and 29.2 °C, respectively.

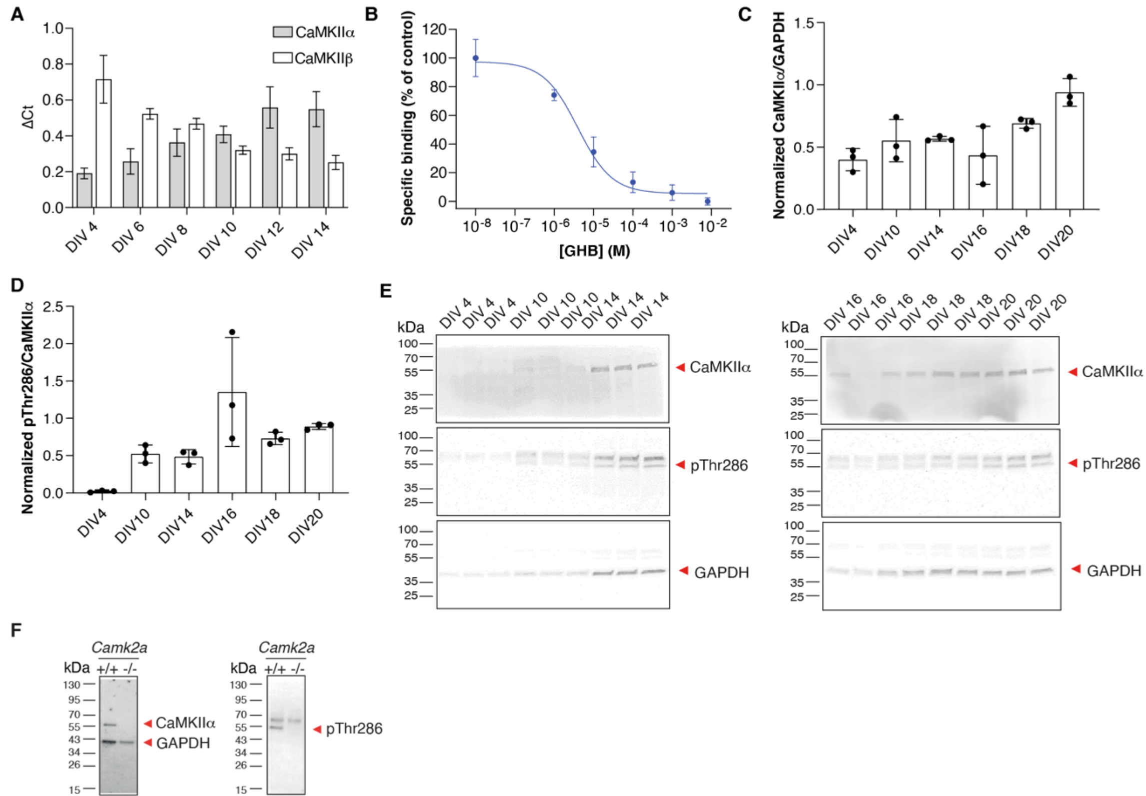

Fig. S7. Validation of cortical neuronal cultures

(A-E) Optimization of neuronal culturing. (A) mRNA levels of *Camk2a* increase during culturing time (grey bars) whereas *Camk2b* decreases (white bars). Data is presented as means ± SEM of three individual cultures in technical triplicates. (B) Concentration-dependent inhibition of [^{3}H]HOCPCA binding to cell homogenate by GHB. Shown is representative data ± SD of technical triplicates. The average IC_{50} value (pIC_{50} ± SEM) obtained was 2.04 μM (5.7 ± 0.07) (three different cultures). Western blots show a temporal increase in protein levels of (C) total CaMKIIα and (D) pThr286. Data is represented as means ± SD from three different wells from the same culture. (E) Validation of signal. Corresponding representative Western blots. (F) Representative Western blots showing the expected absence of CaMKIIα (*left*) and pThr286 bands (*right*) in cortical neuronal cultures from *Camk2a* -/- cf. +/+ cultures. Data is represented as means ± SD from three different wells from the same culture.

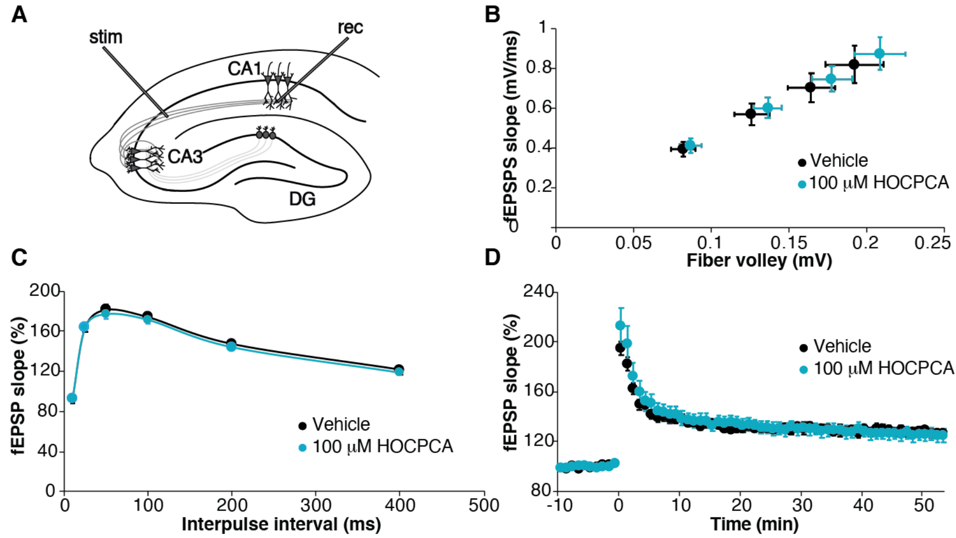

Fig. S8. Lack of effects of HOCPCA in long-term potentiation (LTP)
(A) Schematic overview of LTP induction in the CA3–CA1 pathway. stim, stimulating electrode; rec, recording electrode; DG, dentate gyrus. (B) Basal synaptic transmission is unaffected upon influx of HOCPCA (100 μM) [vehicle (n = 32 from 6 mice), HOCPCA (n = 24 from five mice)]. (C) Paired-pulse facilitation (PPF) is unaffected upon influx of HOCPCA (100 μM) [vehicle (n = 28 from 6 mice), HOCPCA (n = 20 from 4 mice)]. (D) LTP is unaffected upon influx of HOCPCA (100 μM) [vehicle (n = 23 from 6 mice), HOCPCA (n = 22 from five mice)] (mean ± SEM, Repeated Measures ANOVA).

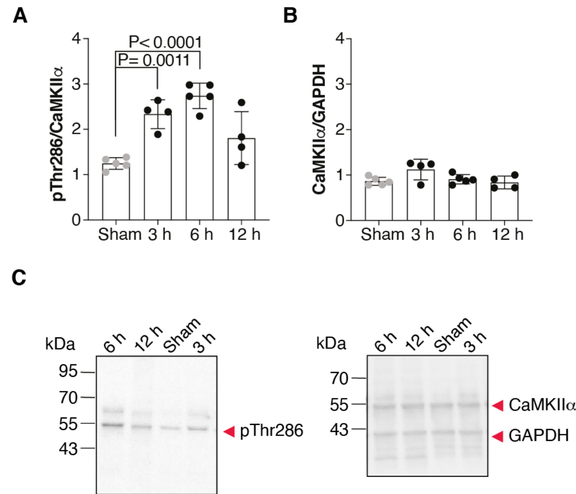

Fig. S9. CaMKIIα Thr286 autophosphorylation post-photothrombosis in mice
(A-C) CaMKIIα pThr286 autophosphorylation and total CaMKIIα expression at different time points after photothrombosis compared to sham-operated animals. Quantification of immunoblots of (A) pThr286 levels normalized to total CaMKIIα expression, and (B) total CaMKIIα expression normalized to GAPDH. (C) Representative Western blots (n = 4-5, three technical repetitions, mean ± SD, One-way ANOVA, post-hoc Dunnett's test).

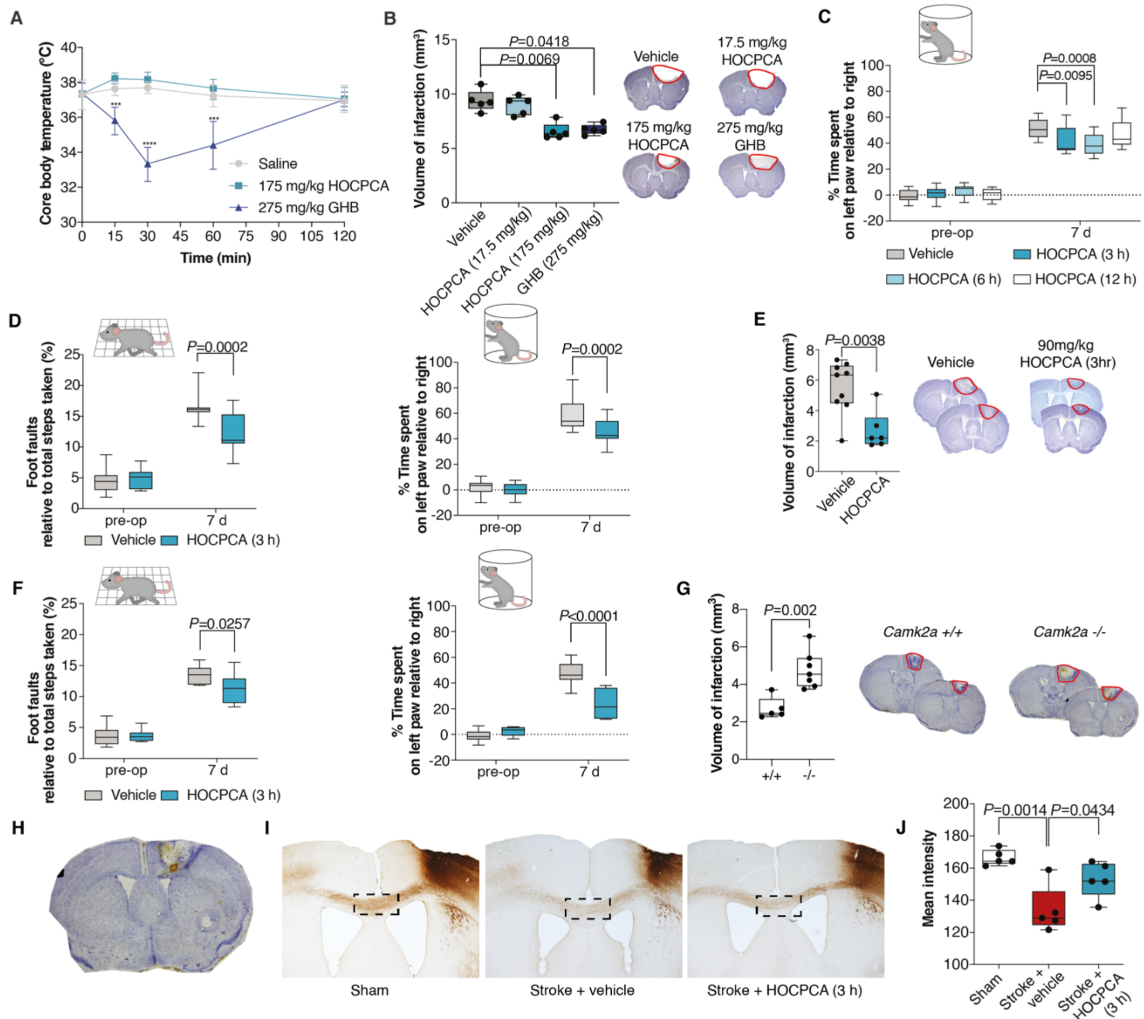

Fig. S10. Neuroprotective effects of GHB & HOCPCA after photothrombotic stroke (A) Temperature recordings in mice after i.p. injections of GHB (275 mg/kg) and HOCPCA (175 mg/kg) compared to saline (means ± SD, $n = 8$, two-way ANOVA with time and treatment as factors followed by Dunnett's test). (B) Neuroprotective effects of a single i.p. dose of HOCPCA (17.5 or 175 mg/kg) and GHB (275 mg/kg) administered 30 min post-photothrombotic stroke cf. vehicle in young male mice ($n = 5$/group). (Quantification of infarct volumes (*left*) and representative cresyl violet stainings (*right*) measured 3 days post-stroke (one-way ANOVA, post-hoc Kruskal-Wallis test; boxes, 25–75%; whiskers, minimum and maximum; lines, median). (C-D) Motor function analysis after HOCPCA treatment. Recovery of motor function was assessed in the grid-walking and cylinder tasks for forelimb asymmetry pre-op (7 days before) and 7 days after photothrombotic stroke. (C) Cylinder task for young male mice treated with a single i.p. dose of HOCPCA (175 mg/kg) at 3 h ($n = 13$), 6 h ($n = 10$) or 12 h ($n = 12$) post-photothrombotic stroke cf. vehicle ($n = 10$). (D) Grid-walking (*left*) and cylinder task (*right*) for aged female mice (20-24 months) treated with single i.p. dose of 1 (175 mg/kg) at 3 h post-photothrombotic stroke ($n = 13$) cf. vehicle ($n = 14$). (E-F)

Neuroprotective effects with HOCPCA (90 mg/kg) ($n = 9$) at 3 h post-photothrombotic stroke cf. vehicle in young male mice ($n = 6$). (E) Quantification of infarct volume (*left*) and representative cresyl violet stainings (*right*) measured 7 days post-stroke (two-tailed Student's *t*-test). (F) Recovery of motor function was assessed by analysis of foot faults in the grid-walking task (*left*) and cylinder task for forelimb asymmetry (*right*) pre-op (7 days before) and 7 days after stroke. (D,F) (two-way ANOVA, post-hoc Dunnett's test with time and treatment as independent factors and time as repeated measures; boxes, 25–75%; whiskers, minimum and maximum; lines, median). (G) Infarcts in young male *Camk2a* +/+ and -/- mice after a photothrombotic stroke (two-tailed Student's *t*-test). (H) Example of cresyl violet staining illustrating hemorrhages in -/- mice. (I) Images of BDA-labeled connections to the premotor cortex for sham (*left*), Stroke + vehicle (mid) and Stroke + HOCPCA (175 mg/kg i.p.) (*right*). (J) Quantification of axonal projections through the corpus callosum. Dotted rectangle represents region of analysis. Box plots for $n = 5$ per treatment group (boxes, 25–75%; whiskers, minimum and maximum; lines, median). (One-way ANOVA, post hoc Tukey's test).

Table S1. List of best hit proteins from non-linear regression analysis ($R^2 > 0.6$) identified from photoaffinity-labeling of 'GHB high-affinity binding sites' in rat hippocampal homogenate

Note that the signature photolabeled, biotin-ligated band fits only convincingly with CaMKIIα at ~55 kDa. Data are illustrated in main Fig. 1 and collected in Supplementary Dataset 1: Full list of proteins identified from photoaffinity-labeling of 'GHB high-affinity binding sites'

Protein Name	Gene Name	UniProt ID	Molecular weight (kDa)	MS/MS counts	Non-linear regression analysis	
					Top plateau	R^2 value
Calcium/calmodulin-dependent protein kinase II alpha	*Camk2a*	P11275	54.115	800	7.97E+09	0.81
Tubulin alpha 1B	*Tuba1b*	Q6P9V9	50.152	680	2.14E+09	0.72
Sodium/potassium-transporting ATPase beta-1	*Atp1b1*	P07340	35.202	446	1.69E+09	0.68
Tubulin beta 4B	*Tubb4b*	Q6P9T8	49.801	1066	1.50E+09	0.76
Calcium/calmodulin-dependent protein kinase II beta	*Camk2b*	P08413	60.402	474	9.76E+08	0.65
Calcium/calmodulin-dependent protein kinase type II subunit delta	*CaMK2d*	P15791	60.081	83	1.08E+08	0.82
Calcium/calmodulin-dependent protein kinase II gamma	*CaMK2g*	P11730	59.038	86	9.09E+07	0.63

Table S2. Saturation binding data of [^{3}H]HOCPCA to CaMKIIα (native and recombinant)

Data are based on a number of independent experiments each performed in technical triplicates
(*n* = 3 for cortical homogenate, *n* = 5 for HEK293T cells). r, rat. Assays were performed at pH 6 using [^{3}H]HOCPCA as selective radioligand.

	Rat cortical homogenate	rCaMKIIα-HEK
K_D (μM) (pK_D ± SEM)	0.26 (6.6 ± 0.06)	1.8 (5.8 ± 0.10)
B_{max} (pmol/mg protein)	43	64

Table S3. Collected inhibitory affinity constants from native and recombinant CaMKIIα binding assays

K_I values were calculated by means of the Cheng-Prusoff equation (*57*). Numbers in parentheses refer to the number of independent experiments each carried out in technical triplicates. n.d. *not determined*. All assays were performed at pH 6 using [^{3}H]HOCPCA as selective radioligand.

	[^{3}H]HOCPCA binding		SPR
	Rat cortical homogenate	**CaMKIIα-HEK**	**6x Hub**
	K_I (µM) (pK_I ± SEM) (n)		K_D (µM) (pK_D ± SEM) (n)
GHB	3.0 (5.5 ± 0.10) (4)	51 (4.3 ± 0.05) (3)	n.d.
HOCPCA	0.13 (6.9 ± 0.07) (4)	1.8 (5.8 ± 0.07) (7)	58 (4.35 ± 0.25) (3)
5-HDC	0.022 (7.7 ± 0.05) (3)	0.80 (6.1 ± 0.05) (3)	0.30 (6.53 ± 0.07) (3)

Table S4. Tabulated 3 crystallography data collection and refinement statistics
Statistics for the highest-resolution shell are shown in parentheses.

	6x Hub with 5-HDC
Wavelength	1.11583
Resolution range	106.47 - 2.20 (2.27 - 2.20)
Space group	C 2 2 21
Unit cell	
a, b, c	101.43, 182.96, 106.47
α, β, γ	90, 90, 90
Total reflections	671271 (58251)
Unique reflections	50585 (4349)
Multiplicity	13.3 (13.4)
Completeness (%)	100 (100)
Mean I/sigma(I)	22.7 (0.8)
Wilson B-factor	61.30
R-merge	0.072 (3.621)
R-meas	0.079 (3.933)
R-pim	0.021 (1.066)
CC1/2	1 (0.292)
Refinement Resolution	91.48 - 2.20 (2.28-2.20)
Reflections used in refinement	50548 (4985)
Reflections used for R-free	1715 (166)
R-work	20.98 (36.35)
R-free	24.37 (36.67)
CC(work)	0.958 (0.568)
CC(free)	0.957 (0.628)
Number of non-hydrogen atoms	7511
macromolecules	7303
ligands	121
solvent	87
Protein residues	899
RMS(bonds)	0.003
RMS(angles)	0.56
Ramachandran favored (%)	96.91
Ramachandran allowed (%)	3.09
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	2.69
Clashscore	3.88
Average B-factor	82.50
macromolecules	82.92
ligands	72.84
solvent	61.19
Number of TLS groups	37