Supplemental Appendix: Methods, Figures and Table Legends

Function-Based Selection of TrkB Activating Antibodies: Characterization of A Full BDNF Agonist Antibody on Human Neurons

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Methods S1

The TrkB and TrkA open reading frames were cloned in the LV2 vector under the Ef1a promoter, made into lentivirus and infected to the CRE-β-lactamase and NFAT-β-lactamase cell line. One hundred individual clones of each reporter line were picked after limiting dilution and inspected by response to the corresponding ligands. The best responders were selected and amplified for future screening assays.

Phage panning and lentivirus preparation

Combinatorial antibody library phage display was performed as described previously [1, 2]. Briefly, histidine-tagged receptor ecto-domain protein was used for phage panning by the "capture out of solution" method. After two rounds of phage selection, ~10^6 colonies were collected and phagemid was extracted. The antibody-encoding fragments were cloned into the LV2 lentiviral vector. These plasmids were used to prepare lentivirus sub-libraries, then infected to the Trk reporter cell lines for functional selection.

Reporter based functional selection

The TrkB reporter cell lines were infected with lentiviral libraries at MOI=2 by spin inoculation. Six h after inoculation, the medium was replaced and incubated for another 40 h. The adherent cells were loaded with CCF4-AM dye, treated with Accutase detachment solution, rinsed with sorting buffer and subjected to FACS. Background signal was determined by the cells infected with lentivirus expressing Td-Tomato florescent protein. Upon activation, the reporter cells express beta-lactamase, cleave CCF4 and disrupt the FRET; excitation at 409 nm produces florescent

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signal at 450 nm, while green florescent signal at 520 nm is diminished. The top 1% of cells was collected and used for antibody fragment recovery. The selection cycles were repeated twice.

Expression, purification and characterization of scFv-Fc fusion proteins

The scFv encoding DNA fragments were cloned into the pFuse-Fc vector, which allows the scFv to be expressed in-frame with the Fc domain of human IgG_1 and secreted by the cells. The plasmids were transfected to Expi293™ cells for 48 h. Supernatants were collected and the antibody fusion protein was purified by a Protein-G affinity column. The purified protein was quantified by Qubit protein kit and tested on the Trk reporter cell lines for concentration-dependent activities.

Molecular Biology Reagents

TrkA H-190 rabbit polyclonal antibody, raised against amino acids 101-290 near the N-terminus of human TrkA was purchased from Santa Cruz; TrkC antibody (Abcam Cat. #33656) was raised against the entire recombinant extracellular domain of rat TrkC; anti-p75 NTR was purchased from Millipore (Cat. #07-476); the epitope was the GST fusion protein corresponding to the intracellular domain (residues 274-425) of rat p75 neurotrophin receptor.

Displacement of ZEB85 binding to TrkB cells by neurotrophins.

293T HEK and TrkB cell lines were resuspended in PBS, washed twice with PBS containing 1% BSA and 1 mM EDTA, and used for antibody competition assays. In brief, $5x10^5$ TrkB cells were pre-incubated in 100 μl of sorting buffer for 10 min at 4°C with the recombinant TrkB-ectodomain (molar ratio 30:1 to ZEB85), BDNF, NGF, NT3 or NT4 in different molar ratios to ZEB85 (4:1 and 12:1). After 10 min, ZEB85 antibody was added and cells were incubated on ice for another 30 min. The cells were then washed twice and stained with anti-Fc PE antibody (Southern Biotech, USA) for 30 min at 4° C. Unmodified 293T HEK cells were used as a negative control for ZEB85 staining. Cells were subjected to flow cytometry analysis to quantify binding.

hES cell neural differentiation

H9 human ES cells were plated on matrigel-coated plates (Corning, VWR) and maintained in mTSER medium (STEMCELL Technologies) supplemented with 10 ng/ml of FGF2 (Peprotech). hES cells were passaged by manual dissociation with 0.02% EDTA in Dulbecco's PBS (0.5mM), pH 7.2 (Sigma) and seeded for an 8-day phase of neuronal induction in SLI medium which contained Advanced DMEM F-12 medium supplemented with GlutaMAX , 1% each of penicillin and streptomycin (ADF, Life Technologies) and 2% NeuroBrew-21 without retinoic acid (Miltenyi Biotec), the SMAD pathway inhibitors LDN193189 (1 µM) and SB431542 (10 µM), and the WNT pathway inhibitor IWR1 (1.5 µM) (Tocris; [3]). Subsequently, neural progenitors were dissociated with Accutase (Life Technologies) and re-seeded for expansion to day 16 in LIA medium which contained ADF medium with 2% NeuroBrew-21 without retinoic acid, LDN193189 (0.2 µM), IWR1 (1.5 µM) and 25 ng/ml of Activin A (Peprotech). Neuronal differentiation was initiated by seeding neural progenitors on a substrate of growth factor-reduced matrigel (Corning, VWR) and poly- L- lysine (Sigma) at a density of 100K cells/cm^2 and cultured for 7 d in SynaptoJuice A medium which contained Advanced DMEM:F12 (with GlutaMAX), 1% penicillin/streptomycin, 2% NeuroBrew-21 with retinoic acid (Miltenyi Biotec), 2 µM PD0332991 (Selleckchem), 10 µM DAPT (Sigma-Aldrich), 0.6 mM $CaCl_2$ (to give 1.8 mM $CaCl_2$ in final complete medium (Sigma-Aldrich)), and 200 mM ascorbic acid (Sigma-Aldrich). The medium was replaced 7 d post-plating by SynaptoJuice B medium which contained equal parts Advanced DMEM/F12 (with GlutaMAX) and Neurobasal A (Life Technologies), 1% penicillin/streptomycin (Life Technologies), 2% NeuroBrew-21 with retinoic acid, 2 µM PD0332991, 3 µM CHIR 99021 (Tocris Bioscience), 0.3 mM $CaCl_2$ (1.8 mM $CaCl_2$ final), and 200 µM ascorbic acid (Sigma-Aldrich). Plated neurons were maintained for up to 14 d before exposure to TrkB ligands.

Construction of cDNA libraries and RNA-seq

RNA-sequencing was performed at the Genome Hub of Cardiff University. At least 500 ng of RNA was sequenced and the RIN number was above 9 as measured with an Agilent Bioanalyzer 2100. The TruSeq Stranded mRNA sample preparation guide and the high-throughput (HT) kit from Illumina were used according to the manufacturer's instructions. RNA-sequencing was performed on an Illumina HiSeq 4000 system at a sequencing depth of 50 million 50-nucleotide single pair-end reads.

qRT-PCR

Total RNA was extracted from neuronal cultures treated for 0-24 h with BDNF, ZEB85 or NT4 using the RNeasy Mini kit (Qiagen, Valencia, CA). First strand cDNA was prepared from RNA using the Supercript III reverse transcriptase (Invitrogen) and random primers (Promega). Quantitative PCR was performed on the StepOne plus PCR system (Applied Biosystem, Weterstadt, Germany), using TaqMan probes and primers from Thermo Fisher (SI Appendix, Table S1). Triplicate wells were analyzed for each condition and standard error of the mean was calculated. The exponential phase of the PCR reaction was used for quantification based on cycle numbers and fluorescence levels higher than the threshold value (Ct value). The fold changes in expression of the target genes relative to the internal control gene (18*S* ribosomal RNA) was calculated using the 2 ΔΔCT method for the timepoints and treatment conditions.

RNA-seq

H9 ES cell-derived neurons were treated for 30 min, 2 h, 12 h or 24 h with BDNF (50 ng/ml), ZEB85 (5 µg/ml) and NT4 (75 ng/ml), RNA extracted and integrity assessed on an Agilent RNA 6000 Pico chip using the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA (500 ng) was used to produce the cDNA library with the Tru-Seq protocol (Illumina) and sequenced with an Illumina HiSeq 4000 system. RNA-seq single-end fastq files were mapped to human assembly genome using the STAR package. Transcript counts were produced with FeatureCounts and data normalized using the Bioconductor package. Gene expression values and differentially expressed genes were obtained using DeSeq2 package in fragments per kilobase of transcript per million mapped reads (FPKM). Gene ontology analysis and a hierarchical clustered heat map was generated using the significantly regulated genes ($p<0.01$ and $|\log2fold|$ higher than 1). Pathway analysis was performed using the QIAGEN Ingenuity Pathway Analysis software suite.

Cell lysis and protein extraction

Neurons were plated in 12-well plates and incubated with the 3 TrkB ligands as indicated. Prior to lysis the cultures were washed with PBS containing 2 mM sodium orthovanadate, and subsequently detached with 500 µl of 0.05% trypsin and orthovanadate for 3 min. Trypsin was neutralized with the addition of 10% Fetal Bovine Serum. After centrifugation, cell pellets were lysed with 100 µl of RIPA lysis buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM

EDTA, 1% Triton-X-100, 0.2% sodium deoxycholate, 0.1 % SDS supplemented with protease and phosphatase inhibitor cocktail mix (Sigma-Aldrich) at 1:100 dilution, 100 mM 1,10-Phenanthroline, 100 mM 6-aminohexanoic acid, 10 mg/ml aprotinin and 2 mM sodium orthovanadate. Cell lysates were kept on ice for 30 min, centrifuged for 5 min at 12,000 rpm and the supernatant transferred to a new tube prior to western blot analysis, or stored at -80^{o} C.

Western blotting

Equal quantities of protein were separated on 4-12% NUPAGE gradient gels (Invitrogen) and transferred to nitrocellulose membranes using the semi-dry Bio-Rad Trans-Blot unit. The blots were probed with primary antibodies (rabbit monoclonal anti-pTrkA Y674/675 / anti-pTrkB Y706/707 (Cell Signaling) diluted 1:2000, or mouse monoclonal anti-synaptophysin (Sigma S5768) diluted 1:1000. Binding of primary antibodies was visualized with donkey anti-Rabbit HRP-conjugated and anti-Mouse HRP-conjugated secondary antibodies (Promega) diluted 1:10,000 followed by Chemiluminescence developing, using the membrane LumiGLO Reserve Chemiluminescent Substrate Kit (KLP). Blots were visualized with the Image Lab software and the Universal Hood III camera system (BIO-Rad). Densitometry analysis of the bands using ImageJ was applied to calculate the relative intensity of the signal between different conditions.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Tween-20 in PBS for 15 min at room temperature, washed with 0.1% Tween-20 in PBS for 5 min and blocked for 1 h with 10% goat serum in 0.1% Tween-20/PBS and incubated for 1 h at room temperature with any of the following antibodies: anti-β-III-tubulin rat polyclonal (Abcam) diluted 1:1000, anti- GAD65-67 rabbit polyclonal (Abcam 11070) diluted 1:500, or c-fos (Santa Cruz, rabbit polyclonal, sc-52) diluted 1:500. Subsequently the cultures were incubated for 1 h at room temperature with the corresponding secondary antibodies (Alexa fluor 488 donkey anti- rabbit and Alexa fluor 488 donkey anti-rat; Invitrogen).

Mouse cortical neuron cultures

Primary cortical neuronal cultures were prepared as described previously [4]. Briefly, cortices were dissected from embryonic day 16 CD1 mouse fetuses and dissociated into single cells following trypsin digestion (Worthington, Lakewood, USA) and DNase I treatment (Roche Applied Science). Neurons were plated on plastic dishes coated with poly-L-lysine (Sigma-Aldrich, UK), at a density of 100,000 cells/ cm2 and then cultured in Neurobasal A supplemented with 2 mM GlutaMAX I, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Crewe, UK). After 14 days *in vitro* (DIV) the neurons were exposed to either BDNF or ZEB85.

Mouse retinal explants

TrkB agonist antibodies and BDNF were assessed for RGC dendritic outgrowth in adult mouse retinal explant cultures as described by Binley et al [5]. Briefly, retinal explants were maintained in culture in the presence or absence of ZEB85 (50 μg/ml), ZEB44 (50 μg/ml) or BDNF (100 ng/ml). After 3 d explants were labeled with DiI/DiO, counterstained with DAPI and fixed with 4% paraformaldehyde. RGCs identified as having a soma in the RGC layer and an axon projecting to the optic nerve, were imaged at 20X as a z-stack (1 μm steps) using a confocal microscope. To quantify these differences, RGCs were traced in 3 dimensions and their arbors quantified by Sholl analysis using Simple Neurite Tracer (Fiji). Area under the Sholl curve (using a trapezoid model) and total dendrite length for each RGC were calculated to confirm the Sholl analysis.

Figure S1. Characterization of cell lines

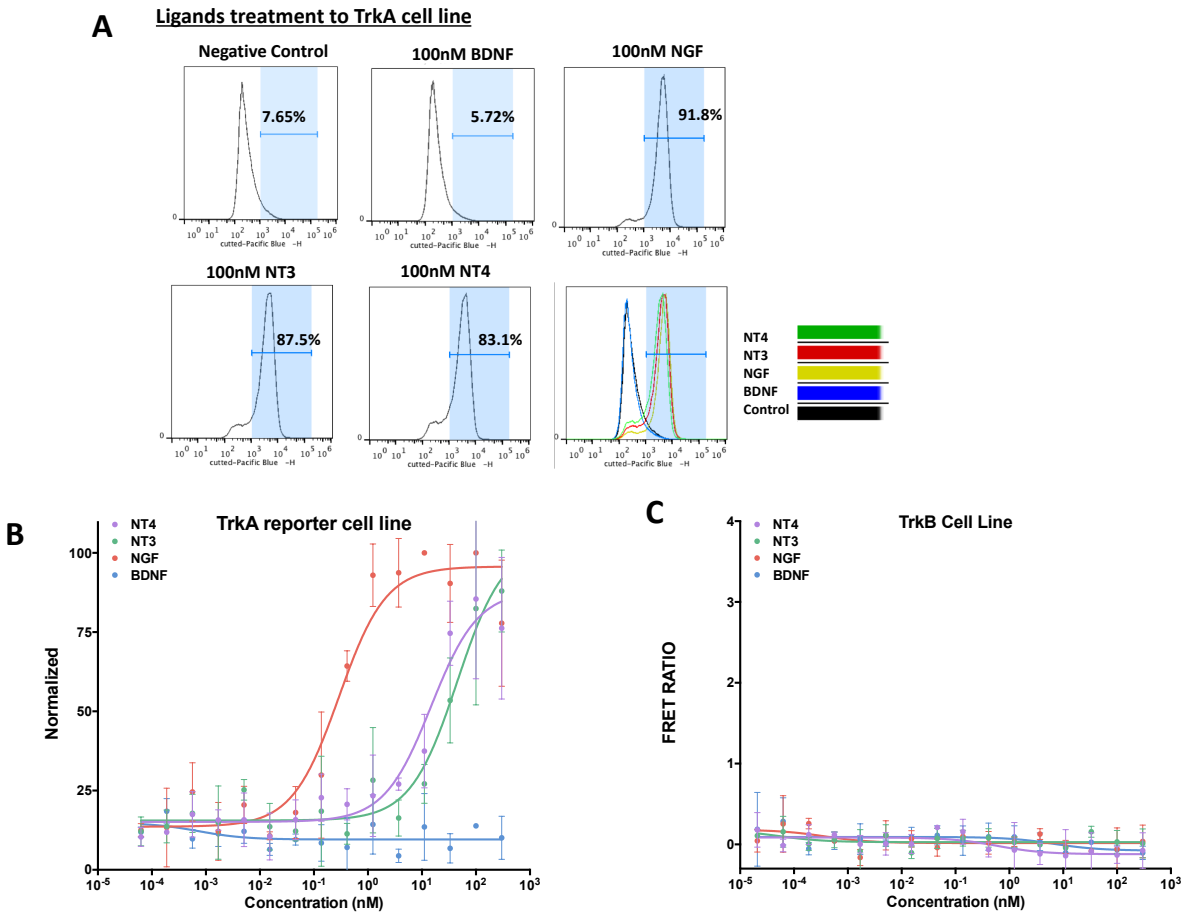


Figure S1. Characterization of cell lines. A) Flow cytometry analysis of TrkA cell line activation by different ligands. **B)** Concentration-response curve of different ligands on TrkA reporter cell line. **C)** Trk ligand activity on HEK293 reporter cell line without Trk receptor overexpression.

Figure S2. Function-based selection scheme.

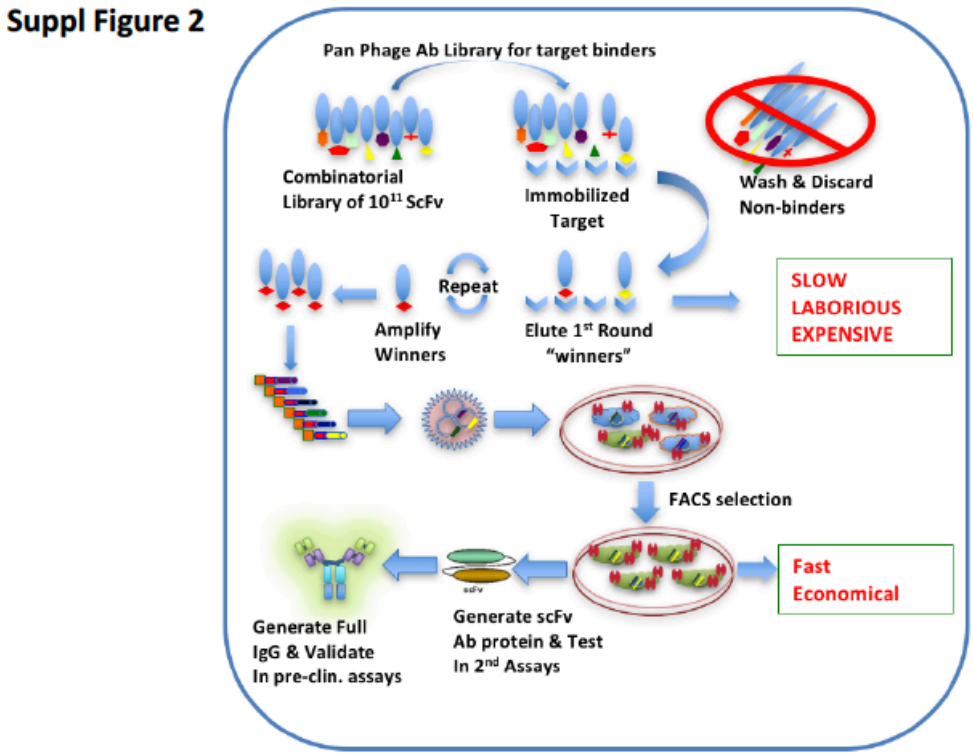


Figure S3. Phylogenetic tree of representative TrkB agonist clone sequences. Analyzed by CLUSTAL O (1.2.4) from EMBL.

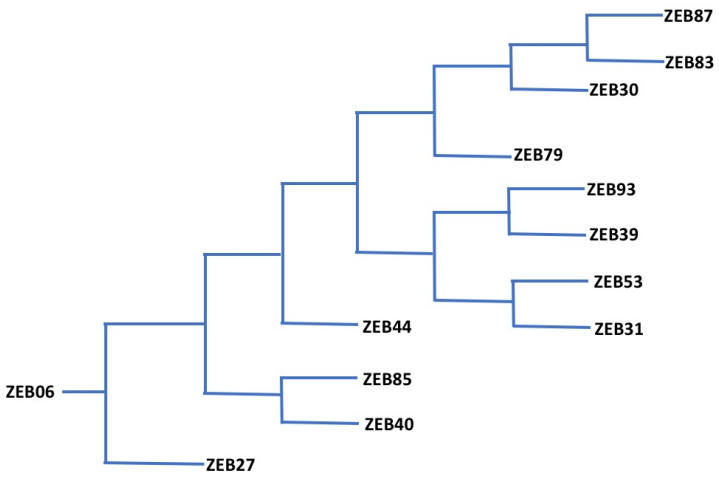
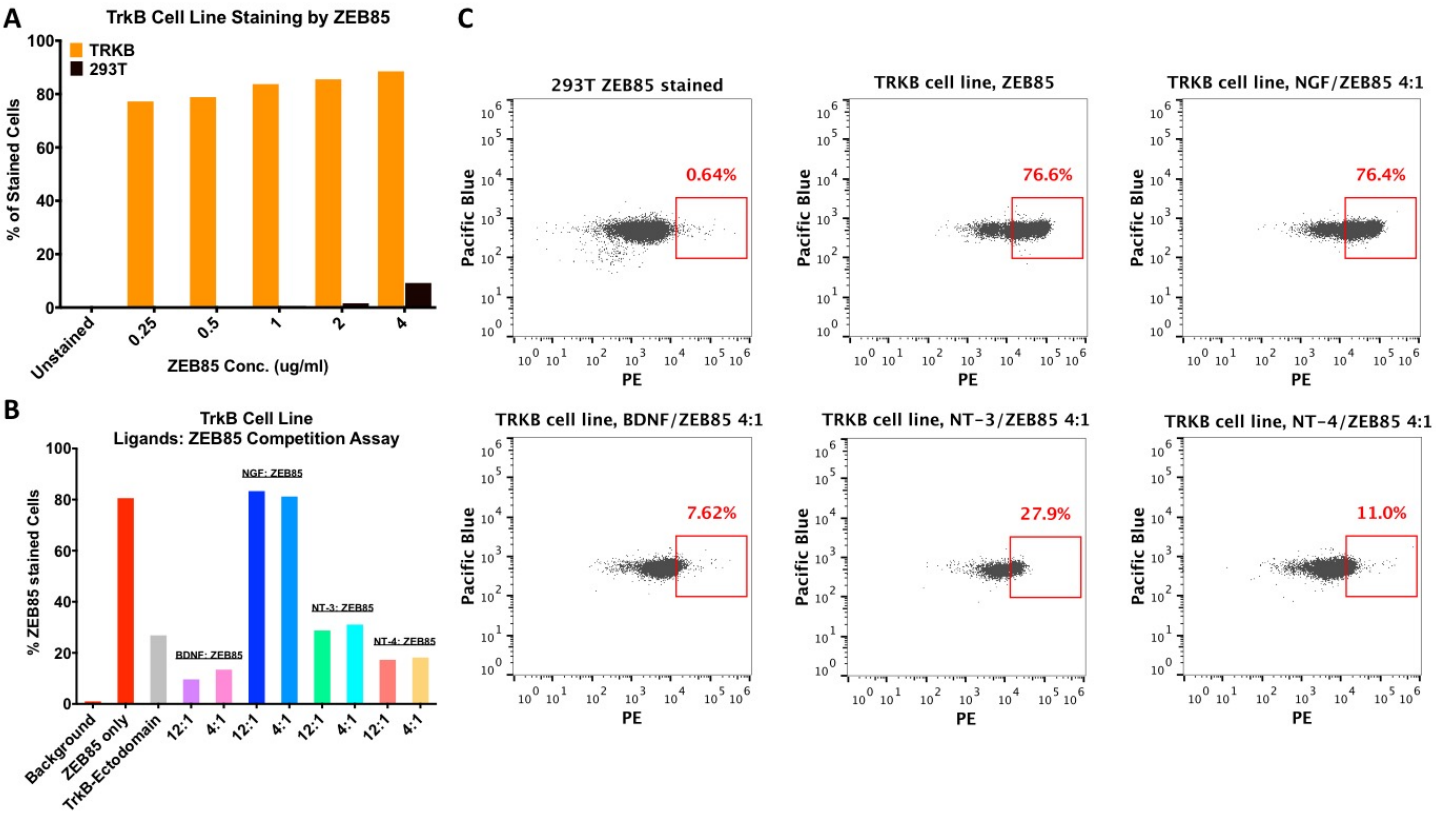


Figure S4. ***ZEB85 binding to hTrkB is displaced by BDNF***



(A) Dose titration of TrkB cell line labeling by ZEB85 antibody, quantified by flow cytometry. Regular HEK293T cells were used as control. **(B)** Binding competition between ZEB85 and natural ligands. Ligands: ZEB85 was calculated in molar ratio. Purified TrkB ectodomain was used in 30-fold excess. **(C)** Examples of flow cytometry dot plots. Readout was by PE conjugated anti-human IgG1 Fc secondary antibody. Pacific blue channel was used as control.

Table S1: Genes tested in Real Time PCR

name of gene	catalogue number (ThermoFisher Scientific)
VGF	Hs00705044_s1
SYNPO	Hs00702468_s1
PALD1	Hs01012869_m1
NPAS4	Hs00698866_m1
EGR2	Hs00166165_m1
EGR1	Hs00152928_m1
EGR3	Hs00231780_m1
Eukaryotic 18S rRNA	Hs03003631_g1

Table S2

RNA -seq: genes involved in synapse function, neuronal survival and apoptosis

Gene	Cont	BDNF 30 m	ZEB85 30 m	NT4 30 m	BDNF 2 h	ZEB85 2 h	NT4 2 h	BDNF 12 h	ZEB85 12 h	NT4 12 h	BDNF 24 h	ZEB85 24 h	NT4 24 h
Arc	1	14	15.5	20.2	25.1	19.9	29.5	4.3	2.7	3.7	5.9	2.7	5.8
SYT2	1	1.2	1.1	1	5.6	4.9	5.4	0.65	0.53	0.35	0.63	0.56	0.6
ACAN	1	0.97	0.88	0.78	65.4	56.6	70.6	8.9	6	24.9	6.5	3.1	7.3
GADD45A	1	1	1.1	1.3	13.7	13.6	13.6	9.2	5.3	7.8	8.5	3.9	7.9
CDKN1A	1	1.1	1.1	1.5	9.9	9.8	10.4	2.7	1.7	3.4	2.4	1.5	2.4
HRK/DP5	1	1	1.1	1.1	0.61	0.64	0.59	0.24	0.38	0.16	0.48	0.58	0.52

Abbrev.:
ARC Activity Regulated Cytoskeleton Associated Protein
SYT2 Synaptotagmin-2
ACAN Aggrecan
GADD45A Growth Arrest And DNA Damage Inducible Alpha
CDKN1A p21Cip1 / p21Waf1
HRK/DP5 Activator of apoptosis harakiri

SI References:

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3. Chambers, S.M., et al., *Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling.* Nat Biotechnol, 2009. **27**(3): p. 275-80.
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