Supplementary Information

Methods.

Neural stem cell culture. Cells were prepared as previously described (Geschwind et al., 2001). Briefly, E11.5 and E12.5 telencephalon and cortex from E14.5 and P1 CD-1 mice (Charles Rivers, Wilmington MA) were dissected in chilled saline 1 (277mM NaCl, 10mM KCl, 2.2mM Na_2HPO_4, 2.2mM KH_2PO_4, 40mM dextrose pH 7.0) or Hank's balanced salt solution (Invitrogen, Carlsbad CA). Cells were dissociated by trituration with a fire-polished pipet and plated at 50,000 cells/mL in DMEM/F12 (Invitrogen) with B27 supplement (Invitrogen), 1000U/mL penicillin/streptomycin (Invitrogen), 20ng/mL basic fibroblast growth factor (bFGF, PreproTech Inc., Rocky Hill NJ), and 5µg/mL heparin (Sigma-Aldrich, St. Louis MO). For P1 cultures, 50ng/mL epidermal growth factor (EGF, PreproTech Inc.) was added. Cells were supplemented with growth factors and heparin twice a week. Neurospheres were passaged every week by digestion with TrypLExpress (Invitrogen) for 10 minutes at 37˚C. Cells were then centrifuged, resuspended in media, and triturated with a P1000 pipet tip to break up remaining cell clusters. This mixture was then filtered through a 40µM cell strainer to ensure a single cell suspension (which was always confirmed during cell counting), and re-plated at 50,000 cells/mL in media as above. For clonal neurosphere assays, dissociated single cells were re-plated at 1,000 cells/mL in flasks, or 96-well plates for analysis, in Neurobasal medium (Invitrogen) with B27, 2mM L-glutamine (Invitrogen), 1,000U/mL penicillin-streptomycin, 20ng/mL bFGF, 2ng/mL EGF, and 2ug/mL heparin. For differentiation, spheres were re-suspended in Neurobasal medium containing B27, 2,500U/mL penecillin-streptomycin, 5mM glutamic acid (Sigma-Aldrich), and 0.5mM L-

glutamine, and plated on 12mm glass coverslips coated with 10µg/mL poly-L-lysine (Sigma-Aldrich). For neuronal culture, freshly dissociated cortex was plated under these conditions at 200,000 cells/mL on 12mm glass coverslips coated with 10µg/mL poly-L-lysine in 24-well plates (Corning). For attached progenitor cultures (Sun et al., 2001), cells were plated on glass coverslips coated with poly-ornithine and fibronectin (PoFn) (Sigma-Aldrich) in Neurobasal + 2% fetal bovine serum (FBS, Invitrogen) for four hours, then media was changed to the Neurobasal media described above without heparin. Cells were fed with 20ng/mL bFGF every other day. P-Ser (L-phospho-O-serine, Sigma-Aldrich) was prepared fresh for each batch of experiments. L-AP4 and UBP1112 were obtained from Tocris (Ellisville MO), D-AP4 was purchased from Alexis (Axxora, San Diego CA), and D-phospho-O-serine was from Bachem California Inc (Torrance CA).

Immunofluorescence. Coverslips were fixed in fresh 4% paraformaldehyde in 0.1M phosphate buffer. After washing, cells were blocked and permeabilized in 10% normal goat serum (Vector Laboratories, Burlingame CA) + 0.1% Triton X-100 (Sigma-Aldrich). Coverslips were then incubated with primary antibody overnight at 4°C in 10% normal goat serum. The primary antibodies used were as follows: Tuj1 (1:500, Covance USA), O4 hybridoma supernatant (variable), GFAP (1:1,000, Dako, Carpinteria CA), BrdU (1:1,000, Roche, Indianapolis IN). The next day, coverslips were washed and incubated with primary antibody isotype-specific Alexa fluor-conjugated secondary antibodies (Molecular Probes, Carlsbad CA), washed, and nuclei counterstained with propidium iodide or hoescht stain.

Cell counting and diameter measurements. For analysis of neuron content in differentiating neurospheres, Tuj1 and O4 staining were always done together, as Tuj1 can display non-specific staining of oligodendrocytes; double-labeled cells were excluded from analysis. In all experiments, 1,000 cells or 10 fields at 20x magnification per coverslip were quantified; at least three coverslips per treatment condition were quantified in each experiment. Neurosphere diameters were measured with MCID software (Imaging Research, St. Chatherine ON). All spheres in at least three wells (of 96-well plates) per condition were measured.

TUNEL assay. Coverslips were processed for TUNEL assay using a Fluorescein *In Situ* Cell Death detection kit (Roche) following manufacturer's instructions. In some cases, coverslips were then blocked, permeabilized, and stained as above with additional markers.

siRNA transfection. Transfections were performed as described previously (Nakano et al., 2005). Cells were plated at 400,000/mL on PoFn-coated plates and incubated overnight. Media was changed to OptiMEM (Invitrogen) immediately before transfection. Cells were transfected with 100nM siRNA duplexes targeting luciferase (Dharmacon, Lafayette CO) or mGluR4 (Ambion, Austin TX) following incubation with Lipofectamine 2000 (Invitrogen) per manufacturer's instructions. Cells were incubated for six hours. For neurosphere formation assays, cells were then typsinized, counted, and re-plated at 1,000 cells/mL in the the Neurobasal growth media described above, without penicillin-streptomycin. For western blot analysis, cells were washed with PBS and fresh Neurobasal growth media, without penicillin-streptomycin and heparin, was added to the wells. Cells were fed with 10ng/mL bFGF daily. The target sequences

used were as follows: simGluR4-1 5'-gcaugucaccauaauuugctt-3'; simGluR4-2 5'-ggucaucggcucauggacatt-3'; siNC 5'-cgtacgcggaatacttcgatt-3'.

RNA collection and RT-PCR. RNA was isolated from cells using Trizol reagent (Invitrogen) following manufacturer's instructions. For cDNA synthesis, 0.5-1μg (matched in each experiment) was digested with RQ1 DNase (Promega, Madison WI) to remove genomic DNA. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) following manufacturer's protocol; reverse transcriptase was omitted from negative control reactions. PCR was performed using 1-3μL cDNA reaction as template, adjusted for equal GAPDH loading. PCR was carried out by heating samples to 95°C for two minutes, followed by 20-35 cycles of 95°C 30 sec., 57°C 30 sec., 72°C 30 sec. with a 2 minute final extension at 72°C. Reactions were analyzed on 2% TAE gels and documented on a Versadoc imaging platform (Bio-Rad, Hercules CA). For validation of primer amplification efficiency, receptor-specific template was generated by running reactions using post-natal day eight mouse brain cDNA as template in reactions as above. Product was purified with a PCR cleanup kit (Qiagen) and quantified on a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington DE). Equimolar amounts of product were then used as template in reactions performed as above and analyzed on 2% TAE gels. Primer sequences used were as follows:

mGluR4forward 5'-TGACCTTCAACGAGAACGGAGAC -3'

mGluR4reverse 5'- ACAAAAGTGACCACCACGAACAG -3'

mGluR7forward 5'- AGCCATCACCATCCAACCTAAG -3'

mGluR7reverse 5'- ACCAGGATTGGTAGTGTTTGTTGTC -3'

mGluR8forward 5'- AGAAAATCCCACGTGAACCAAGAC -3'

mGluR8reverse 5'- GCCATGGAATACACTGCATCAAT -3'

Western blot analysis. Neurospheres and attached progenitors were washed with ice-cold Dulbecco's phosphate-buffered saline (D-PBS) and lysed in M-PER mammalian protein extraction reagent (Pierce) for 15 minutes on ice. Lysates were spun at 14,000rpm for 10 minutes at 4°C to pellet debris, and protein concentrations were measured by Bradford assay (Bio-Rad). 5µg of each lysate was run on a 10% SDS-PAGE gel, transferred onto PDVF membranes, blocked, and incubated overnight with primary antibody. The following primary antibodies were used: phospho-S473 Akt, phospho-S308 Akt, total Akt, phospho-T202/Y204 Erk, total Erk, phospho-T389 S6k1, phospho-T421/S424 S6k1, total S6k1, phospho-S9 GSK3β, phospho-S727 Stat3, phospho-Y705 Stat3, and total Stat3 were from Cell Signaling (Boston MA). β-actin clone AC-15 was purchased from Sigma-Aldrich, and GAPDH was from Ambion. Goat anti-rabbit and goat anti-mouse HRP-conjugated secondary antibodies were from Pierce. Chemiluminescence was developed with ECL reagent (Pierce) or ECL+ (Amersham Biosciences, Piscataway NJ). Blots were stripped with low-pH glycine buffer (400mM glycine, 6mM SDS, 2% Tween-20, pH 2.2).

Statistical analysis. Data are expressed as mean ± SEM, unless otherwise indicated. t-tests were performed using Microsoft Excel to determine statistical significance of treatment sets. For multiple comparisons, one- or two-way ANOVA was performed, as appropriate, and Bonferroni post-hoc t-tests were done to determine significance. Alpha values were 0.05 except when adjusted by the post-hoc tests.

References:

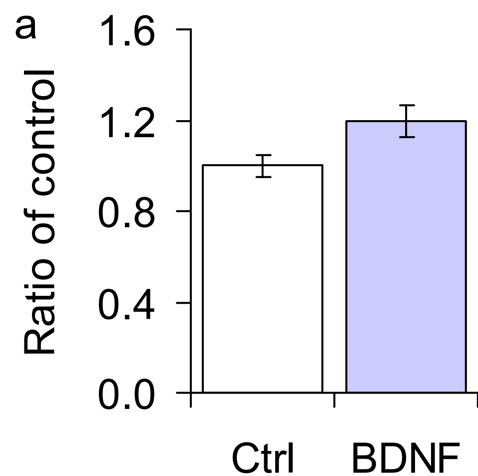
Allen Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science, -. c. N., 2006. Available from: http://www.brain-map.org.

Geschwind, D. H., Ou, J., Easterday, M. C., Dougherty, J. D., Jackson, R. L., Chen, Z., Antoine, H., Terskikh, A., Weissman, I. L., Nelson, S. F., and Kornblum, H. I. (2001). A genetic analysis of neural progenitor differentiation. Neuron *29*, 325-339.

Nakano, I., Paucar, A. A., Bajpai, R., Dougherty, J. D., Zewail, A., Kelly, T. K., Kim, K. J., Ou, J., Groszer, M., Imura, T., *et al.* (2005). Maternal embryonic leucine zipper kinase (MELK) regulates multipotent neural progenitor proliferation. J Cell Biol *170*, 413-427.

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M. Z., Zubiaga, A., Hua, X., Fan, G., and Greenberg, M. E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell *104*, 365-376.

a



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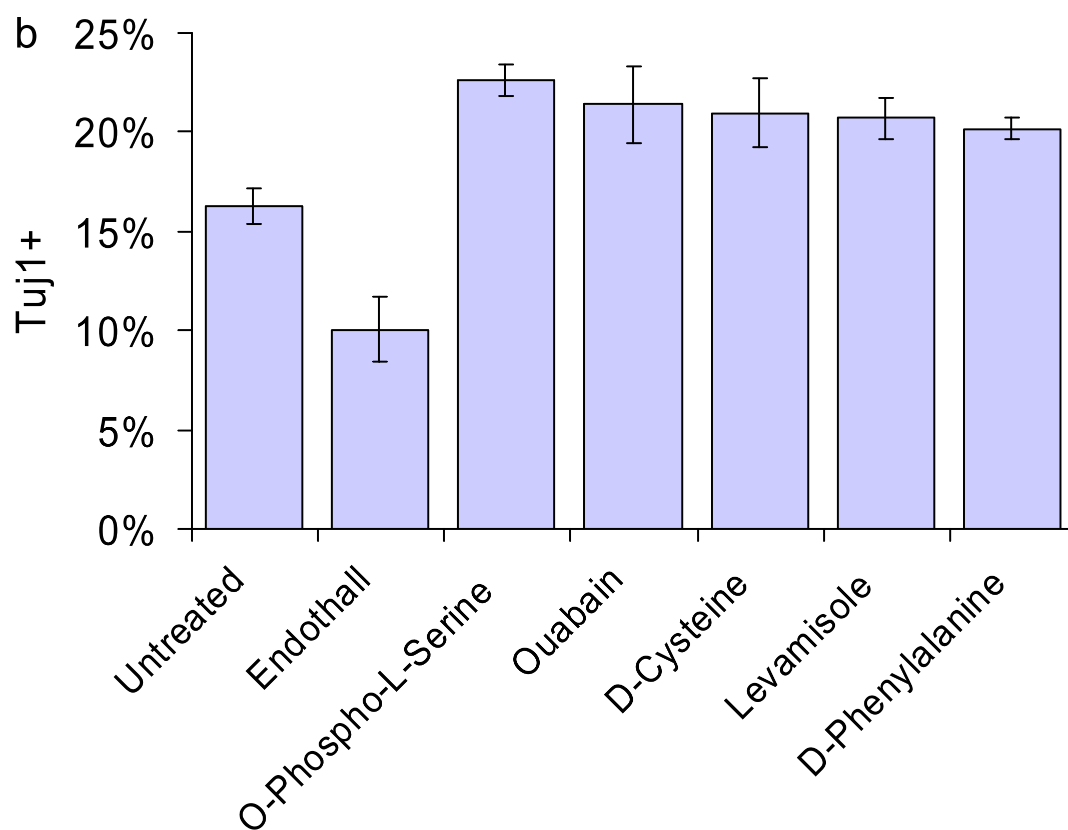
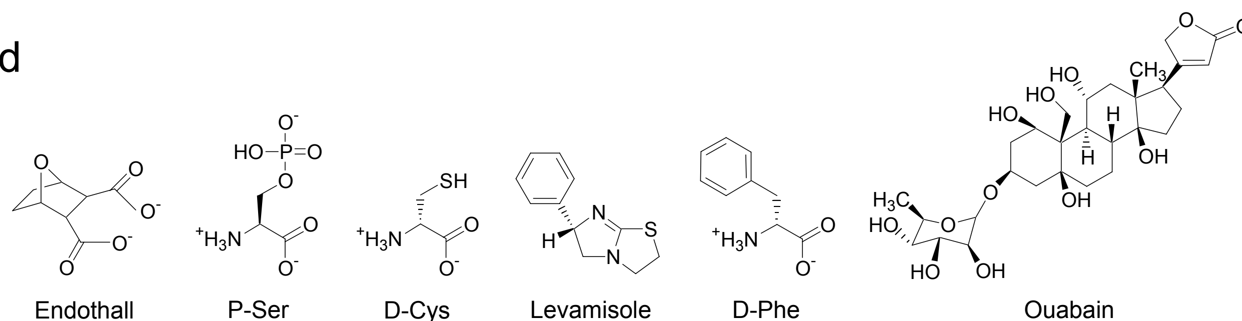


c

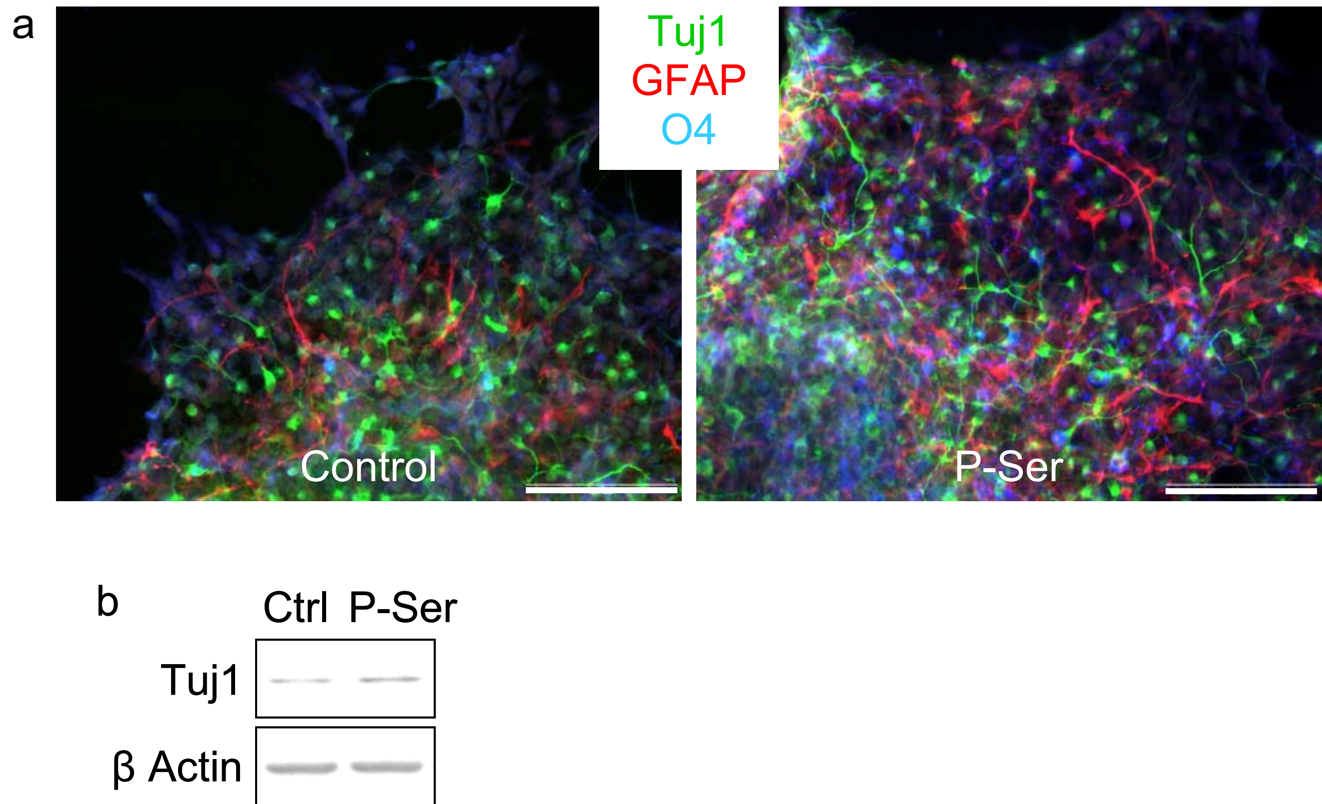
Statistically Significant Hits

Compound	p value	Annotated Activity
Endothall	0.0143	PP2A Inhibitor
O-Phospho-L-Serine	0.00298	Possible endogenous glutamate ligand
Oubain	0.0361	Putative endogenous
D-Cysteine	0.0361	D- amino acid
Levamisole	0.0154	Mammalian alkaline phosphatase inhibitor
D-Phenylalanine	0.0102	D- amino acid

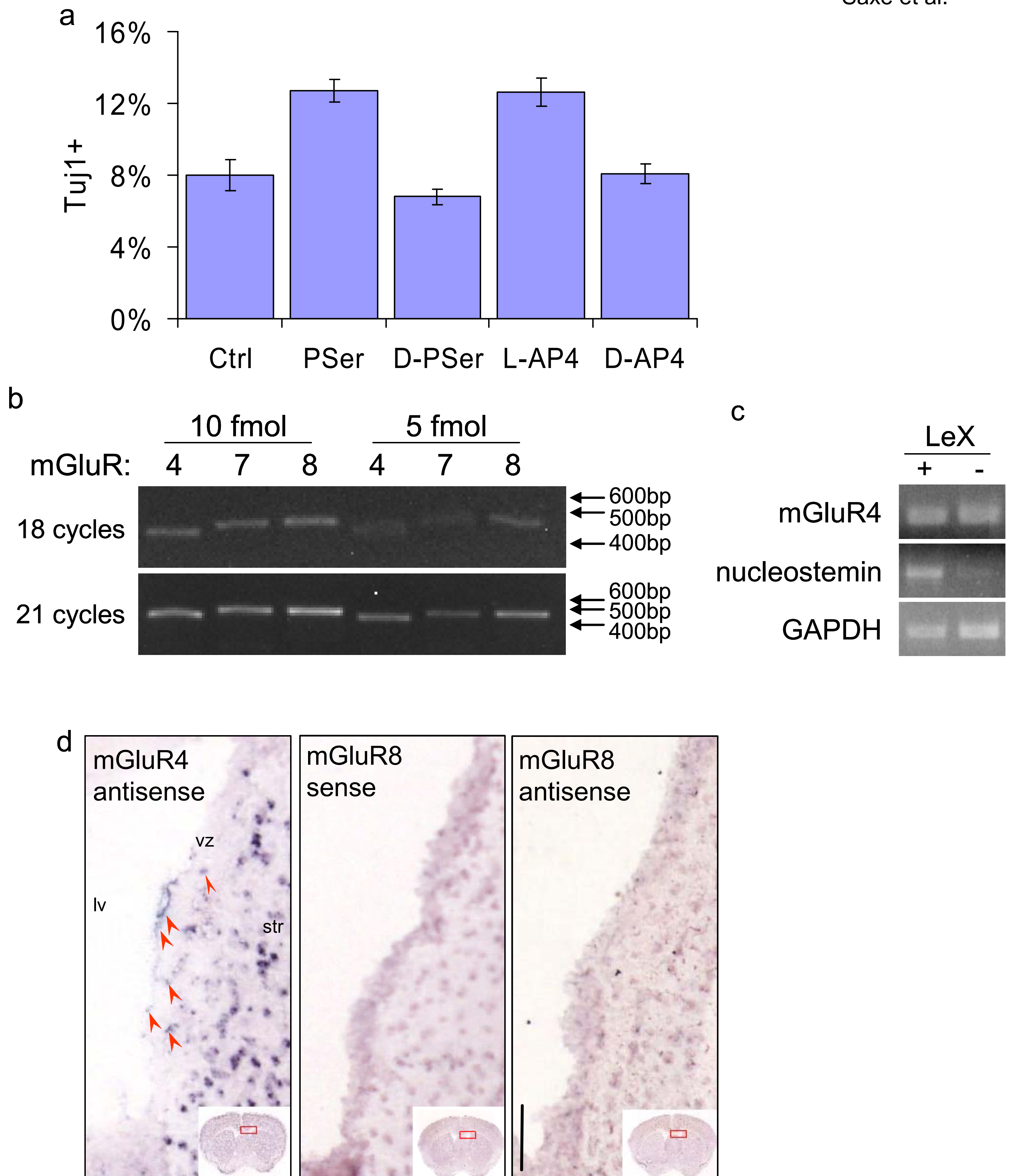
d



Supplementary Figure 1. (a) Validation of small molecule screen. Spheres were plated under assay conditions and treated with water or 10ng/mL BDNF, then processed as described in **Methods** online. p=0.027. **(b)** Secondary screening of hits obtained from primary screen. E11 neurospheres were differentiated in the presence of indicated compounds for three days, then stained for Tuj1 and propidium iodide. Tuj1+ cells were counted and are presented as a percentage of total cells. Data is mean±SEM, P-Ser and control data is the same as reported in **Figure 1b**. **(c)** Compounds from **(b)** which caused a statistically significant increase in the number of Tuj1+ cells. **(d)** Structures of molecules listed in **(c)**.

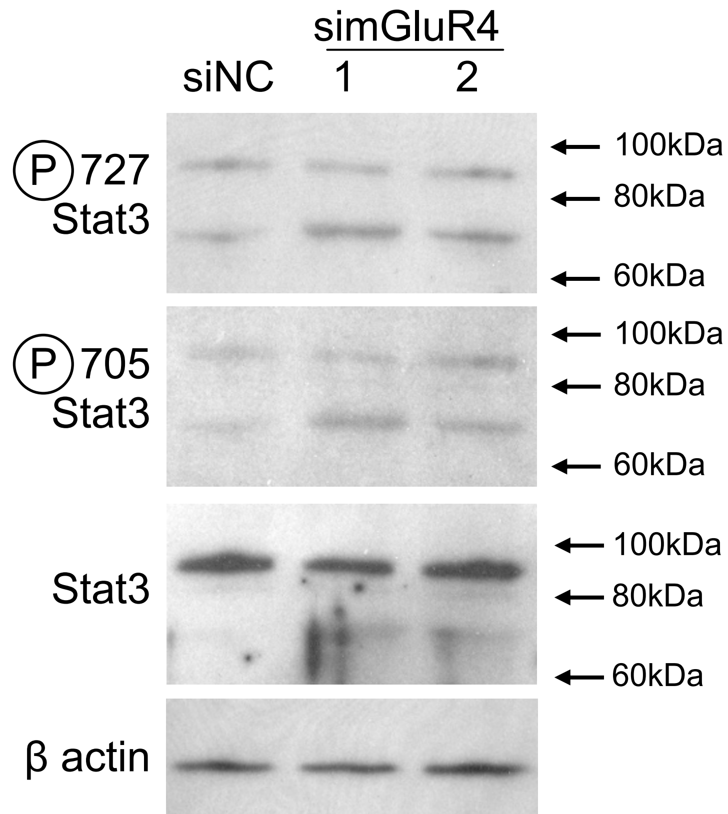


Supplementary Figure 2. (a) Tertiary neurospheres derived from P-Ser treated or control secondary neurospheres are tripotent, scale bar = 100μm. **(b)** Western blot analysis of Tuj1 content in proliferating secondary neurospheres grown with or without 10μM phosphoserine.



Supplementary Figure 3. (a) Secondary E12 neurospheres differentiated for three

days, either untreated or with 10μM of P-Ser ($p < 0.01$ vs. untreated), D-P-Ser ($p > 0.05$), L-AP4 ($p < 0.001$), or D-AP4 ($p > 0.05$). **(b)** Positive control for amplification efficiency of the RT-PCR primers used for mGluR, mGluR7, and mGluR8 analysis. Products were generated with equimolar, indicated amounts of appropriate mGluR template using the primer sequences listed in **Supplementary Methods** online. **(c)** RT-PCR analysis of neurospheres FAC-sorted using the NSC marker LeX. **(d)** *In situ* hybridizations for mGluR4 (antisense) and mGluR8 (sense and antisense) from the Allen Brain Atlas Project (Allen Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science). Images were taken from equivalent sections; the inset shows the entire section with the enlarged area in red. Arrowheads indicate some of the specific staining within the subventricular zone; lv=lateral ventricle, vz=ventricular zone, str=striatum; scale bar=100μM.



Supplementary Figure 4. Western blot analysis of Stat3 phosphorylation in mGluR4-knockdown cells. E12 progenitors were transfected with control siRNA (siNC) or two distinct siRNA duplexes targeting mGluR4 (simGluR4), cultured for three days, and harvested for western blot analysis. Arrows show locations of molecular weight markers.

Supplementary Table 1. List of hits from small molecule screen, "Enhancers", green; "inhibitors", red.

Compound	Library	Annotated Activity
Spaglumic Acid	Orphan Ligand	Endogenous neurotransmitter
(-) Continine	Orphan Ligand	Tobacco smoke constituent
(-) Nicotine	Orphan Ligand	Tobacco smoke constituent
4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone	Orphan Ligand	Tobacco smoke constituent
17 D- amino acids	Orphan Ligand	D- amino acids
Oubain	Orphan Ligand	Putative endogenous
N-acetyltryptamine	Orphan Ligand	Endogenous melatonin agonist
5-methoxytryptamine HCl	Orphan Ligand	Trace amine
L-homocysteinesulfinic acid	Orphan Ligand	Putative neurotransmitter
Agmatine sulfate	Orphan Ligand	Putative endogenous imidazoline ligand
2,4-dihydroxyphenylacetyl-L-asparagine	Orphan Ligand	Possible engodenous glutamate ligand
O-Phospho-L-Serine	Orphan Ligand	Possible endogenous glutamate ligand
6-formylindolo [3,2-b] carbazole	Orphan Ligand	Endogenous
β-alanine	Orphan Ligand	Endogenous
Imidizole-4-acetic acid HCl	Orphan Ligand	Putative endogenous imidazoline ligand
3-methoxytryptamine	Orphan Ligand	Trace amine
Indigo	Orphan Ligand	Endogenous
γ-D-glutamylglycine	Orphan Ligand	Putative endogenous ligand
Ethyl-β-carboline-3-carboxylate	Orphan Ligand	Endogenous β-carboline
L-3,4-dihydrophenethylamine HCl	Orphan Ligand	Dopamine precursor
4-hydroxyphenethylamine HCl	Orphan Ligand	Trace amine
3-hydroxyphenethylamine HCl	Orphan Ligand	Trance amine
1,1'-ethylidene-bis-L-tryptophan	Orphan Ligand	Bioactive tryptophan derivative
DL-4-hyroxy-3-methoxy-mandelic acid	Orphan Ligand	Epinephrine metabolite
4-hydroxy-3-methoxy-phenylacetic acid	Orphan Ligand	Dihydroxyphenylacetic acid metabolite
3-methoxy-L-tyrosine	Orphan Ligand	Tyrosine congener
Adrenochrome	Orphan Ligand	Epinephrine metabolite
6-methoxytryptamine	Orphan Ligand	Trace amine
DL-octopamine HCl	Orphan Ligand	Trace amine
Harmane HCl	Orphan Ligand	Endogenous imidizoline ligand
2-Aminopurine	Enzyme Inhibitor	p58 PITSRLE beta1 inhibitor
HA-1004	Enzyme Inhibitor	PKA and PKG inhibitor
L-p-Bromotetrasmisole oxalate	Enzyme Inhibitor	Tyrosine phosphatase inhibitor
Levamisole HCl	Enzyme Inhibitor	Mammalian alkaline phosphatase inhibitor
AG-126	Enzyme Inhibitor	Tyrosine kinase inhibitor
Tyrphostin46	Enzyme Inhibitor	EGFR, p56, and PDGFR kinase inhibitor

SB203580	Enzyme Inhibitor	p38 MAP kinase inhibitor
ZM336372	Enzyme Inhibitor	Inhibits SAPK2/p38
Endothall	Enzyme Inhibitor	PP2A inhibitor
HA-1077	Enzyme Inhibitor	PKA and PKG inhibitor
LFM-A13	Enzyme Inhibitor	Tyrosine kinase inhibitor
2-Hydroxy-5-(2,5-dihydrobenzylamino)-benzoic acid	Enzyme Inhibitor	Inhibits CAM Kinase II, EGFR kinase, and pp60 kinase
KN-62	Enzyme Inhibitor	CAM Kinase II inhibitor
Tyrphostin AG 1288	Enzyme Inhibitor	Tyrosine kinase inhibitor
GW5074	Enzyme Inhibitor	CRAF1 kinase inhibitor
Indirubin	Enzyme Inhibitor	GSK-3β and Cdk5 inhibitor
Olomoucine	Enzyme Inhibitor	P33 cdk2/cyclin A, p33 cdk2/cyclin E, p34 cdc2/cyclin B, p33 cdk5/p35 inhibitor
Genistein	Enzyme Inhibitor	Tyrosine kinase inhibitor