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SUPPORTING INFORMATION for

Gephyrin promotes autonomous assembly and synaptic localization of GABAergic postsynaptic components without presynaptic GABA release

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2. Supplementary Experimental Methods

Cell lines, Generation of human neurons, Drug incubation, Lentivirus production, Generation of Gephyrin and hPEM-2 KO lines, Quantitative RT-PCR (qRT-PCR), Immunoblotting, Immunostaining, List of antibodies, Image acquisition and analysis, Electrophysiological recordings, and Data presentation.

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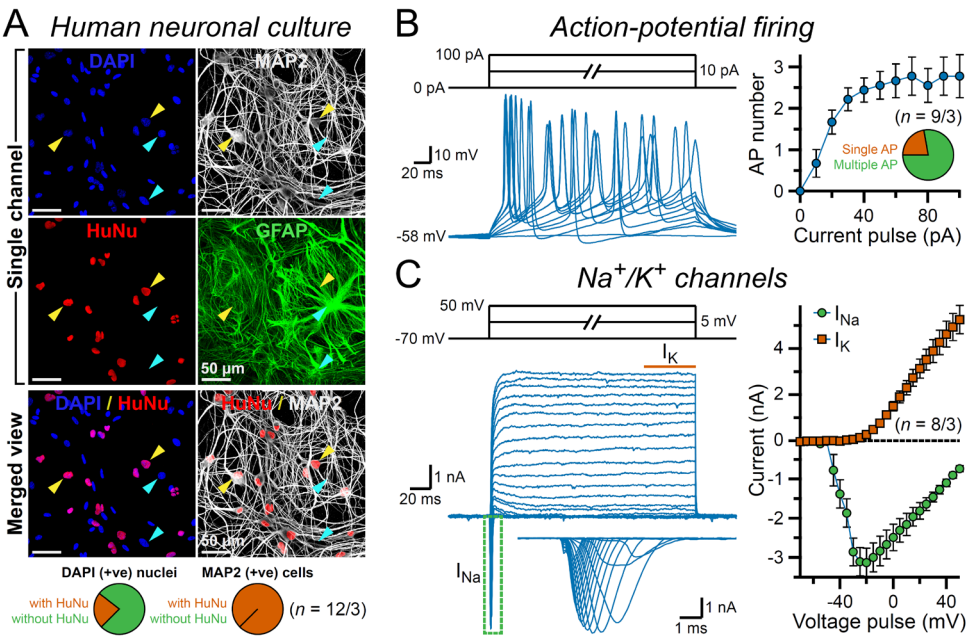
<1> Supplementary Figures & Figure Legends



Supplementary Figure S1: **The iPS cell -derived human neurons rapidly acquire functional maturation.**

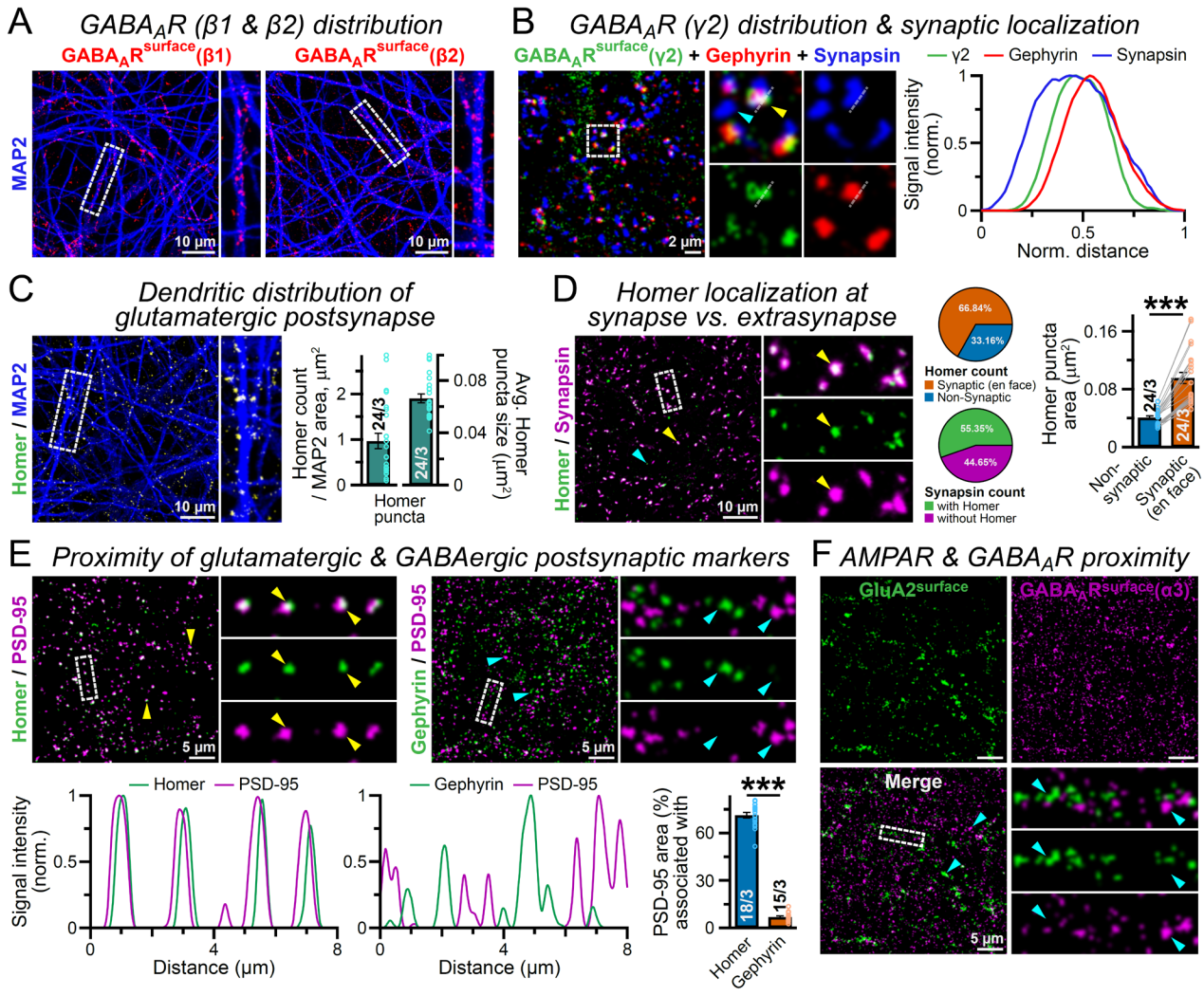
Human iPS cells (i.e., WTC-11 line) were directly reprogrammed into neurons by doxycycline-induced Ngn2 overexpression, co-cultured with mouse primary glia, and subsequently characterized at post-induction day 30.

A. Example images (top; single channel and merged views) of neuronal cultures, co-immunostained for MAP2 (neuronal marker), HuNu (human nuclear marker), and GFAP (glial marker), additionally stained with DAPI (all nuclei); arrowheads = MAP2 +ve neurons co-labeled with HuNu (yellow), or MAP2 -ve astrocytes labeled with GFAP (cyan). Fractions (bottom; pie-charts) of human nuclei (left) or human neurons (right) in total population.

B. Sample recordings (left) in current-clamp mode, and the total number of action-potentials (APs) plotted as a function of injected current amplitude (right); the pie-chart indicates fraction of cells with single or multiple APs.

C. Representative traces (left) in voltage-clamp mode, and the average amplitudes (right) of both Na^+ and K^+ channel currents (I_{Na} and I_K, respectively) measured from day 30 neurons; the inset (left, boxed area in green) region is magnified below to better visualize inward I_{Na} peak, whereas I_K was averaged for 50 ms (orange line).

Summary graphs are plotted as means ± SEM, and provided with number of cells patched / batches analyzed. Results exhibit a robust expression of voltage-gated ion channels in differentiated neurons, already by day 30.



Supplementary Figure S2: **GABAergic postsynapses retain their compositional identity without GABA.**

A. Example images of MAP2-positive dendritic branches co-immunostained for surface epitopes of GABA$_A$R subunits (β1 or β2, left or right, respectively); insets = boxed areas further expanded to ensure better visibility.

B. Representative image (left) with boxed region further expanded in the middle, and merged view further split between individual channels immunolabeled for GABA$_A$R γ2 subunit, Gephyrin, and Synapsin. Arrows point at synapses either containing both Gephyrin and GABA$_A$R γ2 signals (yellow), or those without any GABAergic postsynaptic components (cyan). Signal intensity profiles along the dotted lines (right) denote co-localization.

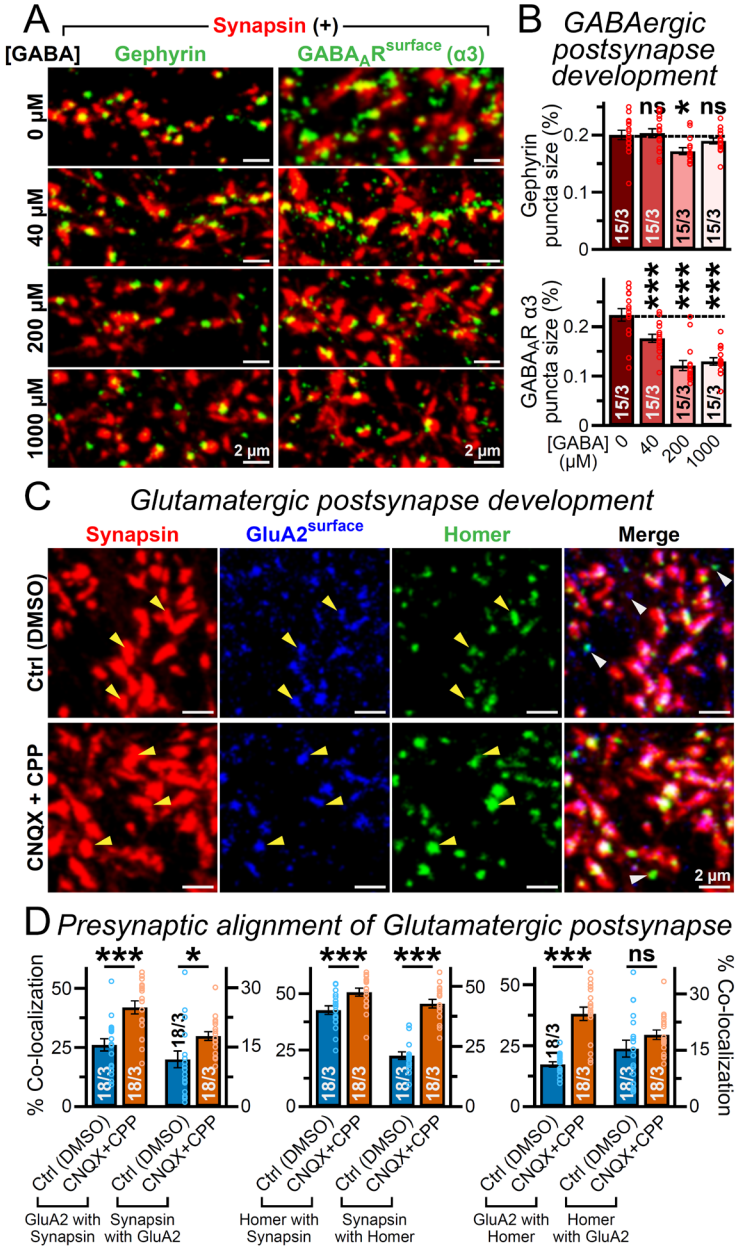
C. Example image (left) of dendritic segments immunolabeled for MAP2 and Glutamatergic postsynaptic scaffold Homer, with boxed section further magnified for clarity; average cluster size and density (right) of Homer signals.

D. Sample image (left) of synapses co-immunostained for presynaptic Synapsin and postsynaptic Homer, with boxed region expanded, and merged view split into individual channels; the arrowheads point at Homer puncta co-localized with Synapsin signal (yellow) or diffused outside (cyan). Pie-charts and average bar-graphs (right) respectively demonstrate the average fraction of Homer and Synapsin puncta aligned with one another, or the size of Homer puncta when positioned outside (non-synaptic) vs. within (synaptic, *en face*) a Synapsin signal.

E. Sample images (top) of human neuronal cultures as immunostained in pairs for Glutamatergic (Homer and PSD-95) and GABAergic (Gephyrin) postsynaptic scaffolds; boxed sections are enlarged to the right for better visibility; signal intensity profiles and average percentages (bottom) of PSD-95 puncta, which co-localize with Homer (yellow arrowheads) but remain physically isolated from adjacent Gephyrin (cyan arrowheads) puncta.

F. Representative image of neuronal cultures co-immunolabeled for both Glutamate and GABA receptors, e.g., AMPAR GluA2 subunit and $GABA_AR$ α3 subunit, respectively. Inset = boxed area enlarged; arrowheads (cyan) depict little spatial overlap between two different postsynaptic markers, suggesting they are mutually exclusive.

Average values indicate means ± SEM, along with individual data-points as color-matched circles (connected for paired comparison in **D**). Numbers on the bar-graphs denote field-of-views imaged / independent biological replicates. Statistical significance (**D** and **E**) was measured using two-tailed Student's t-test, with *** $P < 0.005$.



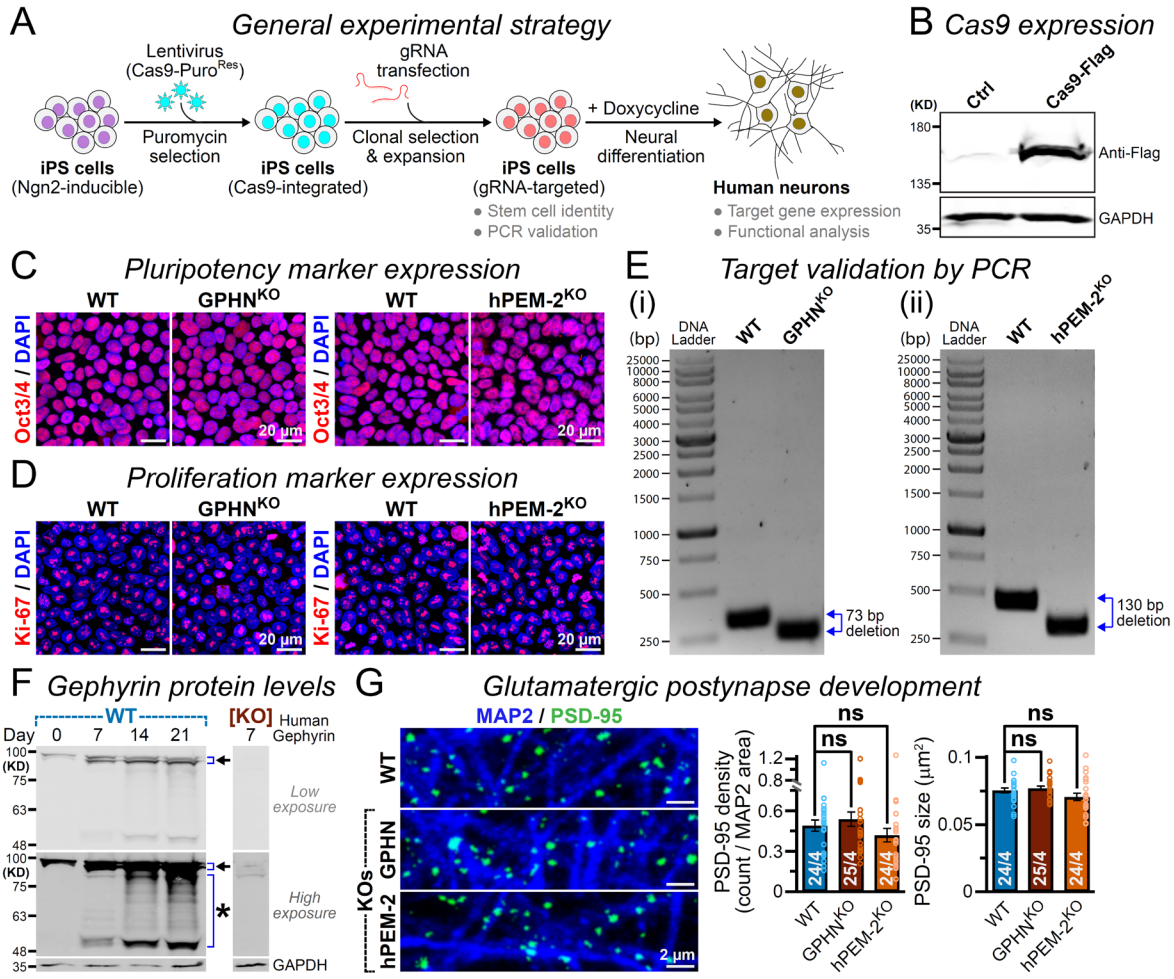
Supplementary Figure S3: **Activity-dependent modulation of postsynaptic organization in human neurons.**

A. Human neurons were continuously incubated with 0, 40, 200, or 1000 µM GABA in the culture media, from day 4-5 to day 36, that activate all (both synaptic and extrasynaptic) $GABA_ARs$ and $GABA_BRs$. Cells were fixed at day 36, and co-immunostained for Synapsin in combination with either Gephyrin (left) or $GABA_AR$ α3 (right).

B. The average size of postsynaptic Gephyrin (top) or $GABA_AR$ α3 (bottom) clusters, at indicated GABA dosages. This agonist-induced global activation of all GABA receptors reduced their aggregation at subsynaptic domains.

C-D. Sample images (**C**, individual channels and merged views) and Mander's coefficients of co-localization (**D**) between presynaptic Synapsin, GluA2-type AMPAR, or Glutamatergic postsynaptic scaffold Homer, following a chronic treatment of CNQX + CPP, from post-differentiation day 4-5 to day 36. This long-term silencing of activity significantly enhanced the association and presynaptic alignment of Glutamatergic postsynaptic compositions. Arrowheads in **C** point at AMPAR or Homer signals located inside (yellow) or outside (white) synapse.

All summary plots (panels **B** and **D**) represent means ± SEM, with total number of images analyzed / independent batches. Individual data-points are included as color-coded open circles. Statistical significances were calculated using two-tailed, unpaired, Students t-test, with *** $P < 0.005$; * $P < 0.05$; ns = not significant ($P > 0.05$).



Supplementary Figure S4: **Generation of Gephyrin and Collybistin KO iPS cell lines by gene targeting.**

A. Schematic of experimental strategy; the WTC-11 iPS cells were infected with a Cas9-expressing lentivirus, transfected with gRNAs against Gephyrin (GPHN) or Collybistin (hPEM-2) endogenous locus, KO validated by PCR screening, expanded, and rapidly differentiated into neurons by doxycycline (see Materials & Methods).

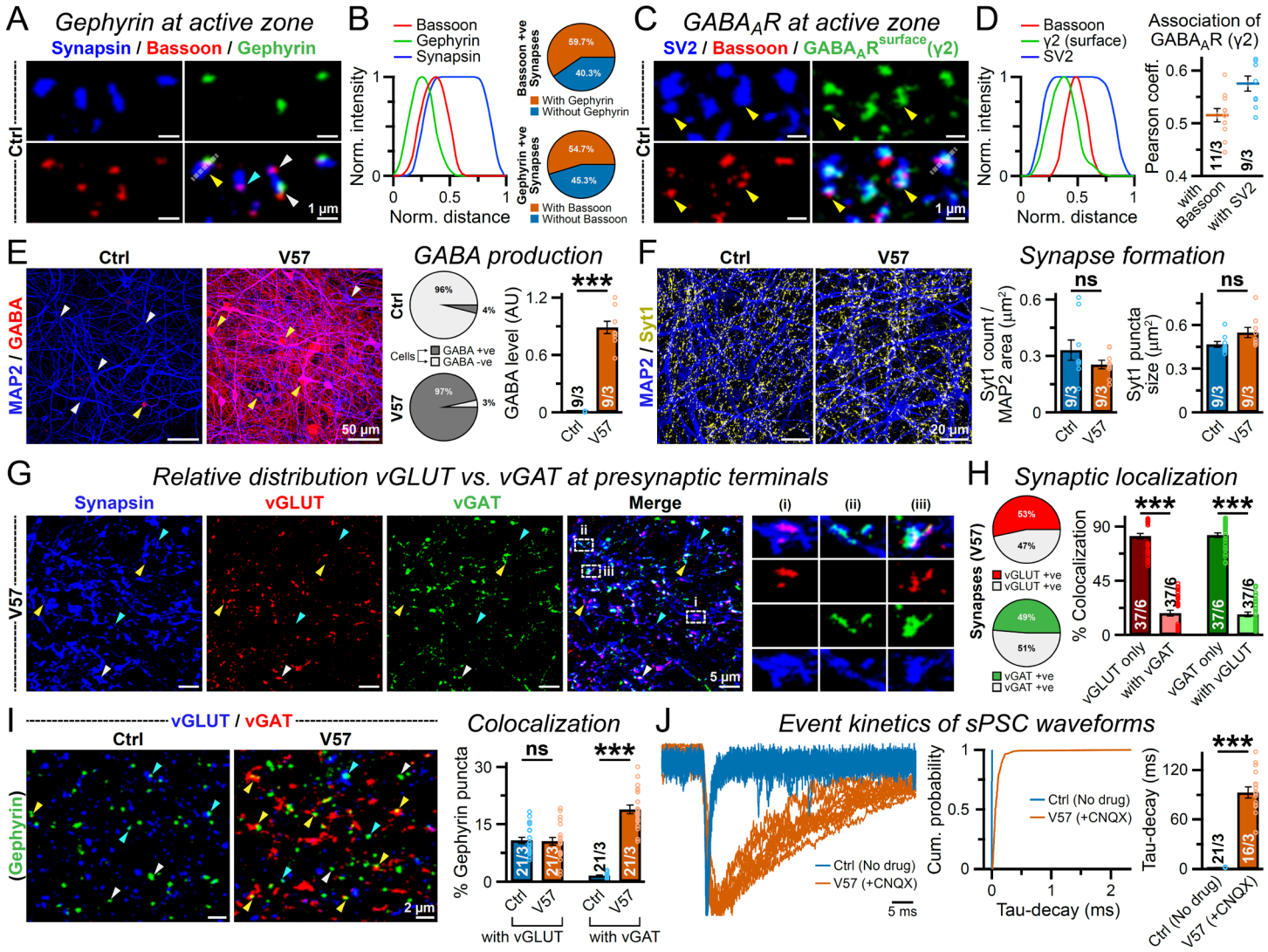
B. Representative western-blot using extracts from control (Ctrl) vs. Cas9-Flag expressing iPS cells, probed with a mouse anti-Flag antibody (catalog # 66008-3-Ig, Proteintech); GAPDH was used as a loading control.

C-D. The nuclei of WT vs. GPHN^{KO} or hPEM-2^{KO} iPS cells were labeled with DAPI, and co-immunostained for pluripotency (**C**, Oct3/4) and proliferation (**D**, Ki-67) markers. All lines were similarly positive for both markers.

E. Genomic DNA was isolated from WT vs. GPHN^{KO} (**i**) or hPEM-2^{KO} (**ii**) cells, PCR reactions were carried out with primers flanking the gRNA target sites (see KO chromatogram), and products were run on an agarose gel.

F. Sample immunoblot of endogenous Gephyrin (top image, arrow) from WT (left lanes) vs. GPHN^{KO} (right lane) neurons at indicated time-points. A high-exposure and low-contrast version of the same (bottom image) reveals additional products of lower molecular weight (asterisk) that express at lower levels. GAPDH = loading control.

G. Example images (left) of MAP2-positive dendrites immunostained for Glutamatergic postsynaptic scaffold PSD-95, its cluster density and average size (right) in WT vs. GPHN^{KO} or hPEM-2^{KO} conditions at day 30. Bar-graphs are means ± SEM, with number of images / experimental batches and data-points plotted as colored circles. Statistical assessment was performed using two-tailed, unpaired, t-test (ns = not significant; $P > 0.05$).



Supplementary Figure S5: **Induction of GABAergic postsynaptic activities by *de novo* GABA synthesis.**

A-B. Example images (**A**) of presynaptic terminals stained for vesicular marker Synapsin and an active-zone protein Bassoon, and GABAergic postsynapses labeled for Gephyrin; Arrowheads point at presynaptic release sites with (yellow) or without (cyan) postsynaptic Gephyrin clusters, or at synapses containing multiple release sites (white) that do or do not contain a corresponding Gephyrin puncta. Signal intensities of all channels were measured across a dotted line in **A**, and scaled to their maximum values (**B**, left); pie-charts (**B**, right) indicate percentages of synaptic (*en-face* with Synapsin) Bassoon and Gephyrin signals that localize with each other.

C-D. Same as **A-B**, except for synaptic vesicle protein SV2, Bassoon, and γ2 subunit of $GABA_A$Rs. The yellow arrowheads elucidate substantial co-localization between all three channels. Pearson's correlation coefficients (**D**, right) further confirm that a considerable number of $GABA_A$Rs effectively pre-aligns with synaptic terminals containing active-zone machineries, already in control (Ctrl, no detectable GABA release) condition at day 30.

E. The Ngn2-induced Glutamatergic human neurons were infected with lentivirus encoding vGAT + GAD65 + GAD67 (i.e., V57 factors) at post-differentiation day 30, and subsequently analyzed at day 51 (see Fig. 6A for details). Cultures were co-immunostained for MAP2 and GABA; sample images (left) with arrowheads indicate neurons with (yellow) or without (white) exogenous GABA; pie-charts and normalized GABA intensities (right) confirm a prominent biosynthesis of this neurotransmitter in majority of neurons, particularly in V57 condition.

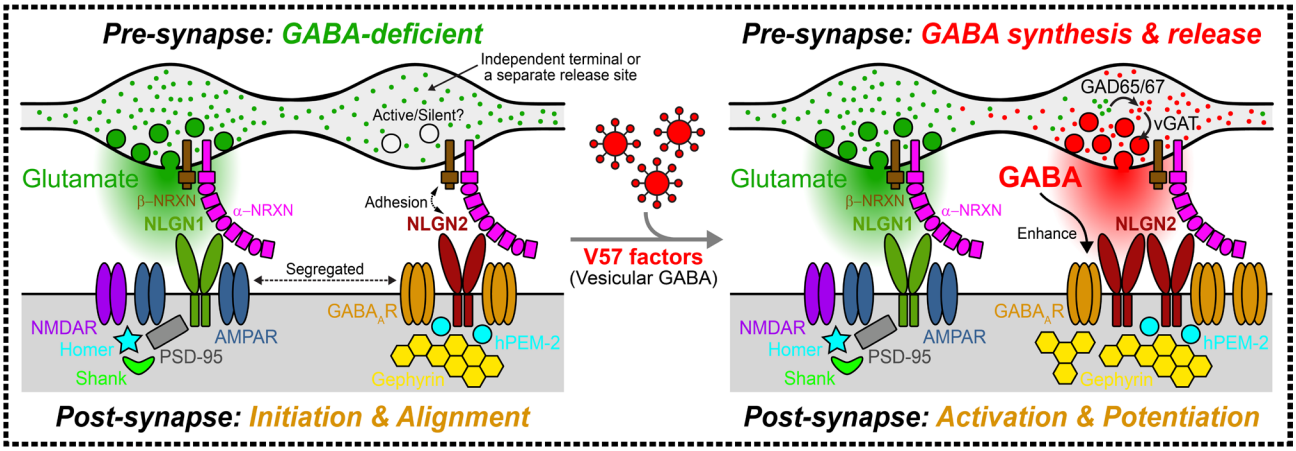
F. Cells were stained for MAP2 and Syt1 (as a proxy for morphologically defined synapses). Example images (left), average density and size (right) of Syt1 puncta suggest no noticeable differences between Ctrl vs. V57.

G-H. The presynaptic terminals (labeled for Synapsin) of V57-transduced neurons were co-immunostained for Glutamatergic vesicle marker vGLUT and GABAergic vesicle marker vGAT. Example images (**G**) of individual channels and merged view; insets = boxed sections (**i**, **ii**, and **iii**) further expanded to the right; arrowheads = synapses with either vGLUT or vGAT (yellow; mutually exclusive terminals), or both (white; mixed terminals), or neither (cyan; unspecified terminals). Pie-charts show the fractions of total synapses containing vGLUT or vGAT (**H**, left), and their limited degrees of association despite being co-expressed in V57 neurons (**H**, right).

I. Sample images (left) and percentages of co-localization (right) between Gephyrin and vGLUT vs. vGAT in Ctrl vs. V57-transduced neurons, at day 51. Arrows point at Gephyrin puncta without any specified synapse (white), or when apposed to Glutamatergic (cyan, vGLUT +ve) vs. GABAergic (yellow, vGAT +ve) terminals.

J. Representative sPSC events (left, 10 traces superimposed for each condition) recorded from Ctrl neurons, vs. when transduced with V57 factors (after bath application of CNQX); cumulative plot and average τ-decays (right) suggest that ectopic expression of the V57 factors can produce slower sPSCs with a different identity.

The summary plots reflect average (means ± SEM) parameters from each experiment, along with total number of field-of-views analyzed (panels **E**, **F**, **H**, and **I**) or neurons recorded (panel **J**) / independent batches (insets in bar-graphs) and individual data-points (color-coded open circles). Statistical comparison between conditions was conducted by two-tailed, unpaired, Student's t-test, with *** $P < 0.005$; ns = not significant ($P > 0.05$).



Supplementary Figure S6: **Proposed hypothesis for GABAergic postsynapse formation and activation.**

The schematic model suggests that a number of GABAergic postsynaptic proteins can self-aggregate through Gephyrin's intra- and intermolecular interactions and assemble at subsynaptic domains apposing GABA-less presynaptic specifications, but remain physically separated from their Glutamatergic counterparts (left); these pre-existing postsynaptic complexes could be functionally stimulated and further potentiated by *de novo* GABA biosynthesis and vesicular release from presynaptic terminals, with a delayed expression of V57 factors (right).

<2> Supplementary Experimental Methods

Cell lines: The HEK 293T (containing SV40 T-antigen) cells were commercially available from Takara Bio USA (catalog # 632180). The human iPS cells carrying doxycycline-inducible Ngn2 transgene (i.e., the WTC-11 line, [1]) was a gift from Dr. Michael E. Ward, National Institute of Neurological Disorders and Stroke (NINDS).

Generation of human neurons: WTC-11 cells were maintained in mTeSR™1 / mTeSR™ Plus media (StemCell Technologies) under feeder-free condition. Media was changed every day. When cell density reached 60-70%, they were dissociated with phosphate-buffered saline (PBS) + 0.5 mM EDTA and replated at ~1:6 dilution onto Matrigel -coated wells. During passage, cultures were additionally supplemented with ROCK-inhibitor Y-27632 (2.5 μM, MedChem Express) overnight, but excluded for later media changes. For neural differentiation, these cells were first plated at 1:15 dilution, cultured with N3 media (compositions: DMEM/F12 [Thermo Fisher] + N2 [Thermo Fisher] + B27 [Thermo Fisher] + insulin [20 μg/ml, Sigma-Aldrich] + penicillin-streptomycin mix [1%, Thermo Fisher], and then reprogrammed into neurons by doxycycline (2 μg/ml, Sigma-Aldrich) exposure.

For prolonged culture, the day 4-5 neurons were gently dissociated using PBS + EDTA (1mM) or accutase (Innovative Cell Technologies), and then replated together with primary mouse glia (passage 1-2, derived from WT C57BL/6J mice) on Matrigel (Corning, Sigma-Aldrich) pre-coated glass coverslips placed inside individual wells of 24-well dishes, in the N3 media also containing doxycycline and 10% fetal bovine serum (FBS, Atlas Biologicals). Media was half-exchanged every 3-4 days with FBS concentration gradually decreased to ~2.5%, and additionally supplemented with 5-fluorodeoxyuridine (FdU, 10 μM) to block glial growth after reaching 70-80% density. From day 15 onward, N3 media was progressively switched to Neurobasal Plus media (Thermo Fisher) supplemented with B27 (Thermo Fisher) + 5% FBS + 1% penicillin-streptomycin (Thermo Fisher).

Drug incubation: For chronic drug-treatment of neuronal cultures (Fig. 3, and Supplementary Fig. S3), the cells were exposed to drug cocktails blocking specific combinations of postsynaptic receptors [i.e., Picrotoxin (PTX, 100 μM; $GABA_AR$ and GlycineR antagonist, Tocris Bioscience), CGP55845 (CGP, 10 μM; $GABA_BR$ antagonist, Tocris Bioscience), 3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, 50 μM; NMDAR antagonist; Tocris Bioscience), or cyanquixaline (CNQX, 100 μM; AMPAR antagonist; Tocris Bioscience)]. The media was half-exchanged with drugs every 2-3 days, from post-differentiation day 4-5 to 28-36, and analyzed afterwards.

Lentivirus production: Three helper plasmids (i.e., pRSV-REV, pMDLg/pRRE, and VSV-G; 7.5-8 μg each) and expression vectors encoding either vGAT, GAD65, or GAD67 (driven by the human Synapsin-1 promoter, 15-20 μg each) were co-transfected with polyethylenimine (PEI) into 70-80% confluent HEK 293T cells plated on 10 cm dishes. Around 10-12 hours post-transfection, the culture medium (DMEM, Genesee Scientific) was exchanged completely, and supernatants containing virus particles were collected after 24, 48, and 72 hours. The supernatant was pooled and spun at ~800 x g for 6-8 minutes to remove HEK cell debris. Viral particles were concentrated by spinning the supernatant at ~120,000 x g for 2 hours at 4°C (using a Beckman L8-70M ultracentrifuge, equipped with SW41Ti rotor). The lentiviral pellets were stored overnight at 4°C, resuspended into ~100 μl DMEM media, mixed uniformly, aliquoted, frozen, and stored at -80°C prior to experimental usage.

Generation of Gephyrin and hPEM-2 KO lines: We produced lentiviral particles encoding a polycistronic product that linked FLAG epitope-tagged full-length Cas9 protein with a puromycin resistance cassette via self-cleaving 2A peptide (Cas9-T2A-$Puro^{resistance}$), cloned under a constitutive active cytomegalovirus (CMV) promoter. WTC-11 human iPS cells were sparsely infected with the virus, selected with puromycin (1 μg/ml) for 5 consecutive days, then expanded and stored at -80°C (Supplementary Fig. S4A). Western-blot assay confirmed stable FLAG-tagged Cas9 expression in the puromycin-resistant cell population (Supplementary Fig. S4B).

Next, these Cas9-expressing cells were seeded (approximately 50% confluency) onto a 6-well plate and transfected with pre-annealed trans-activating CRISPR RNA (tracrRNA) plus gRNAs (see KO chromatograms) using the Lipofectamine™ 3000 kit (catalog # L3000-015, Invitrogen). Around 24 hours post-transfection, these colonies were detached using PBS + EDTA, triturated thoroughly, dissociated into single cells by passing them through 70 μm cell strainers (Fisher Scientific, catalog # 22363548), and then reseeded onto a 6-well plate at

an extremely low density (~1% confluency). After three additional days in culture, the small colonies developed from single cell clones were hand-picked and grown individually in isolation. Genomic DNA was extracted from the subclones of each colony using Monarch Genomic DNA Purification Kit (New England Biolabs, catalog # T3010). Candidate clones for Gephyrin and hPEM-2 KOs were identified by PCR with primer pairs flanking the two intended gRNA target sites, followed by commercial Sanger sequencing (Supplementary Fig. S4E, see KO chromatograms). The successfully targeted clones were further expanded, aliquoted, and stored at -80°C for all future experiments. For Gephyrin^{KO} line, both alleles were similarly affected resulting in clean chromatogram peaks flanking the Cas9 deletion point. For hPEM-2^{KO} line, chromatogram peaks surrounding the deletion site corresponded to a single allele on the X-chromosome of WTC-11 iPS cells (male line). Cas9-integrated clones without gRNA transfection were considered as WTs, to be sequenced, maintained, and differentiated in parallel.

Quantitative RT-PCR (qRT-PCR): The WT vs. hPEM-2^{KO} neurons (at post-induction day 7) were lysed using TRIzol reagent (ambion, catalog # 15596018, Thermo Fisher), followed by phase separation using chloroform, RNA precipitation by isopropanol, washes with 70% ethanol, and reconstitution of the RNA pellet in autoclaved nanopure water. Complementary DNAs (cDNAs) were synthesized from mRNAs using Invitrogen SuperScript III First-Strand Synthesis System (catalog # 18080-051, Thermo Fisher) and oligo (dT) primers. The cDNA concentrations were measured by a NanoDrop machine and adjusted between WT and KO conditions. qRT-PCR reactions were carried out on a CFX-96 C-1000 thermocycler (Bio-Rad) using SYBR Green master-mix (Catalog # M-915, GoldBio) following the manufacturer's standard protocol. Both the target gene (i.e., hPEM-2) and GAPDH were assessed in triplicate wells (technical replicates) for each experimental batches, their cycle thresholds (Ct values) were averaged, and then normalized with corresponding GAPDH expression levels.

Immunoblotting: Neuronal cultures were lifted with PBS + EDTA, spun at ~800 x g for 5 minutes to precipitate, and the pellets were lysed with RIPA buffer (150 mM NaCl, 5 mM EDTA, 25 mM Tris pH 7.4, 1% Nonidet P-40 substitute, and 0.5% sodium deoxycholate) supplemented with Halt™ protease inhibitor cocktail (PIC; catalog # 78429, Thermo Fisher). Protein extracts were mixed with 10% Sodium dodecyl-sulfate (SDS) loading buffer, ran on 5-10% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. The membranes were then blocked with 5% bovine serum albumin (BSA) in TBS containing 0.1% Tween-20 detergent (TBS-T) while rocking for at least 1 hour at 37°C, and incubated overnight at 4°C with corresponding primary antibodies (see below) diluted in blocking buffer. The blots were washed thoroughly for 4-5 times with TBS-T over an hour, subsequently incubated with fluorescent-dye conjugated secondary antibodies (1:2000; DyLight 680/800, Invitrogen) diluted in blocking buffer (TBS-T, containing BSA) for 1-2 hours, washed again 4-5 times with TBS-T, and imaged immediately using a LI-COR Odyssey CLx system. Brightness and contrast of all protein bands were uniformly adjusted using the Image Studio Lite (version 5.2) software. Protein contents were estimated by plotting total-intensity profiles for each lane and calculating the peak areas underneath.

Immunostaining: Differentiated human neurons were washed once with PBS and fixed immediately with 4% paraformaldehyde (PFA, dissolved in PBS) for 30-45 minutes at room temperature, followed by an additional PBS wash. The cultures were subsequently blocked in PBS + 10% cosmic calf serum (CCS, HyClone) for 1 hour at 37°C while rocking, and then incubated with primary antibody combinations (see below) for 2 hours at 37°C or overnight at 4°C, washed 4 times with blocking buffer and incubated with Alexa Fluor dye -conjugated secondary antibodies (405/488/555/647, Invitrogen) for 1-2 hours at 37°C. Coverslips were washed twice with blocking buffer and twice with PBS, and mounted upside down on glass-slides using Fluoromount-G (Southern Biotech). To detect extracellular epitopes on $GABA_AR$ and AMPAR subunits localized on dendritic membranes, the cultures were first immunostained under non-permeabilized conditions, i.e., without any Triton X-100. For assays where permeabilization was required, Triton X-100 (0.1%) was applied afterwards to the blocking buffer and for all following steps, including washes as well as in primary or secondary antibody dilutions.

List of antibodies: Antibodies selected for western-blot and/or immunostaining assays included appropriate combinations of chicken anti-MAP2 (1:1000; catalog # Ab5392; Abcam), guinea pig anti-Synapsin1/2 (1:500; catalog # 106004; Synaptic Systems), mouse anti-Synapsin1 (1:500; catalog # 106011, Clone: 46.1; Synaptic

Systems), rabbit anti-Synapsin1/2 (1:500; catalog # 106002; Synaptic Systems), guinea pig anti-vGLUT1 (1:500; catalog # 135304; Synaptic Systems), rabbit anti-vGAT (1:500; catalog # 131003; Synaptic Systems), mouse anti-vGAT (1:500; catalog # 131011, Clone: 117G4; Synaptic Systems), rabbit anti-GAD65 (1:500; catalog # A0971; Abclonal), rabbit anti-GAD67 (1:500; catalog # A2938; Abclonal), guinea pig anti-GABRG2 (1:500; catalog # 224004; Synaptic Systems), rabbit anti-NLGN2 (1:500; catalog # 129203; Synaptic Systems), rabbit anti-GABRA2 (1:500; catalog # A1803; Abclonal), rabbit anti-GABRA3 (1:500; catalog # A11636; Abclonal), rabbit anti-GABRB1 (1:500; catalog # A19681; Abclonal), rabbit anti-GABRB2 (1:500; catalog # A11558; Abclonal), mouse anti-Gephyrin (1:500; catalog # 147011; Clone: mAb7a; Synaptic Systems), mouse anti-Gephyrin (1:500; catalog # 147111; Clone: 3B11; Synaptic Systems), mouse anti-β-Dystroglycan (1:500; catalog # MABT1546, Clone: 7D11; Sigma-Aldrich), mouse anti-Homer (1:500; catalog # 160011, Clone: 2G8; Synaptic Systems), rabbit anti-Bassoon (1:500; catalog # 141003; Synaptic Systems), rabbit anti-GluA2 (1:500; catalog # MAB397, Clone: 6C4; Sigma-Aldrich), fluorescence-labeled anti-PSD-95 (1:500; catalog # N3702-AF647-L; Synaptic Systems), mouse anti-GAPDH (1:5000; catalog # 60004-1-Ig, Proteintech), mouse anti-Oct3/4 (1:500; catalog # sc-5279; Santa Cruz Biotechnology), mouse anti-Ki-67 (1:500; catalog # 550609, BD Biosciences), rabbit anti-GABA (1:1000; catalog # A2052; Sigma-Aldrich), mouse anti-Syt1 (1:500; catalog # 105011; Synaptic Systems), mouse anti-SV2A (1:500; catalog # SV2; DSHB), rabbit anti-GFAP (1:500; catalog # AB5804, Sigma-Aldrich), and mouse anti-HuNu (1:1000; catalog # MAB1281, Sigma-Aldrich). When applicable, the cell nuclei were briefly stained with DAPI (1:50000; catalog # D1306; Thermo Fisher) for 5-10 minutes in washing buffer.

Image acquisition and analysis: The confocal images were captured using an inverted STELLARIS 5 (Leica Microsystems) laser scanning microscope (405/488/561/638) equipped with Power HyD detectors and and processed with a Leica Application Suite version X (LasX) software. Series of optical z-sections (~0.5-1 µm thickness) were acquired with 20x (dry, 0.75 na), 40x (oil immersion, 1.3 na), and 63x (oil immersion, 1.4 na) plan-apochromatic objectives. All super-resolution images (see Fig. 2E-I) were collected (dimension in x/y/z axis: 0.04 × 0.04 × 0.18 µm) using a Zeiss LSM 880 microscope (Zen 2.3 black edition, software v.14.0.9.201) equipped with plan-apochromat 63× oil-immersion objective (1.4 na) and Airyscan Gallium Arsenide Phosphide (GaAsP) detector, that reported to have spatial resolution of 120 nm in x/y- and 350 nm in z-plane [2].

All confocal images were analyzed using the ImageJ (FIJI, NIH) software. To quantify various parameters of synaptic puncta along the neuronal processes, images were generally superimposed as maximum-intensity z-projections (10-20 optical slices). Synaptic signals from different regions-of-interest (ROIs) were normalized with respect to corresponding MAP2-labeled dendrite areas. Co-localization parameters between two synaptic markers were assessed by first thresholding individual channels appropriately to eliminate background signals for individual experimental batches, and then measuring the Mander's coefficients from each optical section using either DiAna (object-based distance analysis) or JACoP (pixel-based area analysis) plugin. For super-resolution microscopy, image processing and analysis modules within the ZEN 2.3 (blue edition) software (v.2.3.69.1000) were used to extract maximum-intensity profiles, and measure fluorescence intensities.

Electrophysiological recordings: Whole-cell patch-clamp recording of human neurons were performed similarly to those described before [3, 4]. In brief, the neurons were patched using an internal solution containing (in mM) 135 $CsCl_2$, 1 EGTA, 1 NaGTP, 2 QX-314, and 10 HEPES (pH 7.4 adjusted with CsOH, 310 mOsm), and extracellular bath-solution containing (in mM) 140 NaCl, 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES NaOH (pH 7.4 adjusted with NaOH, 300 mOsm). All recordings were conducted at room temperature, and at a V_{hold} of -70 mV, using an integrated patch-clamp amplifier (IPA, Sutter Instruments) controlled by customized Igor Pro 8 (WaveMetrics) data acquisition system. Evoked PSCs were triggered by repetitive field stimulation with bipolar matrix electrodes (FHC, MX21AEW-RT2) connected to A365RC isolated pulse stimulator (World Precision Instruments). The AMPAR- and $GABA_AR$-mediated PSCs were pharmacologically isolated by using indicated combination of blockers (100 µM PTX, 50 µM CPP, and 50-100 µM CNQX) for other receptor types.

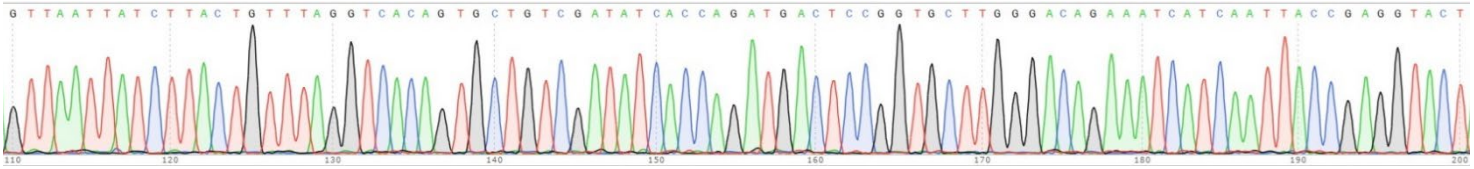
Data presentation: For all figure panels, the average values (bar-graphs, filled symbols [circles or squares] in cumulative plots, or pie-charts) reflect means ± SEM (i.e., standard-deviation of a given parameter divided by

square-root of sample numbers), and are presented along with the number of independent observations, e.g., cultures examined (for immunoblots or qRT-PCR), neurons patched (for electrophysiology), or field-of-views imaged (for immunostaining) / number of experimental replicates. Individual data-points are included as color-matched open symbols, and connected with straight-lines for paired comparisons. The types and strengths of all statistical evaluations between experimental groups are mentioned in corresponding figure legends, which included either unpaired (multiple measurements from each batch) or paired (batchwise assessments), two-tailed, Student's t-test (*** $P < 0.005$; ** $P < 0.01$; * $P < 0.05$; ns = non-significant, $P > 0.05$). Two-way analysis of variance (ANOVA) with replication was performed for multipoint (time-course, cumulative plot) comparisons.

<3> DNA (gRNA, qPCR) Sequences

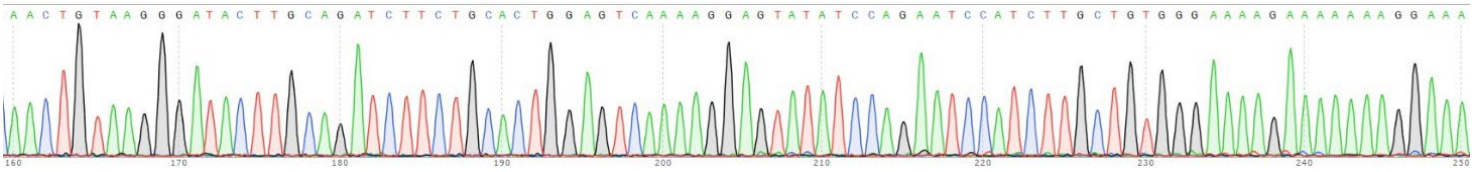
Generation of Gephyrin (Chr. 14) KO:	
Target exon	11
gRNA target #1	GGAGATGACTCCGGTGCTTG
gRNA target #2	TGCTGTCGATATCACCAAGG
Deleted DNA (73 bp)	AGGTGGCTAGAAGACATCGCATGTCTCCTTTTCCTCT GACATCTATGGACAAAGCCTTTATCACAGTCCTGGA
Sequencing Primer Fwd	AGGCTGTATTTTGGAGGCAGG
Sequencing Primer Rev	TTGACCATTGCCCTTTCTTTTCT

Fwr strand chromatogram (PCR): *Point of deletion* ▼



Generation of hPEM-2 (Chr. X) KO:	
Target exon	5
gRNA target #1	ATGATTGACATTGCTATCGA
gRNA target #2	TGAGTATTGTAACAACCACC
Deleted DNA (130 bp)	TGAGTATTGTAACAACCACCTGGATGCTTGCATGGAGCTCTCC AAACTGATGAAGGACAGCCGCTACCAGCACTTCTTTGAGGCCT GTCGCCTCTTGCAGCAGATGATTGACATTGCTATCGATGGTTTC
Sequencing Primer Fwd	TCAAGACTTCTTTTGGGGTCC
Sequencing Primer Rev	GCCTCATAGCCAACAGGGTA

Rev strand chromatogram (PCR): *Point of deletion* ▼



qPCR primers:		
hPEM-2	**Fwd (paired with Rev#1 or Rev#2)**	ATGATTGACATTGCTATCGA
	Rev #1 [Set 1, Product size = 234 bp]	CCAGTCTAGGACAGAAGCC
	Rev #2 [Set 2, Product size = 418 bp]	CCATGTCAATGCGGCCTTTGT
GAPDH	**Fwd (paired with Rev)**	TTGAGGTCAATGAAGGGGTC
	Rev [Product size = 117 bp]	GAAGGTGAAGGTCGGAGTCA

<4> Supplementary References

1. Wang, C., et al., *Scalable Production of iPSC-Derived Human Neurons to Identify Tau-Lowering Compounds by High-Content Screening.* Stem Cell Reports, 2017. **9**(4): p. 1221-1233.
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3. Cast, T.P., et al., *An Autism-Associated Mutation Impairs Neuroligin-4 Glycosylation and Enhances Excitatory Synaptic Transmission in Human Neurons.* J Neurosci, 2021. **41**(3): p. 392-407.
4. Chanda, S., et al., *Generation of induced neuronal cells by the single reprogramming factor ASCL1.* Stem Cell Reports, 2014. **3**(2): p. 282-96.