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Supplemental Information

Networks of Cultured iPSC-Derived Neurons Reveal the Human Synaptic Activity-Regulated Adaptive Gene Program

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Figure S1

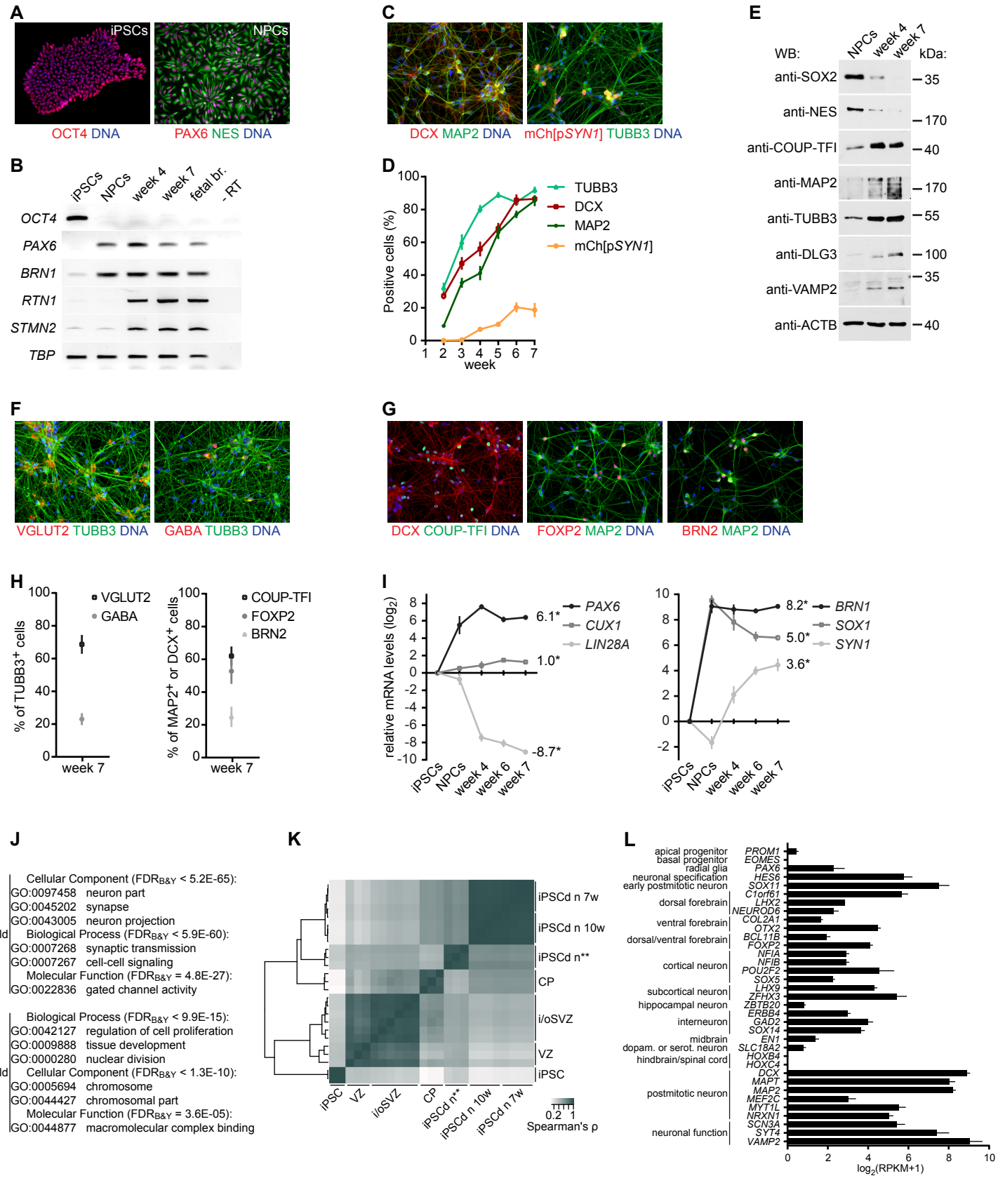
A
iPSCs
NPCs
OCT4 DNA
PAX6 NES DNA
C
DCX MAP2 DNA
mCh[pSYN1] TUBB3 DNA
E
WB:
NPCs
week 4
week 7
kDa:
anti-SOX2
35
anti-NES
170
anti-COUP-TFI
40
anti-MAP2
170
anti-TUBB3
55
anti-DLG3
100
anti-VAMP2
35
anti-ACTB
40
B
iPSCs
NPCs
week 4
week 7
fetal br.
-RT
OCT4
PAX6
BRN1
RTN1
STMN2
TBP
D
Positive cells (%)
week
TUBB3
DCX
MAP2
mCh[pSYN1]
F
VGLUT2 TUBB3 DNA
GABA TUBB3 DNA
G
DCX COUP-TFI DNA
FOXP2 MAP2 DNA
BRN2 MAP2 DNA
H
% of TUBB3+ cells
% of MAP2+ or DCX+ cells
week 7
VGLUT2
GABA
COUP-TFI
FOXP2
BRN2
I
relative mRNA levels (log2)
PAX6
CUX1
LIN28A
6.1*
1.0*
-8.7*
BRN1
SOX1
SYN1
8.2*
5.0*
3.6*
iPSCs
NPCs
week 4
week 6
week 7
J
Cellular Component (FDRB&Y < 5.2E-65):
GO:0097458 neuron part
GO:0045202 synapse
GO:0043005 neuron projection
>8-fold
Biological Process (FDRB&Y < 5.9E-60):
GO:0007268 synaptic transmission
GO:0007267 cell-cell signaling
Molecular Function (FDRB&Y = 4.8E-27):
GO:0022836 gated channel activity
Biological Process (FDRB&Y < 9.9E-15):
GO:0042127 regulation of cell proliferation
GO:0009888 tissue development
GO:0000280 nuclear division
>8-fold
Cellular Component (FDRB&Y < 1.3E-10):
GO:0005694 chromosome
GO:0044427 chromosomal part
Molecular Function (FDRB&Y = 3.6E-05):
GO:0044877 macromolecular complex binding
K
iPSCd n 7w
iPSCd n 10w
iPSCd n**
CP
i/oSVZ
VZ
iPSC
iPSC
VZ
i/oSVZ
CP
iPSCd n**
iPSCd n 10w
iPSCd n 7w
Spearman's ρ
0.2
1
L
apical progenitor
basal progenitor
radial glia
neuronal specification
early postmitotic neuron
dorsal forebrain
ventral forebrain
dorsal/ventral forebrain
cortical neuron
subcortical neuron
hippocampal neuron
interneuron
midbrain
dopam. or serot. neuron
hindbrain/spinal cord
postmitotic neuron
neuronal function
PROM1
EOMES
PAX6
HES6
SOX11
C1orf61
LHX2
NEUROD6
COL2A1
OTX2
BCL11B
FOXP2
NFIA
NFIB
POU2F2
SOX5
LHX9
ZFHX3
ZBTB20
ERBB4
GAD2
SOX14
EN1
SLC18A2
HOXB4
HOXC4
DCX
MAP1
MAP2
MEF2C
MYT1L
NRXN1
SCN3A
SYT4
VAMP2
log2(RPKM+1)

Figure S1. Molecular characterization of hiPSCd neuron differentiation. Related to Experimental Procedures
(A) Immunofluorescence staining of iPSCs and neuronal precursor cells (NPCs) with antibodies recognizing the indicated proteins. Human iPSCs (hiPS D1, see Experimental Procedures) were differentiatiated into PAX6- and nestin (NES)-expressing NPCs using a spin embryoid body protocol (Kim et al., 2011) with dual SMAD inhibition (Chambers et al., 2009).
(B) RT-PCR analysis of mRNA levels in iPSCs, NPCs and NPCs differentiated for 4 and 7 weeks, or in human fetal brain with primers targeting the indicated genes. A reaction without reverse transcription (-RT) was used as a control.
(C) Immunofluorescence staining of hiPSCd neurons differentiated for 7 weeks from NPCs with antibodies recognizing the indicated proteins. Expression of DCX and MAP2, or mCherry (direct fluorescence) under the control of the human *SYN1* promoter (mCh[p*SYN1*], lentiviral infection of NPCs) and TUBB3 is shown. Of note, expression of EGFP was very similar to expression of mCherry when NPCs were infected with a lentivirus encoding for EGFP under the control of p*SYN1*. See also Figure 3G.
(D) Quantification of cells positive for the indicated proteins in cultures of hiPSCd neurons differentiated for 2 to 7 weeks from NPCs. Three or four biological replicates were used for the quantification at each time point (mean ± SEM).
(E) Western blot analysis of protein expression using lysates from NPCs and hiPSCd neurons differentiated for 4 or 7 weeks from NPCs with antibodies recognizing the indicated proteins.
(F and G) Immunofluorescence staining of hiPSCd neurons differentiated for 7 weeks from NPCs with antibodies recognizing the indicated antigens.
(H) Quantification of cells double-positive for TUBB3, MAP2 or DCX and the indicated antigen in cultures of hiPSCd neurons differentiated for 7 weeks from NPCs. TUBB3 and VGLUT2, TUBB3 and GABA, DCX and COUP-TFI, MAP2 and FOXP2, or MAP2 and BRN2 were assessed. Three biological replicates were used for the quantification at each time point (mean ± SEM).
(I) RT-qPCR analysis of mRNA levels in hiPSCs, NPCs and hiPSCd neurons differentiated for 4, 6 or 7 weeks from NPCs with primers targeting the indicated genes. mRNA levels relative to the levels measured from iPSCs are shown for each gene. The values marked with asterisks designate the $\log_2$ fold changes measured with RNA-Seq in the respective genes' mRNA levels in hiPSCd neurons differentiated for 7 weeks from NPCs relative to the levels measured from iPSCs. Two or three biological replicates were used at each time point (mean ± SEM).
(J) GO term over-representation analysis with ToppGene (Chen et al., 2009) among genes up- or downregulated more than 8-fold in hiPSCd neurons differentiated for 7 weeks from NPCs relative to the expression levels in iPSCs. Most significantly enriched GO terms are shown. See also Table S1.
(K) Spearman correlation between the transcriptomes of the iPSCs (n = 2) and hiPSCd neurons (iPSCd n) produced in this study (7w and 10w, 7th and 10th week of differentiation from NPCs, respectively, both n = 4), hiPSCd neuron samples from Wen et al., 2014 (iPSCn**, four weeks of differentiation, n = 3), and human fetal (13th to 16th week postconception) ventricular zone (VZ, n = 3), inner or outer subventricular zone (i/o SVZ, n = 6) or cortical plate (CP, n = 3) samples from Fietz et al., 2012. 4497 genes with log-transformed RNA-Seq read counts per million standard deviation > 1 were used for the analysis. Correlation between all our hiPSCd neuron samples, ρ 0.968 to 0.998. Our hiPSCd neurons *vs* hiPSCd neurons produced by Wen et al. (ρ up to 0.712). Our hiPSCd neurons *vs* human 13th to 16th week postconception fetus cortical plate, which contains mostly postmitotic neurons (ρ up to 0.646). Our hiPSCd neurons *vs* human 13th to 16th week postconception fetus ventricular and subventricular zones, which contain proliferating cells (ρ up to 0.509).
(L) mRNA levels of selected marker genes in hiPSCd neurons differentiated for 7 weeks according to RNA-Seq (n = 4, mean ± SEM). Marker genes were selected based on single-cell transcriptomes of *in vivo* and *in vitro* human neurons (Camp et al., 2016), and additionally, some classical brain region-specific markers, used for example to identify regional specificity of human PSC-derived neurons (Imaizumi et al., 2015), were included. This data indicates that the hiPSCd neuron cultures are a heterogeneous population of neurons representing dorsal as well as ventral developing forebrain.

A brief description of markers whose expression was assessed in panels other than L: *OCT4* (*POU5F1*) and *LIN28A*, pluripotency markers; *PAX6* and *BRN1* (*POU3F3*), neuron/brain development-associated genes; *RTN1* and *STMN2*, neuron-specifically expressed genes; DCX and MAP2, postmitotic neuron markers; TUBB3, neuronal lineage-expressed protein; SOX2 and NES, NPC-expressed proteins; COUP-TFI (NR2F1), developing forebrain-enriched protein; DLG3, VAMP2 and SYN1, synaptic proteins; VGLUT2 (SLC17A6), excitatory neuron marker; GABA, inhibitory neuron marker; FOXP2 and BRN2 (POU3F2), forebrain-enriched/cortical layer markers; *SOX1*, NPC marker; *CUX1*, cell differentiation regulator.

Figure S2

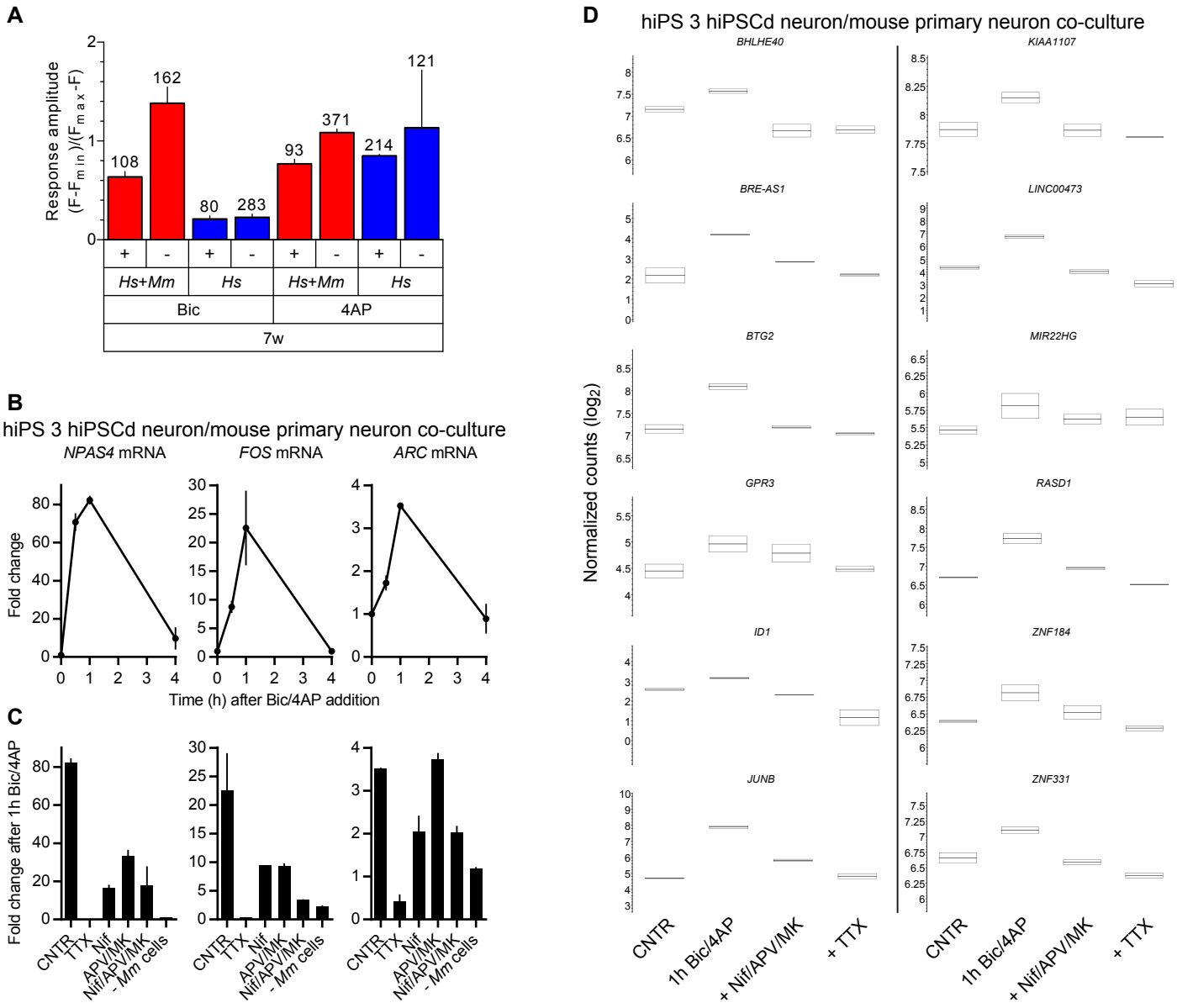

Figure S2.

(A) Rhod2 Ca^{2+} imaging of responses to bicuculline (Bic, 50 µM) or 4-aminopyridine (4AP, 250 µM).

Related to Figure 2

Ca^{2+} response amplitudes (peak minus baseline, mean ± SEM) in the number of cells indicated for $EGFP^{+}$ (+) and $EGFP^{-}$ (-) cells in the 7w *Hs*+*Mm* or *Hs* cultures and in the 10w *Hs* cultures. 7w and 10w, NPCs were differentiated for 7 weeks and 10 weeks, respectively. *Hs*+*Mm*, co-culture of hiPSCd neurons with mouse primary hippocampal neurons; *Hs*, human iPSCd neuron-only culture.

(B - D) Excitatory synaptic input to hiPS 3-derived hiPSCd neurons upregulates human IE genes.

Related to Figures 3 and 4

RT-qPCR (B and C) and nCounter (D, Kulkarni et al., 2011) analyses of changes in human IE gene mRNA levels after treatment with Bic/4AP (bicuculline, 50 µM and 4-aminopyridine, 250 µM) of hiPS 3-derived 7 week hiPSCd neuron/mouse primary hippocampal neuron co-cultures.

(B) Temporal profiles of IE gene mRNA levels in response to enhancement of synaptic activity.

(C and D) Analysis of the effects of blocking action potentials (TTX, tetrodotoxin, 1 µM), VGCCs (C, Nif, Nifedipine, 10 µM) or NMDARs (C, APV/MK, 2-amino-5-phosphonovalerate, 50 µM and MK801, 10 µM), or both VGCCs and NMDARs (Nif/APV/MK) on the increase in IE gene mRNA levels in response to Bic/4AP-tretament. In C, IE gene mRNA level changes in hiPS 3-derived hiPSCd neurons differentiated for 7 weeks and maintained without addition of mouse primary hippocampal neurons (- *Mm* cells) are also shown. In D, + shows that the indicated substances were present in the culture medium in addition to Bic/4AP.

Two biological replicate measurements were performed for all data points (B and C, mean ± SEM; C, data range with average).

Figure S3

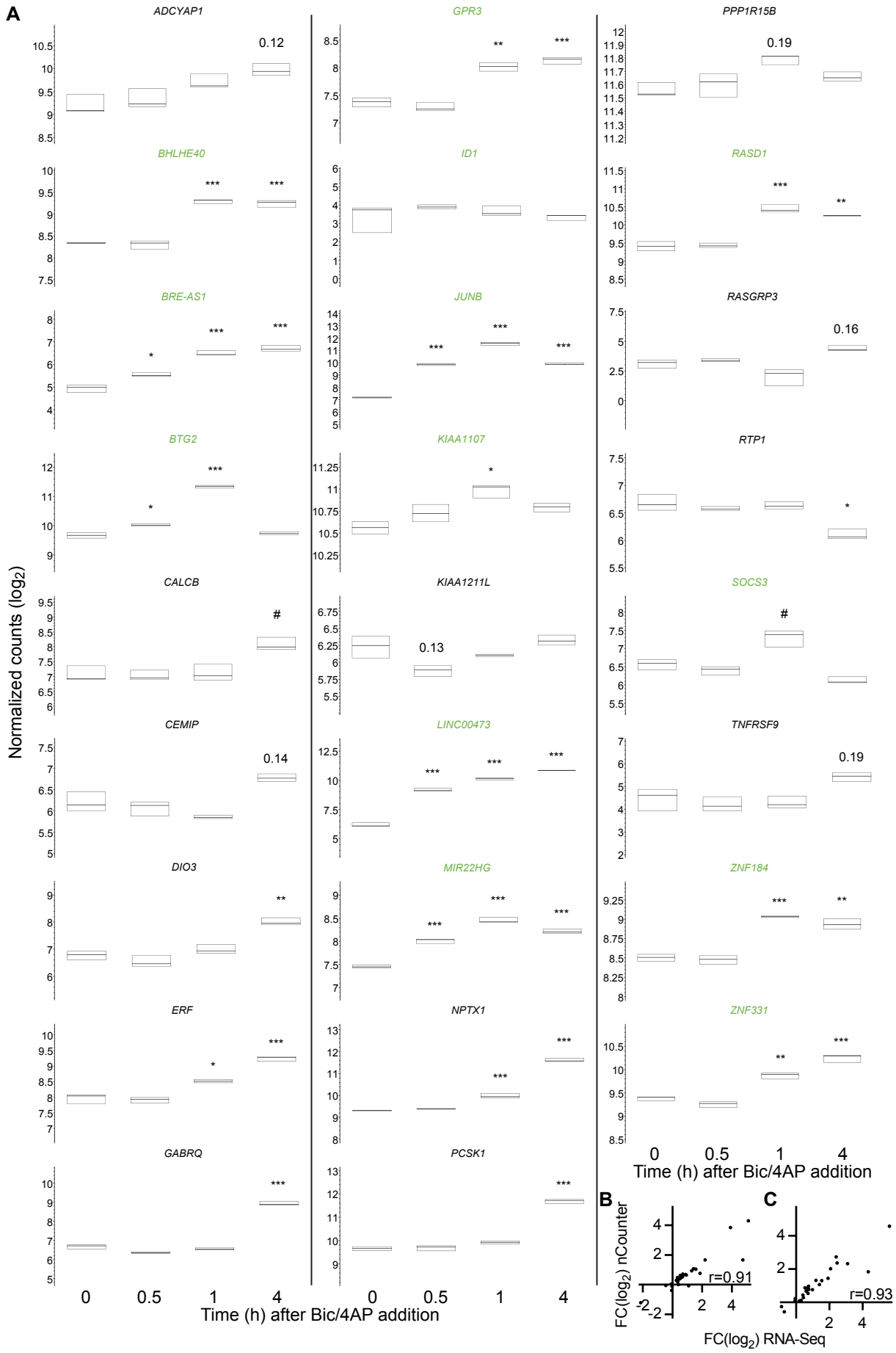
A
ADCYAP1
GPR3
PPP1R15B
BHLHE40
ID1
RASD1
BRE-AS1
JUNB
RASGRP3
BTG2
KIAA1107
RTP1
CALCB
KIAA1211L
SOCS3
CEMIP
LINC00473
TNFRSF9
DIO3
MIR22HG
ZNF184
ERF
NPTX1
ZNF331
GABRQ
PCSK1
Normalized counts (log2)
Time (h) after Bic/4AP addition
B
C
FC(log2) nCounter
FC(log2) RNA-Seq
r=0.91
r=0.93

Figure S3. Analysis of RNA levels of the indicated human genes with the nCounter method (Kulkarni et al., 2011). Related to Figure 4

(A) 7 week hiPSCd neuron/mouse primary hippocampal neuron co-culture was left untreated or treated with Bic/4AP (bicuculline, 50 μM and 4-aminopyridine, 250 μM) for the time indicated. RNA expression levels were normalized to the levels of three "housekeeping" genes: *EIF2B4*, *FCF1*and *HPRT1* (normalized counts). The names of human IE genes according to RNA-Seq data are in green. Three biological replicate measurements were performed for each data point. Box plots show data range with median. Significance (one-way ANOVA and Dunnett's multiple comparison test) is indicated compared to untreated control, #$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Values above boxes are p values (shown if < 0.2 and > 0.1).

(B and C) Correlation of gene expression fold change $\log_2$ [FC($\log_2$)] values of 26 human genes measured using the nCounter method after a one-hour Bic/4AP-treatment (B) or after a four-hour Bic/4AP-treatment (C) with the corresponding values measured with RNA-Seq. The dots denote the mean values of three biological replicate experiments. Pearson's r two-tailed $p < 0.0001$. The mean fold changes in gene expression obtained with the two methods at both the one hour and the four hours time point are very similar, verifying the reliability of the mixed-species RNA-Seq analysis.

Figure S4

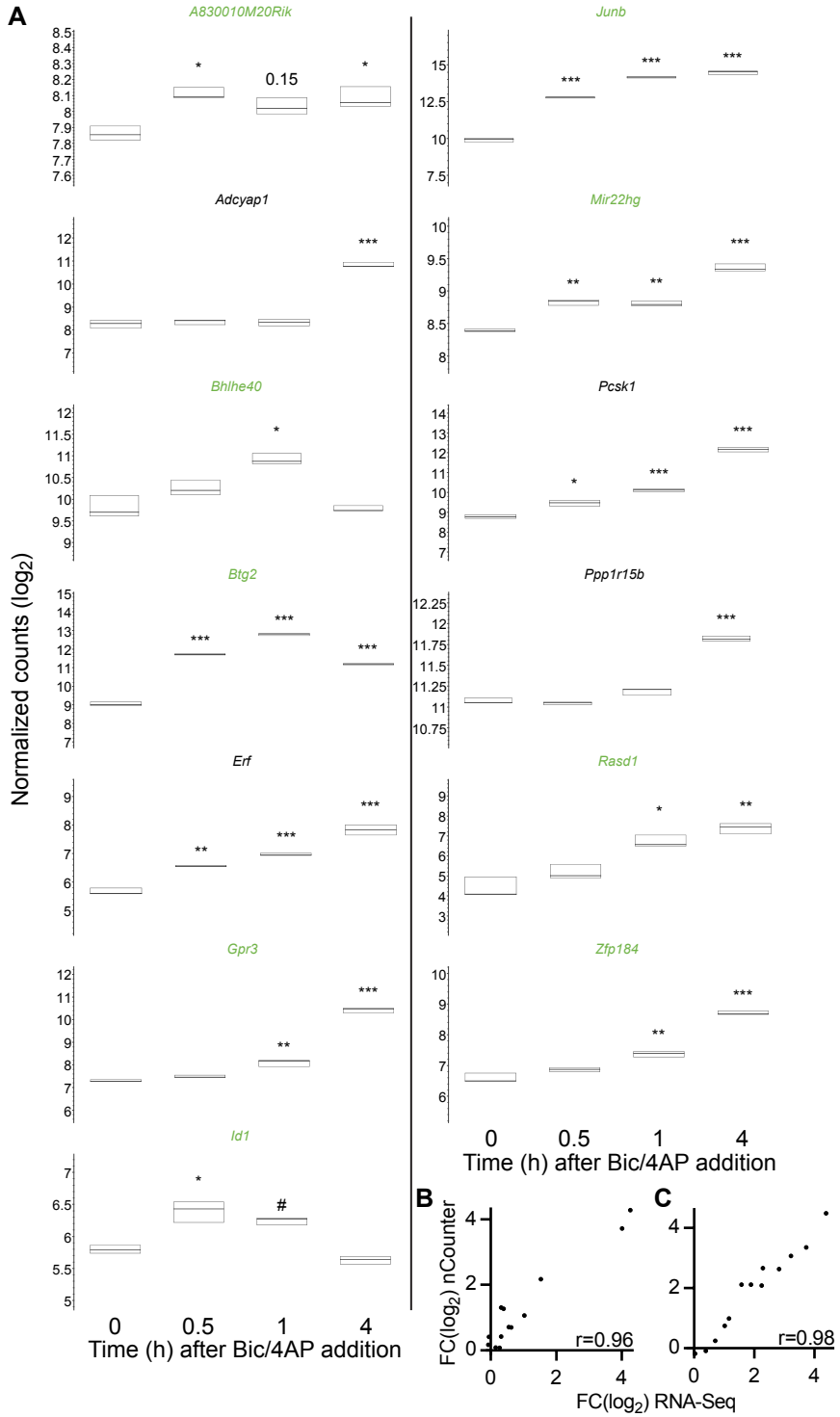

Figure S4. Analysis of RNA levels of the indicated mouse genes with the nCounter method (Kulkarni et al., 2011). Related to Figure 4

(A) 7 week hiPSCd neuron/mouse primary hippocampal neuron co-culture was left untreated or treated with Bic/4AP (bicuculline, 50 µM and 4-aminopyridine, 250 µM) for the time indicated. RNA expression levels were normalized to the levels of three "housekeeping" genes: *Eif2b4*, *Fcf1* and *Hprt1* (normalized counts). The names of the mouse orthologs of human IE genes are in green. Three biological replicate measurements were performed for each data point. Box plots show data range with median. Significance (one-way ANOVA and Dunnett's multiple comparison test) is indicated compared to untreated control, #$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 0.15, p value for the respective data point. All 9 mouse orthologs of human IE genes analysed, including genes that were not significantly induced at the one hour time point in RNA-Seq analysis, showed significantly upregulated RNA levels at 30 minutes and/or one hour after Bic/4AP addition.

(B and C) Correlation of gene expression fold change $\log_2$ [FC($\log_2$)] values of 13 mouse genes measured using the nCounter method after a one-hour Bic/4AP-treatment (B) or after a four-hour Bic/4AP-treatment (C) with the corresponding values measured with RNA-Seq. The dots denote the mean values of three biological replicate experiments. Pearson's r two-tailed $p < 0.0001$. The mean fold changes in gene expression obtained with the two methods at both the one hour and the four hours time point are very similar, verifying the reliability of the mixed-species RNA-Seq analysis.

Figure S5

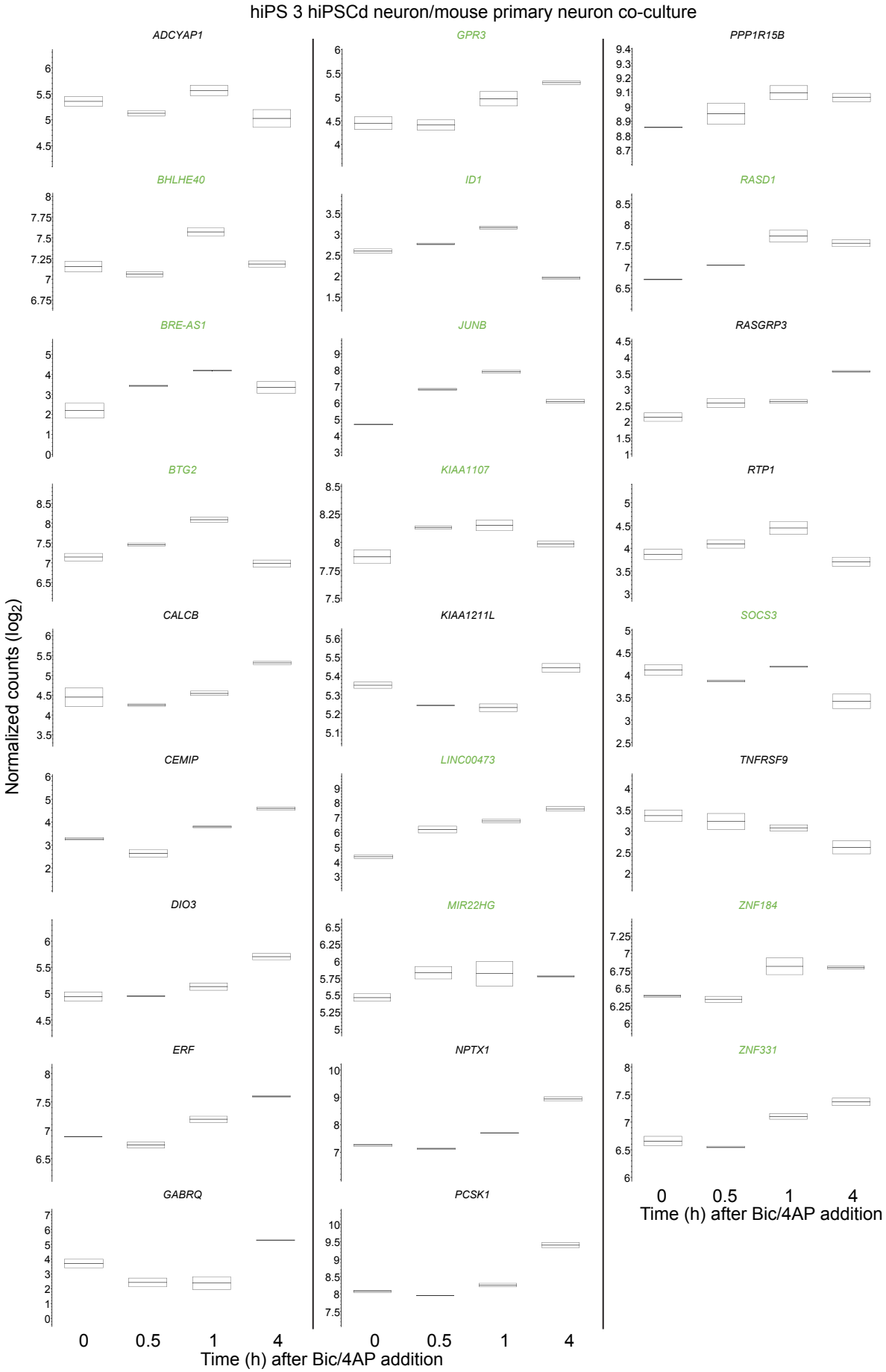
hiPS 3 hiPSCd neuron/mouse primary neuron co-culture
ADCYAP1
GPR3
PPP1R15B
BHLHE40
ID1
RASD1
BRE-AS1
JUNB
RASGRP3
BTG2
KIAA1107
RTP1
CALCB
KIAA1211L
SOCS3
CEMIP
LINC00473
TNFRSF9
DIO3
MIR22HG
ZNF184
ERF
NPTX1
ZNF331
GABRQ
PCSK1
Normalized counts (log2)
0
0.5
1
4
Time (h) after Bic/4AP addition

Figure S5. Analysis of RNA levels of the indicated human genes with the nCounter method (Kulkarni et al., 2011) in hiPS 3-derived 7 week hiPSCd neuron/mouse primary hippocampal neuron co-culture. Related to Figure 4
The cells were left untreated or treated with Bic/4AP (bicuculline, 50 μM and 4-aminopyridine, 250 μM) for the time indicated. RNA expression levels were normalized to the levels of three "housekeeping" genes: *EIF2B4*, *FCF1* and *HPRT1* (normalized counts). The names of the human IE genes according to RNA-Seq data obtained with the hiPS D1-derived hiPSCd neurons are in green. Two biological replicate measurements were performed for each data point. Box plots show data range with average.

Figure S6

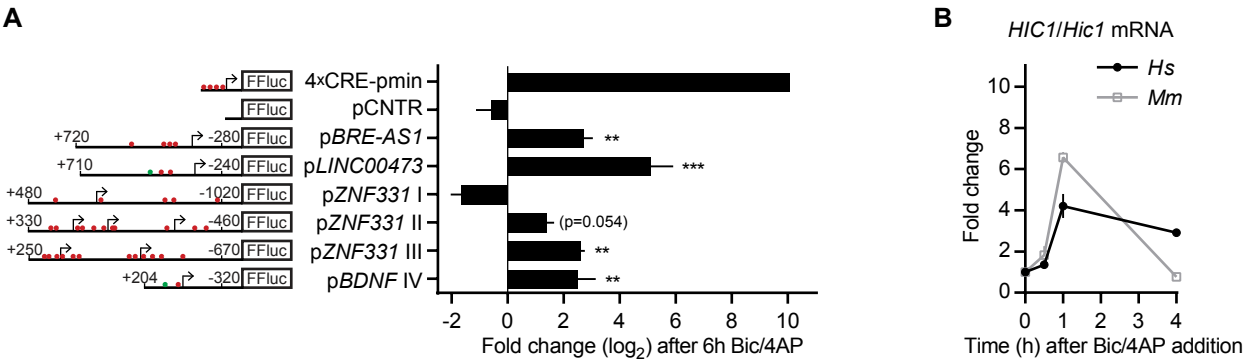

Figure S6. Assessment of potential lineage-specific control of synaptic activity-regulated genes.
Related to Figures 5 and 7

(A) Luciferase reporter assay with the firefly luciferase (FFluc) under the control of the indicated promoter or without a promoter (pCNTR) in mouse primary cortical neurons. Change in FFluc activity in response to Bic/4AP-treatment (bicuculline, 50 µM and 4-aminopyridine, 250 µM) is shown. Arrows denote transcription start sites (TSSs). Numbers specify bps upstream (-) or downstream (+) of the most 5' or 3' TSS, respectively. Red or green dots represent CRE and CREhs or PasRE cis-elements, respectively. n = 3; mean ± SEM; one-way ANOVA and Dunnett's multiple comparison test, **$p < 0.01$, ***$p < 0.001$. The asterisks indicate significance compared to pCNTR.

(B) RT-qPCR analysis of *HIC1*/*Hic1* mRNA levels in hiPS 3-derived hiPSCd neuron/mouse primary hippocampal neuron co-culture. Changes in human (*Hs*) *HIC1* and mouse (*Mm*) *Hic1* mRNA levels after treatment with Bic/4AP (bicuculline, 50 µM and 4-aminopyridine, 250 µM) for the time indicated are shown. n = 2, mean ± SEM.

Figure S7

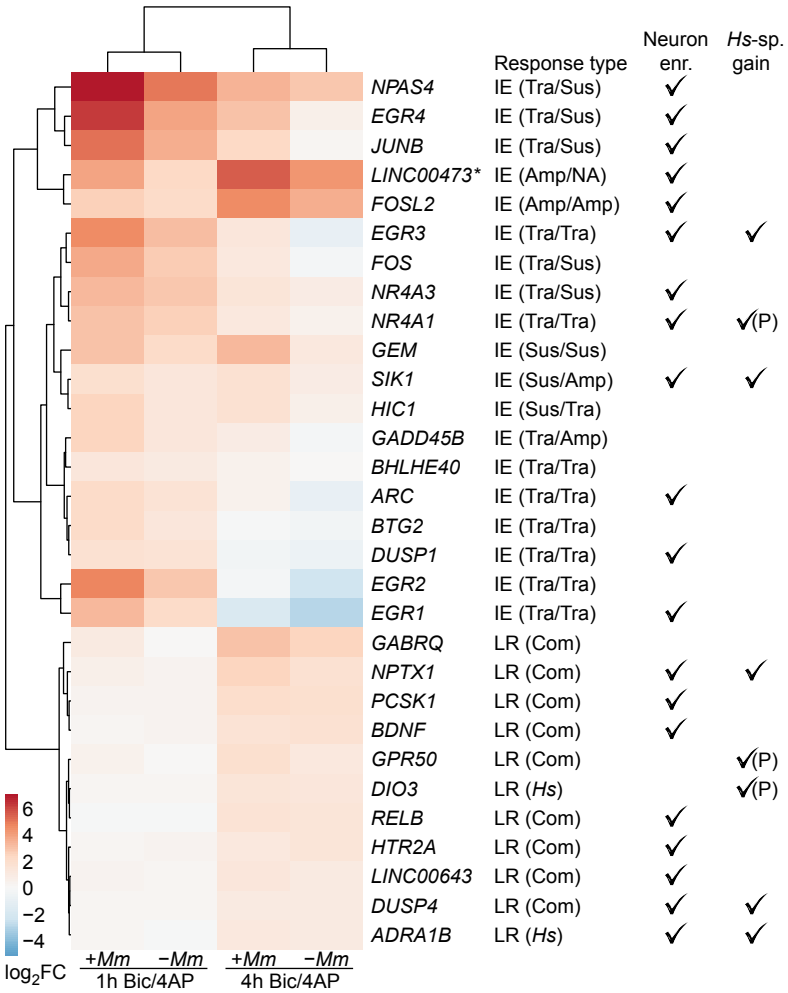

Figure S7. Core set of human iPSC-derived neuron synaptic activity-regulated genes.
Related to Figures 4 and 6, and to Discussion
Heat map of the expression level changes of 30 human genes with the most consistent responses [> two-fold expression change with $p_{adj} < 0.01$ both in the mixed-species culture (+*Mm*) and in the hiPSCd neuron-only culture (-*Mm*)] to synaptic activity in hiPSCd neurons after one hour and/or four hours of Bic/4AP-treatment. The temporal profiles of the expression level changes (Response type) in the mixed-species culture is specified for both the human gene and its mouse ortholog with a separation of the two by a slash (human/mouse). IE, immediate-early; LR, late response; Tra, transient upregulation; Sus, sustained upregulation; Amp, amplified upregulation; NA, not applicable; *Hs*, upregulated hiPSCd neuron-specifically; com, upregulated in hiPSCd neurons and in mouse neurons. Each genes' status in regard to neuron-enriched open chromatin-associated promoter mark (Neuron enr.) and human-specific promoter or enhancer epigenetic regulatory gain (*Hs*-sp. gain) in the human brain *in vivo* (see main text for details) is indicated. (P) specifies promoter gain. The asterisk indicates that there is no ortholog in rodents. Mean gene expression level changes (log_2 fold change, log_2FC, determined with RNA-Seq; n = 4 or n = 3, for one hour or four hours of Bic/4AP-treatment, respectively) were used to generate the heat map together with Euclidean distance Ward clustering.

Table S1 (Excel worksheet). Related to Experimental Procedures
(Table S1A) Results of the ToppGene Suite analysis of GO category over-representation among genes more than 8-fold upregulated after generation of NPCs from iPSCs and 7 weeks of neuronal differentiation of NPCs (2106 genes).
(Table S1B) Results of the ToppGene Suite analysis of GO category over-representation among genes more than 8-fold downregulated after generation of NPCs from iPSCs and 7 weeks of neuronal differentiation of NPCs (2105 genes).

Table S2 (Excel worksheet). Related to Figure 4
(Table S2A) DESeq2 results showing human genes with significantly ($p_{adj} < 0.1$) changed RNA expression levels in response to one hour of Bic/4AP-treatment of 7 weeks differentiated hiPSCd neurons in co-culture with mouse primary hippocampal neurons.
(Table S2B) DESeq2 results showing human genes with significantly ($p_{adj} < 0.1$) changed RNA expression levels in response to one hour of Bic/4AP-treatment of 10 weeks differentiated hiPSCd neurons.

Table S3 (Excel worksheet). **Human synaptic activity-regulated immediate early genes.** Related to Figure 4
Shown are DESeq2 results for human IE genes in response to one hour of Bic/4AP-treatment of 7 weeks differentiated hiPSCd neurons in co-culture with mouse primary hippocampal neurons.

Table S4 (Excel worksheet). Results of the ToppGene Suite analysis of GO category and transcription factor binding site over-representation among human synaptic activity-regulated IE genes. Related to Figure 4

Table S5 (Excel worksheet). Related to Figure 6
(Table S5A) DESeq2 results showing human genes with significantly ($p_{adj} < 0.1$) changed RNA expression levels in response to four hours of Bic/4AP-treatment of 7 weeks differentiated hiPSCd neurons in co-culture with mouse primary hippocampal neurons.
(Table S5B) DESeq2 results showing human genes with significantly ($p_{adj} < 0.1$) changed RNA expression levels in response to four hours of Bic/4AP-treatment of 10 weeks differentiated hiPSCd neurons.

Table S6 (Excel worksheet). **Human synaptic activity-regulated late response genes.** Related to Figure 6
Shown are DESeq2 results for human late response genes in response to one hour of Bic/4AP-treatment of 7 weeks differentiated hiPSCd neurons in co-culture with mouse primary hippocampal neurons.

Table S7

hiPSCd n.-specific LR genes	FC *Hs* (+*Mm*)	FC *Hs* (-*Mm*)	neuron-enriched	*Hs* -specific gain
RTL1	4.36	2.26	no	no
RASGRP3	3.79	2.14	no	yes(promoter)
AGPAT9 (*GPAT3*)	3.07	2.11	yes	no
DIO3	2.92	2.56	no	yes(promoter)
TNFRSF9	2.64	4.08	no	no
NAB2	2.36	1.55	yes	no
ADRA1B	2.35	2.11	yes	yes
NKD2	1.86	1.52	yes	yes
F2RL1	1.77	1.43	no	no
CALCB	1.67	2.65	no	yes(promoter)
NPHP4	1.67	1.41	yes	yes
CEMIP	1.65	3.05	yes	no
DNMBP	1.60	1.24	yes	no
TUNAR	1.59	1.59	yes	no
PRPF4	1.47	1.27	no	no
CAMTA1	1.47	1.34	yes	yes
MAK16	1.39	1.32	no	no
ZNF330	1.36	1.26	no	no
ATG16L1	1.36	1.31	yes	no
POLD3	1.35	1.35	no	yes
GORASP1	1.31	1.22	yes	no
CPOX	1.29	1.41	yes	no
FAM134C	0.79	0.81	yes	yes
SORBS2	0.77	0.78	yes	yes
PLCH1	0.73	0.73	no	no
JMY	0.72	0.74	yes	yes
ARID5B	0.70	0.69	no	no
CNTN3	0.69	0.71	no	no
DNAJC27-AS1[a]	0.64	0.59	no	no
RTP1	0.61	0.46	no	no
TMEM187[a]	0.57	0.70	yes	yes

[a]no ortholog in rodents

Table S7. hiPSCd neuron-specifically synaptic activity-regulated late response genes. Related to Figure 6
31 human late response (LR) genes detected here to be significantly regulated by synaptic activity only in hiPSCd neurons (hiPSCd n.) without published evidence for neuronal activity-dependent regulation of the mouse ortholog. Mean expression level changes (fold change, FC, determined with RNA-Seq) in response to four hours of Bic/4AP-treatment of the hiPSCd neuron/mouse primary neuron co-culture [*Hs*(+*Mm*)] and the hiPSCd neuron-only culture [*Hs*(-*Mm*)] is shown. Each genes' status in regard to neuron-enriched open chromatin-associated promoter mark (neuron-enriched) and human-specific promoter or enhancer epigenetic regulatory gain (*Hs*-specific gain) is indicated, (promoter) indicates promoter gain (see main text for details).

Supplemental Experimental Procedures

Human iPSCs. Both iPSC lines used in this study, hiPS D1 (Horschitz et al., 2015) and iPS 3 (Utikal et al., 2009), have been fully characterized to be pluripotent by standard methods, including teratoma formation assay. In this study, if not indicated otherwise, experiments were performed with hiPS D1-derived neurons.

Generation of NPCs. iPSC colonies, grown on Matrigel (BD Biosciences) in mTesR1 (STEMCELL Technologies), were pretreated with the ROCK inhibitor Y-27632 (10 µM, EMD Millipore) and dissociated with Accutase (eBioscience). 10,000 cells were aggregated to form embryoid bodies (EBs) in V-bottom 96-well plates (Thermo Fisher Scientific) by centrifugation at 400 RCF in mTeSR1 with Y-27632 (30 µM). After 16 h EBs were transferred into neural induction (NI) medium [DMEM:F12, Glutamax (2 mM), N2 (1 %), B27 -vitamin A (4%), β-mercaptoethanol (50 µM) (all Thermo Fisher Scientific), Dorsomorphin (500 nM, Tocris), recombinant mouse Noggin (100 ng/mL, R&D Systems), SB431542 (10 µM, Tocris) and Pen/Strep (Thermo Fisher Scientific, 1:200)] as free floating EBs for two days. Then, the EBs were plated onto Matrigel-coated dishes in NI medium with FGF2 (20 ng/mL, PeproTech). After four days, neural rosettes were microdissected from EBs and replated onto Matrigel in NI medium with FGF2 for another four days. Until this stage the medium was changed every day. Then, cells were treated with Accutase and replated as a monolayer on poly-ornithine (0.1 mg/mL) and laminin (10 µg/mL) coated (PO/Lam, both Sigma Aldrich) dishes in NI medium with FGF2. After two days, or when fully confluent, cells were Accutase-treated and split 1:3 onto PO/Lam dishes in NPC medium [DMEM:F12, Glutamax (2 mM), N2 (1 %), B27 -vitamin A (2 %), β-mercaptoethanol (50 µM), Pen/Strep (1:200), EGF (10 ng/mL) (all Thermo Fisher Scientific) and FGF2 (10 ng/mL, PeproTech)]. These cells were termed passage 1 (P1) NPCs. NPCs were maintained on PO/Lam dishes in NPC medium, split 1:3 or 1:4 with Accutase when confluent and used for neuronal differentiation at passage 4 to 10. NPCs can be frozen in NPC medium without Pen/Strep in the presence of 10 % DMSO.

Neuronal differentiation. When plating cells to differentiate, CHIR99021 (2.5 µM, Cellagen Technology) was added to the NPC medium without EGF and FGF2 to increase viability of cells. Cells were maintained in this medium for 7 days with full medium change every other day. Then, cells were treated with Accutase and replated 75,000 cells/cm^2 on PO/Lam dishes or coverslips in neuronal differentiation (ND) medium [Neurobasal (Thermo Fisher Scientific), Glutamax (1 mM), B27 -vitamin A (2 %), ascorbic acid (200 µM, Sigma Aldrich), Pen/Strep (1:200) and BDNF (10 ng/mL, PeproTech)] and allowed to differentiate for up to 9 more weeks with full medium changes three times per week. NPCs differentiated for 7 days can be frozen in NPC medium without EGF, FGF2 and Pen/Strep in the presence of 10 % DMSO, re-thawed and plated into ND medium to differentiate.

hiPSCd neuron/mouse primary hippocampal neuron co-culture. All medium was changed at mouse cell day *in vitro* (DIV) 1, 3, 6 and half of the medium was changed at DIV 8.

Lentivirus production. 60 % confluent HEK293FT (Invitrogen) cells on a 15 cm cell culture dish in high glucose DMEM (Thermo Fisher Scientific), supplemented with 10 % heat-inactivated fetal bovine serum (Thermo Fisher Scientific), non-essential amino acids (Thermo Fisher Scientific, 1:100) and sodium pyruvate (Thermo Fisher Scientific, 1:100), were transfected with plasmids pLP1 (11 µg), pLP2 (5.75 µg), pVSVG (6.5 µg) and pRRL (17.75 µg) (Follenzi et al., 2000) containing either EGFP or mCherry under the control of the human *SYN1* promoter using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. DNA (µg):Lipofectamine 2000 (µl) ratio of 1:2.5 in a total of 625 µl DMEM was used for preparing the transfection solution. Medium was changed 16 h after transfection. Then, the medium was collected twice after 24 h and 48 h. After centrifugation (4 °C, 2500 × g, 10 min) the collected medium was filtered (0.45 µm pore, PVDF, Millex-HV, Merck Millipore), purified (Speedy Lentivirus Purification, abm) and concentrated (Amicon-Ultra 100K column, Merck Millipore), all according to the manufacturer's recommendations, respectively. Viral particles in PBS were aliquoted and frozen in a cell freezing container for storage at -80 °C.

NPC infection. 75 % confluent NPCs (P2 to P4) were infected overnight with the addition of a 1:500 dilution of concentrated lentivirus into NPC medium. Infected NPCs were used for neuronal differentiation after no more than two passages.

Immunocytochemistry. Cells grown on glass coverslips for the time indicated were fixed with 4 % PFA/4 % sucrose in PBS, treated with 50 mM NH_4Cl in PBS, permeabilized with 0.25 % – 0.5 % Triton X-100 in PBS (or with 0.1 % saponin in PBS if anti-VGLUT2 was used, then all subsequent solutions contained 0.1 % saponin), blocked with ICC

blocking buffer (2 % BSA in PBST) and incubated with the following primary antibodies diluted in ICC blocking buffer: goat anti-BRN2 (C-20) (Santa Cruz Biotechnology, sc-6029, 1:50), mouse anti-COUP-TFI [H8132] (Abcam, ab41858 1:200), goat anti-DCX (C18) (Santa Cruz Biotechnology, sc-8066, 1:100), rabbit anti-FOXP2 (Abcam, ab16046, 1:400), rabbit anti-GABA (Sigma-Aldrich, A2052, 1:2000), rabbit anti-JUNB (C37F9) (Cell Signaling Technology, #3753, 1:200), mouse anti-MAP2 (Millipore, MAB3418, 1:500), mouse anti-NES (10C2) (Millipore, MAB5326, 1:400), goat anti-OCT4 (N-19) (Santa Cruz Biotechnology, sc-8628, 1:100), rabbit anti-PAX6 (Covance, PRB-278P, 1:200), mouse anti-TUBB3 (Covance, MMS-435P, 1:500) and rabbit anti-VGLUT2 (Synaptic Systems, 135 402, 1:500). Dylight488- or Dylight594-conjugated anti-IgG secondary antibodies, all produced in donkey by Dianova (anti-goat, 705-515-003; anti-mouse, 715-485-150; or anti-rabbit 711-585-152) were diluted in ICC blocking buffer 1:1000. Coverslips were mounted using Mowiol 4-88 (Merck Millipore) with Hoechst 33258 (Serva). Images were obtained with DM IRB/E inverted microscope (Leica) with a 40× objective, or with TCS SP2 confocal laser-scanning microscope (Leica) equipped with DM IRE2 inverted microscope (Leica) with a 100× objective (JUNB immunostainings).

RNA isolation, cDNA synthesis, RT-PCR and RT-qPCR. At the time of differentiation indicated and/or after the treatment specified, total RNA was purified from cultured cells (3.5 cm dish) with RNeasy Mini (hiPSCd neuron/mouse primary neuron co-cultures) or Micro (hiPSCd neuron-only cultures) Kit (Qiagen) along with on-column RNase-Free DNase (Qiagen) digestion of DNA according to manufacturer's instructions. cDNA was produced with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) using 100 - 1000 ng of total RNA. Semiquantitative RT-PCR was performed with NEB Taq DNA Polymerase (New England Biolabs). RT-qPCR analysis was performed in triplicates with Power SYBR Green Master Mix (Thermo Fisher Scientific) and 7300 Real-Time PCR System (Applied Biosystems). *TBP* and *GAPDH* or *Gapdh* were used for expression normalization in hiPSCd neuron differentiation and synaptic activity-regulated transcription experiments, respectively. All primers used here are listed in a table available at the end of the Supplemental Experimental Procedures.

Western blotting. Cells grown on 3.5 cm dishes for the time indicated were washed once with ice-cold PBS, collected and pelleted in PBS at 4 °C and lysed on ice in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1 % Triton X-100, 0.5 % Na-deoxycholate and 0.1 % SDS, supplemented with 1 mM PMSF and 1× Roche complete EDTA-free protein inhibitor cocktail) for 30 min. After centrifugation with a table-top centrifuge at 4 °C with 13,000 rpm for 10 min, the supernatant was collected on ice. 50 µg of protein/sample was separated in 10 % SDS-PAGE and transferred to nitrocellulose blotting membrane (GE Healthcare). Membranes were blocked in blocking buffer (5 % skim milk in PBST). The primary antibodies used and their dilutions in blocking buffer applied were: mouse anti-ACTB (Santa Cruz Biotechnology, sc-47778, 1:2000), mouse anti-COUP-TFI [H8132] (Abcam, ab41858 1:800), mouse anti-DLG3 (N19-2) (antibodies-online, ABIN1304941, 1:2000), mouse anti-MAP2 (Millipore, MAB3418, 1:2000), mouse anti-NES (10C2) (Millipore, MAB5326, 1:2000), goat anti-SOX2 (Y-17) (Santa Cruz Biotechnology, sc-17320, 1:400), mouse anti-TUBB3 (Covance, MMS-435P, 1:2000) and rabbit anti-VAMP2 (anti-Synaptobrevin 2) (Synaptic Systems, 104202, 1:2000). HRP-conjugated anti-IgG secondary antibodies used, diluted in blocking buffer 1:5000, were from Dianova: donkey anti-goat (705-035-147), goat anti-mouse (115-035-003) or goat anti-rabbit (111-035-144). Chemiluminescence, produced with the ECL Western Blotting Detection system (GE Healthcare), was detected by exposing membranes to High Performance Chemiluminescence film (GE Healthcare).

RNA-Seq. Poly(A)+ RNA was isolated from total RNA with the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). cDNA libraries were prepared with NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). NEBNext Multiplex Oligos for Illumina (New England Biolabs) were used. All the preparatory work was performed by CellNetworks Deep Sequencing Core Facility in Heidelberg, Germany. The libraries were sequenced in EMBL Genomics Core Facility in Heidelberg, Germany, on HiSeq 2000 or NextSeq 500 (Illumina) to generate 50 bp single-end reads for iPSC and 7 weeks- or 10 weeks-differentiated hiPSCd neuron samples and 50 bp or 75 bp paired-end reads for 7-weeks-differentiated hiPSCd neuron/mouse primary neuron co-culture samples.

Differential gene expression analysis. Before the final read alignment and differential gene expression analysis, in order to discriminate human and mouse reads, the sequencing data from hiPSCd neuron/mouse primary neuron co-culture samples was processed similar to what has been described for tumour xenograft RNA-Seq data analysis (Bradford et al., 2013). Paired-end reads were aligned independently, without pairing, to either the human genome assembly GRCh37/hg19 or to the mouse genome assembly GRCm38/mm10 with Bowtie2 version 2.1.0 (Langmead and Salzberg, 2012). Unaligned read pairs were joined and split again (Blankenberg et al., 2010) to eliminate pairs

with one aligned read. Then, genome-unaligned paired-end reads were aligned to either GRCh37/hg19 or GRCm38/mm10 transcriptomes (UCSC Genes/knownGene), respectively, with Bowtie2 (2.1.0). The reads that were not aligned to the human genome and transcriptome, or not aligned to the mouse genome and transcriptome, were then handled as reads obtained from single-species samples and used as mouse or human RNA-seq data input to TopHat, respectively. Both, processed mixed-species sample reads and human-cells-only sample reads, were aligned to gene model annotations of the human genome assembly GRCh37/hg19 (or, if appropriate, the mouse genome assembly GRCm38/mm10) with TopHat version v2.0.9 (Kim et al., 2013). Mapped reads per gene were counted strand-specifically with HTSeq-count (HTSeq version 0.6.0 or 0.6.1) in the union overlap resolution mode (Anders et al., 2015). All aligning and counting were done via the Galaxy platform (Goecks et al., 2010; http://usegalaxy.org/; http://galaxeast.fr/). Differentially expressed genes were determined with the DESeq2 package version 1.8.1 (Love et al., 2014) in R (3.2.1) environment (http://www.R-project.org/). Cook's distance flagging functionality was disabled. Benjamini-Hochberg adjusted $p < 0.1$ was considered significant for synaptic activity-regulated gene expression analysis.

Spearman correlation between transcriptomes. Analysis was performed using R (3.2.1). Gene expression levels in RNA-seq read counts per million were used to compare datasets. Counts with added pseudocount of 1 were log-transformed. 4497 genes with variable expression levels between samples, defined by standard deviation > 1 within all samples shown in Figure 1K, were used to calculate Spearman correlation coefficients.

Hierarchical clustering. Size factor normalized RNA-Seq counts from not treated and one hour and four hours Bic/4AP-treated samples were scaled per gene in the range of 0 to 1, and used to compute a Euclidean distance matrix. Agglomerative hierarchical clustering was performed using R (3.2.1) with Ward's clustering criterion (method *ward.D2*).

GO term enrichment analyses. Over-represented GO terms within gene lists were acquired with ToppGene (Chen et al., 2009).

Patch clamp recordings. Cells were viewed with differential interference contrast optics and infrared illumination through a 40x objective (LUMPLFL40xW, N.A. 0.8, Olympus) on a wide field upright microscope (BX51WI, Olympus) equipped with a digital camera (sCMOS, Andor, BFi OPTiLAS) connected to a computer monitor through a PC interface using Andor IQ2 software. $EGFP^+$ or $mCherry^+$ cells were identified using appropriate LEDs (CoolLEDs, Andover, UK) and filters (AHF Analysetechnik). Patch electrodes (3-4 MΩ) were made from borosilicate glass (1.5 mm, WPI) and filled with intracellular solution [HEPES pH 7.35 (10 mM), KGluconate (122 mM), KCl (12 mM), NaCl (8 mM), EGTA (5 mM), $CaCl_2$ (0.25 mM), Na_3-GTP (0.5 mM), Mg_2-ATP (4 mM), K2-phosphocreatine (10 mM)]. Extracellular solution was an artificial cerebrospinal fluid, ACSF [NaCl (125 mM), KCl (3.5 mM), $MgCl_2$ (1.3 mM), NaH_2PO_4 (1.2 mM), $CaCl_2$ (2.4 mM), glucose (25 mM), $NaHCO_3$ (26 mM), gassed with 95 % O_2 and 5 % CO_2]. Whole-cell patch clamp recordings were made with a Multiclamp 700A amplifier, digitised through a Digidata 1322A A/D converter and acquired using pClamp 10 software (Molecular Devices). Spontaneous postsynaptic currents were recorded in voltage clamp at a -70 mV holding potential. Stock solutions of Bic (Enzo Life Sciences) were dissolved in DMSO and gabazine-hydrobromide (SR95531, Biotrend), 4AP (Sigma), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX, Biotrend) and TTX (Biotrend) in water. Cell numbers are given in the figures and their legends.

Ca^{2+} imaging. For multi-cellular Ca^{2+} imaging experiments, cells were loaded at 36 °C with membrane permeable Rhod2-AM [1.5 µM (stock dissolved at 1.5 mM in pluronic acid and DMSO)] for 30 min followed by 10 min washout for de-esterification of the dye. Somatic Ca^{2+} levels were quantified as $(F-F_{min})/(F_{max}-F)$ where F represents the average fluorescence intensity, F_{max} represents the maximal F after incubation in 50 µM ionomycin, and F_{min} represents the minimal F after subsequent application of a 1:500 dilution of saturated manganese solution.

Nanostring nCounter analysis (Kulkarni et al., 2011). Custom designed nCounter Elements code set targeting human-specifically 30 human genes and mouse-specifically 17 mouse genes was used. Probe sequences are available upon request. The nSolver Analysis Software (version 2.5) was used to analyse RNA expression data produced by CellNetworks nCounter Core Facility in Heidelberg, Germany. Geometric means were used to perform background subtraction, to compute normalization factors for positive control normalization and perform code set content

normalization. Count ratio (log_2) was used for assessing differential expression between the analysed conditions with one-way ANOVA and Dunnett's multiple comparisons tests.

Luciferase reporter assays. Human *BRE-AS1*, *LINC00473* and *ZNF331* and mouse *Hic1* genomic proximal promoter sequences were PCR-amplified (Phusion High-Fidelity DNA Polymerase, New England Biolabs), cloned into the pGL4.15[luc2P/Hygro] vector (Promega) and verified by sequencing. *pBRE-AS1* was the only cloned promoter not fully verified by sequencing because of sequencing problems. Instead, it was confirmed to be correct by control cuts with several restriction enzyme combinations. The primers used for amplification of genomic DNA are listed in a table available at the end of the Supplemental Experimental Procedures. pGL4.29[luc2P/CRE/Hygro] (Promega), containing four CRE *cis*-elements and a minimal promoter (4×CRE-pmin), and pGL4.15[luc2P/Hygro]/p*BDNF* IV, containing the human *BDNF* promoter IV (Pruunsild et al., 2011), or pGL4.15[luc2P/Hygro] (Promega) without promoter (pCNTR), were used as positive or negative controls, respectively, for synaptic activity-induced transcriptional activity. pGL4.83[hRlucP/Puro] plasmid containing the human *EF1α* promoter in front of Renilla luciferase (Rluc) (Sepp et al., 2012) was used for normalization. At DIV 8 mouse (P0) primary hippocampal or cortical neurons in 48-well plates, maintained thus far in Neurobasal-A supplemented with rat serum (1 %), B27 (2 %), L-glutamine (0.5 mM) and Pen/Strep (1:200) (all Thermo Fisher Scientific), were changed to transfection medium (TM) consisting of salt-glucose-glycine solution [HEPES pH 7.4 (10 mM), NaCl (114 mM), $NaHCO_3$ (26.1 mM), KCl (5.3 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2 mM), glycine (1 mM), glucose (30 mM), sodium pyruvate (0.5 mM) and phenol red (0.001 %)] and minimum Eagle's medium (with Earle's salt and without L-glutamine, Thermo Fischer Scientific), 9:1; supplemented with insulin (6.3 µg/mL), transferrin (5.7 µg/mL) and sodium selenite (7.5 ng/mL) (ITS, Sigma Aldrich). Lipofectamine 2000 (Thermo Fisher Scientific) was used for transfection at DIV 8 according to the manufacturer's instructions. Cells were transfected with a pGL4.15[luc2P/Hygro] plasmid (Promega) containing the indicated cloned promoter in front of FFluc, or with the pGL4.29[luc2P/CRE/Hygro] plasmid, or the pGL4.15[luc2P/Hygro] plasmid (both Promega) (all 0.5 µg/well), together with the pGL4.83[hRlucP/Puro] plasmid containing the human *EF1α* promoter in front of Renilla luciferase (Rluc) (5 ng/well) for normalization. If mCherry, CaMBP4, ICER or delTAD-NPAS4 was co-expressed, 0.25 µg/well of either pmCherry-N1 (Clontech), rAAV/CaMBP4-mCherry (Lau and Bading, 2009), pcDNA3.1/ICER (Pruunsild and Timmusk, 2012) or pcDNA3.1/delTAD-NPAS4 (Pruunsild et al., 2011), respectively, along with 0.25 µg/well of FFluc construct and 5 ng/well of Rluc construct were co-transfected. DNA (µg):Lipofectamine 2000 (µl) ratio of 1:2 in total of 25 µl/well TM was used for preparing the transfection solution. At DIV 10, Dual-Glo Luciferase Assay System (Promega) was used to measure FFluc and Rluc levels. Background measured from non-transfected cells was subtracted and FFluc levels were normalized to Rluc levels. Data presented in Figure 5B were autoscaled using the data obtained with the pGL4.29[luc2P/CRE/Hygro] construct. All data presented as fold change after Bic/4AP-treatment was log-transformed for statistical analyses.

Gene classification based on promoter/enhancer histone modifications. Data for neuron-enriched H3K4me3 promoter marks in the human prefrontal cortex *in vivo* is from Cheung et al. (Cheung et al., 2010) and for human-specific promoter or enhancer H3K27ac and/or H3K4me2 gains during brain cortex development *in vivo* is from Reilly et al. (2015). UMMS Brain Hist Track and 8 custom tracks with peaks for human-specifically increased H3K27ac or H3K4me2 marks in the human cortex at postconception week (pcw) 7 or 8.5, and in the frontal or occipital cortex at pcw 12 (Reilly et al., 2015) were used to display data in the UCSC Genome Browser. We assigned a gene to have a neuron-enriched H3K4me3 promoter mark if according to the data by Cheung *et al*. the mark was enriched in all neuronal samples compared with lymphocyte samples and overlapped a TSS. We categorized a gene to have a human-specific epigenetic promoter gain if in at least two of the total of 8 ChIP-Seq tracks with peaks for human-specifically increased H3K27ac or H3K4me2 marks (Reilly et al., 2015), the peaks were coinciding and overlapped a TSS. A gene was considered to have a human-specific epigenetic enhancer gain if it was located in a gain-enriched genomic hotspot (Reilly et al., 2015), and/or in at least two of the 8 tracks with human-specifically increased H3K27ac or H3K4me2 ChIP-Seq peaks, the peaks were coinciding and not overlapping a TSS, but were within 30,000 bps up- to 15,000 bps downstream of any of the TSSs of the gene, and were not in an adjacent gene. Exceptionally, the gain mark inside an adjacent gene was counted if the gene of interest and the adjacent gene had a bidirectional, "head-to-head" promoter. RefSeq genes (as in Cheung et al., 2010) and Genecode version 10 genes (as in Reilly et al., 2015) were used to estimate chance levels of the respective promoter marks. A set of 85 random genes were included in the comparison of proportions of genes with human-specific epigenetic enhancer gains between different gene sets.

Statistical analyses. t-tests and ANOVA were performed using GraphPad Prism or Origin. For statistical analysis qPCR, nCounter and luciferase assay data were log-transformed and cell proportions obtained with immunocytochemistry were arcsine-transformed. Exact binomial tests and Fisher's pairwise comparisons of proportions with Holm's p adjustment were performed using R (3.2.1).

Primers used in this study

Target	Sense	Antisense	Amplicon (bps)
		Expression	
OCT4	GGGAAGGTATTCAGCCAAAC	CTTTCTCTTTCGGGCCTGCAC	174
PAX6	GCACCAGTGTCTACCAACCAA	CCCAACATGGAGCCAGATGTGAA	73
BRN1	TTGGCGCTGGGCACACTCTA	CCTTGACGCTCACCTCGATAG	223
RTN1	ACGGGCATCGTGTTTGGGAGT	TGCCTCTTAGCGCCTGGGATT	521
STMN2	GCTCTTGCTTTTACCCGGAAC	ATTGTTTCAGCACCTGGGCCT	219
TBP	GCCTTGTGCTCACCCACCAACAATTT	GGTACATGAGAGCCATTACGTC	221
CUX1	CAAAGGCCAGGCTGACTATGA	TCTCCAGCAACAGCACCTCCA	128
LIN28A	GAGGCGGCCAAAAGGAAAGAG	ATTCCTTGGCATGATGATCTAGACCT	92
SOX1	CGCTGACACCAGACTTGGGTT	ACAAAAGTGGGCTTCGCCTCT	154
SYN1	GGAGAAATTGACATTAAAGTAGAACAG	CTTCTGTCCCCAGTTTCTTATGC	319
NPAS4 (*Hs*)	GTGAGGCTACAGGCCAAGAC	AGGGCAGCATGGTCGGAGTG	190
Npas4 (*Mm*)	CAGGGCGACAGTATCTACGAT	CAACGGAAAAGGCGATCAGCA	104
FOS (*Hs*)	TGCAGCCAAATGCCGCAAC	TCGGTGAGCTGCCAGGATG	154
Fos (*Mm*)	GGCAGAAGGGGCAAAGTAGAG	TGTCAGCTCCCTCCTCCGATTC	117
ARC (*Hs*)	AAGTCGCACACGCAGCAGAGCA	AGGCGGGCGTGAATCACTGGA	77
Arc (*Mm*)	AGACCTGACATCCTGGCACC	GCTCTGCTCTTCTTCACTGGTA	65
GAPDH (*Hs*)	CAAAATCAAGTGGGGCGATGCT	TTGGCTCCCCCCTGCAAATGA	102
Gapdh (*Mm*)	CACTCTTCCACCTTCGATGCC	GGGTGGGTGGTCCAGGGTT	159
HIC1 (*Hs*)	ATGCGGTTCACGCGCCAGTAC	CTGATGAGGTTGCGTTGCTGT	116
Hic1 (*Mm*)	ATGCGCTTCACCCGCCAGTAT	CTGATGAGGTTGCGCTGTTGA	116
		Promoter	
p*BRE-AS1* (BglII)	ACGTACAGATCTGATGACACCTGAGGGAATGAA	ACGTACAGATCTAAGTTCCAGGTACCTGGACTG	1024
p*LINC00473* (BglII)	ACGTACAGATCTTGGCCTCTTAAGGGTCTTTTG	ACGTACAGATCTGGCGAGTGTGTGGGGTTCCTC	974
p*ZNF331 I* (BglII)	ACGTACAGATCTTGAACCACTTTTCCCAGGAAT	ACGTACAGATCTGCGTAGAGCCTCTCACGTCAC	1524
p*ZNF331 II* (BglII)	ACGTACAGATCTGGACTACCGGGTGCGTCTGCG	ACGTACAGATCTTCGGGGATGCTTTTTCTGAAG	1524
p*ZNF331 III* (BglII)	ACGTACAGATCTGCGTGTCAGGATCTGTGTGCA	ACGTACAGATCTTTTAACGATTCGTCTTCCTCC	1448
p*Hic1* (*Mm*, HindIII)	ACGTACAAGCTTTCTCCTGCCAAAGAAACGCAGC	ACGTACAAGCTTTGCCGGCGGATCCAGGGGGGGA	1677
		Mutagenesis	
p*Hic1* (*Mm*, +EGR1 site)	GTCAGGAGGTGCGGGGCGGAGGCGGAGCGTGGGC	GCCCACGCTCCGCCTCCGCCCCGCACCTCCTGAC	

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