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Supplemental Information

The Cerebrospinal Fluid Provides a Proliferative Niche for Neural Progenitor Cells

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Inventory of Supplemental figures and tables.

We present 3 Supplementary Figures and 3 Supplementary Tables as further support of the main Figures 1-7 and Table 1.

Supplemental Figure S1, related to Figure 1, presents data characterizing the *Pten*$^{loxP/loxP}$*/NestinCre*$^{-/-}$ mouse and *Pten*$^{loxP/loxP}$*/Pals1*$^{loxP/+}$*/Emx1Cre*$^{+/-}$ mice. The following data are presented in the figure panels:

A. Brain sizes of *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ and control mice.
B. H&E staining of samples presented in (A).
C. Higher magnification images of H&E staining shown in (B).
D. Analysis of progenitor exit from cell cycle using Ki67 and BrdU immunostaining in embryonic *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ and control mice.
E. Analysis of ratio of apical to basal progenitors in *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ and control mice.
F. Cdc42 staining in *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ and control mice at E17.
G. Brain sizes of *Pten*$^{loxP/loxP}$*/Pals1*$^{+/+}$*/Emx1Cre*$^{+/-}$, *Pten*$^{loxP/+}$*/Pals1*$^{loxP/+}$*/Emx1Cre*$^{+/-}$, *Pten*$^{loxP/loxP}$*/Pals1*$^{loxP/+}$*/Emx1Cre*$^{+/-}$, and control mice at E14.5
H. H&E staining of samples presented in (G).

Supplemental Figure S2, related to Figure 4, presents data characterizing the role of embryonic CSF in supporting cortical explant survival and proliferation of neurospheres, an in vitro model of neural stem cells. The following data are presented in the figure panels:

A. Representative images of E16 rat explants cultured with artificial or 100% E17 CSF and immunostained with cleaved caspase 3.
B. Quantification of embryonic CSF's support of cortical explant survival compared to adult CSF.
C. Representative images of E16 explants cultured with E13, E17, P6, or adult CSF.
D. Quantification of long-term neurospheres maintained by E17 CSF.
E. Quantification of relative size of long-term neurospheres maintained by E17 CSF.
F. Quantification of circularity of long-term neurospheres maintained by E17 CSF.
G. Representative images of long-term neurospheres maintained by E17 CSF.

H. Neurospheres cultured with E17 CSF maintained responsiveness to Egf and Fgf.

Supplemental Figure S3, related to Figure 5, presents Igf2 expression in mouse brain and CSF.

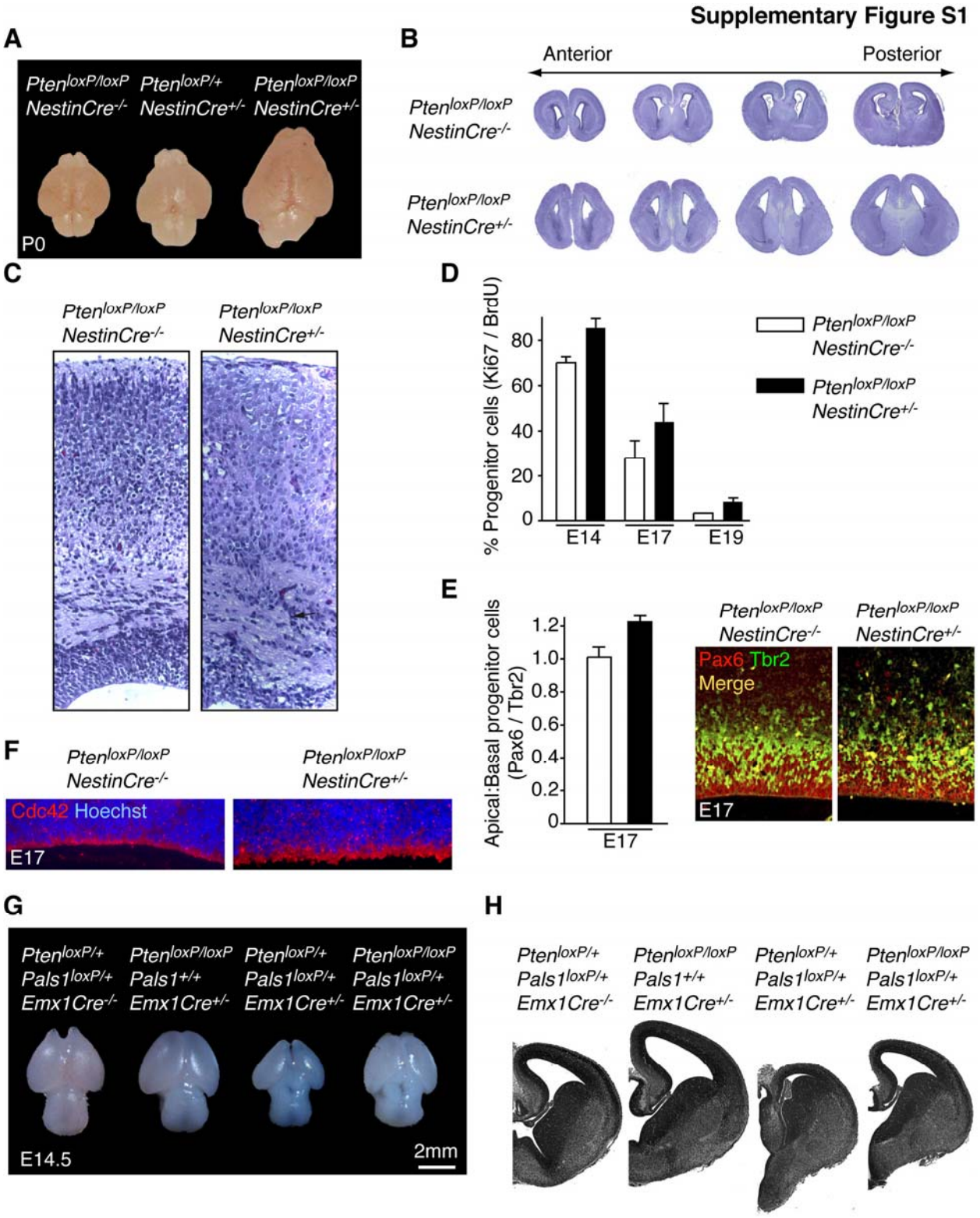
- **A.** *Igf2* in situ hybridization at E14.5.
- **B.** Mouse CSF Igf2 expression by immunoblotting.

Supplemental Table S1, related to Figure 3, presents tandem mass spectrometry (LC-MS/MS) analyses of rat E17 CSF. See separate Excel spreadsheet.

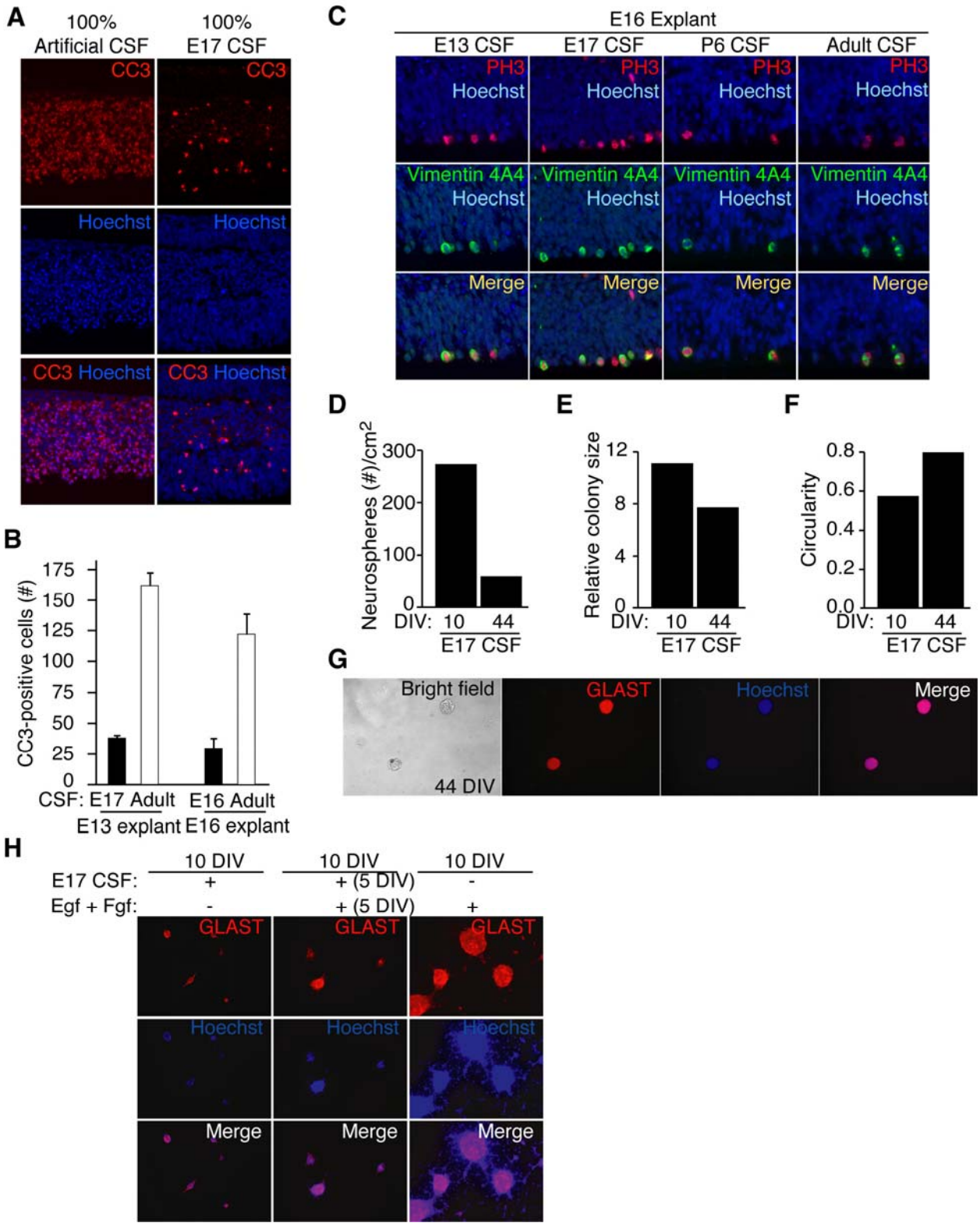
Supplemental Table S2, related to Figure 4, presents data in which the CSF fraction containing proteins from 10-100kDa contained activities essential for neurosphere growth.

Supplemental Table S3, related to Figure 4, presents data in which media conditioned with E17 choroid plexus provided enhanced support for neurosphere formation compared to media conditioned with embryonic cortex, adult choroid plexus, or adult brain.

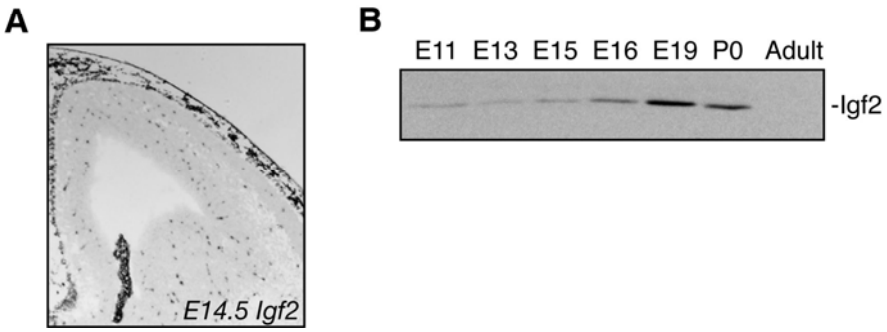
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1, related to Figure 1. *Pten* deletion results in increased brain size, progenitor proliferation, and partially restores the *Pals1*-deficienct brain phenotype. (A) Representative brains from *Pten*$^{loxP/+}$*/NestinCre*$^{+/-}$ and *Pten*$^{loxP/loxP}$*/Emx1Cre*$^{+/-}$ mice at P0. Loss of Pten function in the developing neural tube increased brain size. **(B)** H&E stained serial sections of *Pten*$^{loxP/+}$*/NestinCre*$^{+/-}$ (top) and *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ (bottom) brains. Sections separated by approximately 160μm showed that both the radial thickness and the tangential extent of the cerebral cortex were increased in brains lacking Pten expression. **(C)** Higher magnification images of sections from panel **(B)**. The cortical plate appears disorganized in *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ mice. **(D)** *Pten*$^{loxP/+}$*/NestinCre*$^{+/-}$ and *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ pulsed with BrdU for 24 hours were analyzed at E14, E17, or E19 for proliferation. A greater percentage of BrdU positive cells remained in the cell cycle in *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ mutants compared to control littermates at all ages examined (E14: Control 70.1 ± 2.8%, *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ 85.5 ± 5.3%; E17: Control 28 ± 6.9%, *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ 44 ± 6.1%; E19: Control = 3.4%, *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ 8.4 ± 1.8%; t-test, $p<0.05$). **(E)** *Pten*$^{loxP/+}$*/NestinCre*$^{+/-}$ and *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ mutants were analyzed for numbers of apical (Pax6) and intermediate progenitors (Tbr2) and revealed a relative increase in numbers of Pax6-positive apical progenitors in *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$ mutants at E16 and E17 (Numbers of positive staining cells per section at E17: *Pten*$^{loxP/+}$*/NestinCre*$^{+/-}$ Tbr2 = 424 ± 43, Pax6 = 432 ± 52, Tbr2/Pax6 = 99 ± 0.06; *Pten*$^{loxP/loxP}$*/Emx1Cre*$^{+/-}$ Tbr2 = 529 ± 54, Pax6 = 651 ± 26, Tbr2/Pax6 = 0.80 ± 0.05. A similar trend was observed for Tbr2 positive progenitors labeled with BrdU 24 hours earlier at E16, where 36.0 ± 1.8% of BrdU positive cells were Tbr2 positive in *Pten*$^{loxP/+}$*/NestinCre*$^{+/-}$ compared with 29.4 ± 1.3% in *Pten*$^{loxP/loxP}$*/NestinCre*$^{+/-}$. Collectively, these data demonstrate a

shift in *Pten*-deficient mutants from epithelial, Pax6-positive progenitors that divide asymmetrically to produce neurons or intermediate progenitors, towards symmetric divisions that produce two radial glia. Data are presented as mean ± SD. **(F)** Apical marker expression including Cdc42 was disrupted in E17 $Pten^{loxP/loxP}/NestinCre^{+/-}$ mice compared to littermate controls. **(G)** Conditional *Pten* deletion ($Pten^{loxP/loxP}/Emx1Cre^{+/-}$) resulted in an enlarged cerebral cortex already by 14.5. The small brain size of *Pals1* heterozygotes appeared more normal in the double mutants by E14.5. **(H)** H&E staining of brains shown in panel (G).

Supplemental Figure S2, related to Figure 4. Embryonic CSF supports cortical explant survival and proliferation. **(A)** E16 explants cultured for 24 hours in 100% E17 CSF or 100% artificial CSF were immunostained with the apoptotic cell death marker cleaved caspase 3 (CC3). Explants cultured in 100% embryonic CSF has decreased CC3 immunoreactivity compared to explants grown in ACSF. **(B)** CC3-positive dying cells were quantified in E13 explants cultured for 24 hours in 100% E17 CSF or 100% adult CSF and in E16 explants cultured for 24 hours in 100% E16 CSF or 100% adult CSF. Embryonic CSF supports embryonic tissue viability and survival (E13 explant + E17 CSF = 39.5 ± 3.9; adult CSF = 163.5 ± 10.4; Mann-Whitney, $p<0.05$, n=3; E16 explant + E16 CSF = 33.7 ± 4.4; adult CSF = 122.1 ± 19.4; Mann-Whitney, $p<0.05$, n=3 and n=4, respectively). **(C)** Representative images of E16 explants cultured with E13, E17, P6, or adult CSF quantified in **Figure 4D-F**). **(D)** Dissociated cells from primary neurospheres cultured in E17 CSF for 44 DIV maintain GLAST-positive neural progenitors when cultured in embryonic CSF, n=2. Quantification of number of spheres/cm^2 when cultured for 10 DIV vs. 44 DIV. **(E)** Quantification of relative colony size of spheres cultured for 10 DIV vs. 44 DIV. **(F)** Quantification of circularity of spheres cultured for

10 DIV vs. 44 DIV. **(G)** Representative images of neurospheres quantified in **D-F**. **(H)** Left and middle panels: Dissociated cells from primary neurospheres cultured in E17 CSF for 5 DIV and then supplemented with Egf and Fgf. GLAST-positive-staining cells cultured in E17 CSF maintain responsiveness to Egf and Fgf suggesting that stem cells cultured in CSF maintain undifferentiated and uncommitted state. Right panels: Dissociated cells from primary neurospheres cultured in Egf and Fgf for 10 DIV.

Supplemental Figure S3, related to Figure 5. Igf2 expression in mouse choroid plexus and CSF. **(A)** *Igf2* in situ hybridization at E14.5. *Igf2* levels are highest in the choroid plexus, leptomeninges, and vasculature. **(B)** Igf2 is transiently expressed in mouse CSF.

Supplementary Table S2

Filtration analysis of E17 CSF	
Sample	**Neurosphere formation**
< 3 kDa	-
< 10kDa	-
< 30kDa	+
< 100kDa	+++
E17 CSF	+++

Supplementary Table S2. CSF factors that stimulate neurosphere formation are likely small proteins ranging from 10-100kDa. Neurospheres derived from E14 rat brain were dissociated and plated at clonal density in CSF fractions of various sizes and assayed for the presence of neurospheres after 10 DIV; n=3.

Supplementary Table S3

Tissue-conditioned media	
Sample	**Neurosphere formation**
E14 cortex	+
E17 cortex	+
E17 choroid plexus	+++
Adult cortex	-
Adult choroid plexus	++

Supplementary Table S3. Actively secreted factors from choroid plexus stimulate neurosphere formation. Neurospheres derived from E14 rat brain were dissociated and plated at clonal density in either cortex- or choroid plexus-conditioned medium. The presence of neurospheres was assayed after 10 DIV; n=3.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Immunostaining

Paraffin sections (5μm) of brains or explants were dehydrated and subjected to antigen retrieval with Antigen Unmasking Solution (Vector), followed by blocking (PBS/5% serum), permeabilization, and antibody incubation. Alternately, cryosections (14-50μm) were blocked, permeabilized, and incubated with antibodies. Immunostained cells counted in serial explants are expressed as numbers of cells along the ventricular surface as detailed in figure legends. All samples were counterstained with Hoechst 33258 (Sigma). For quantification of cells in distinct cortical laminae, the radial extent of the cortex was either divided into six equally sized bins (Figure 1D) and quantified, or all positive staining cells were counted in an area of cortex of equal width across all samples (Figure 5H). All quantification was carried out using ImageJ software.

Neurospheres

E14, E17, or adult rat cortex was dissected in sterile HBSS and dissociated by the Papain Dissociation System (Worthington Biochem. Corp). Primary spheres were generated in either CSF or DMEM/F12 supplemented with heparin, N2, Fgf2 (10ng/ml), and Egf (20ng/ml), and collected after 7-9 DIV. In the latter case, primary spheres were then re-suspended in media without Egf or Fgf, dissociated, plated at a final density of 2,500 cells/cm^2, and cultured as described in text. Fresh media was supplemented at 4 DIV. Cells were fixed in 4% paraformaldehyde and stained for GLAST.

Cortical cell cultures

Rodent E13.5 cortices were isolated and dissociated by the Papain Dissociation System (Worthington Biochem. Corp). Cells were cultured in NBM supplemented with penicillin-streptomycin, glutamine, N2, and Fgf (10ng/ml) for 24 hours. For Igf signaling experiments, cells were deprived of growth factors for 6 hours, followed by a 5 minute pulse of 20% ACSF, 20% embryonic CSF, or Igf2 (20ng/ml). For P-LRP6 experiments, cells were left untreated, or treated with 20% CSF, 10% L-cell conditioned media, or 10% Wnt3a conditioned media (ATCC) for 2 hours.

Tissue-conditioned media

Rat tissue was dissected in approximately equal mass, washed in HBSS, and placed in 250 μl of embryonic neurosphere media without Egf or Fgf at 37°C for 2-3 days. Dissociated primary neurospheres were added to the centrifuged conditioned media free of tissue content. The numbers of secondary neurospheres generated were assayed at 10 DIV.

Indicator cell experiments

Luciferase reporter constructs were generated using synthetic responsive elements (3xDR5-RARE (Balkan et al., 1992) or 2xBMP responsive element (Korchynskyi and ten Dijke, 2002)), fused to a thymidine kinase minimal promoter and inserted into the Luciferase reporter of pGL3 (Promega). Reporter cassettes were inserted into a vector containing a constitutive neomycin cassette, and electroporated into L-M^{tk-} (RA) or 3T3 (BMP) cell lines, and grown in DMEM containing 10% charcoal-stripped calf serum, antibiotic/antimycotic solution (Gibco/Invitrogen) and 750μg/ml of G418. Clonal cell lines were produced by dilution cloning. Samples were

screened by adding CSF (20%) to assay media (as above, but with 5% charcoal-stripped calf serum and 25mM HEPES, pH 7.4). Cells were treated for 48 hours, with the sample refreshed after 24 hours. Effective ligand concentration was calculated by assaying wells using a luciferase assay, and comparing activity to assays treated with a dilution series of known concentrations of all-trans RA (Sigma) or recombinant mouse BMP4 (R&D Systems) prepared in ACSF.

Electron microscopy

Scanning electron microscopy (SEM): E12.5 mouse cortex fixed in 2% glutaraldehyde was processed for SEM according to standard procedures. Samples were examined using a Carl Zeiss 1450 VP Scanning Electron Microscope. Transmission electron microscopy (TEM): Pre-embedding and immunogold labeling were carried out E17 rat embryos perfused with 0.5% glutaradlehyde / 2.5% paraformaldehyde and postfixed overnight. Briefly, 100µm vibratome sections were blocked with 5% BSA / 5% goat serum in PBS, immunostained with anti-Igf2 antibodies (Abcam) followed by incubation with a secondary antibody conjugated to colloidal gold, enhanced with silver, postfixed with 0.5% osmium tetroxide, and embedded. Ultrathin sections were stained on copper grids stained with lead citrate and examined in a JEOL 1200EX Transmission electron microscope.

In Situ hybridization

Non-radioactive in situ hybridization was performed as described (Berger and Hediger, 2001), using digoxigenin (DIG)-labeled cRNA probes generated from a TA vector (Invitrogen) clone of Igf2 and Igf1R.

Brain size measurements

Cortical perimeter was measured using ImageJ by tracing the edges of both hemispheres. Cortical surface area was measured similarly using ImageJ.

Biochemical assays

Igf1 concentration in rat CSF was measured using the Active Mouse/Rat Igf-1 ELISA (Diagnostic Systems Laboratories) according to the manufacturer's instructions. Igf2 concentration in human CSF was measured using the Non-extraction Igf2 ELISA (Diagnostic Systems Laboratories) according to the manufacturer's instructions.

RNA quantification

Expression of Bmp and RA synthetic and catabolic enzymes relative to Gapdh was measured in embryonic rat choroid plexus by Sybr-green qPCR. The following primer sets were used: Bmp2 forward (F): CGGGACCCGCTGTCTTCTAGTGTTG, Bmp2 reverse (R): GGGCACCACGACGTCCTTGCTG; Bmp4 (F): GGAGGCGCGAGCCATGCTAGTTTG, Bmp4 (R): CCCGGTTCCCTGGCTCTGCTCTTC; Bmp5 (F): GAAGACACGGGCCTCAGTCAAAGCAG, Bmp5 (R): CCATCCCAGATCGCGGAAACTCAC; Bmp7 (F): GGCAGGGAGTCCGACCTCTTCTTG, Bmp7 (R): CTTGGAGCGGTTCTGGCTGCGTTG; Raldh1 (F): TTCCTCCTGGCGTGGTGAACATTG, Raldh1 (R): ACGCAGCATTGGCCTTGATGGTAG; Raldh2 (F): GCAGGGGCAGCAATAGCGTCTCAC, Raldh2 (R): GCGCCTCTTGGCTCTTTCCACAC; Raldh3 (F): GCGGCCTCCAGGGTGTTTGTG, Raldh3 (R): CCGCCGTGAGCCCATAGTCAGTG; Rdh10 (F): GCTGGTGCGGCCCAAGGAGAAG,

Rdh10(R): CCAGCGTTATTGACCAGGACCGAGAC; Ttr (F): GCCCTGGGGGTGCTGGAGAATC, Ttr (R): GAGCAGGGCTGCGATGGTGTAGTG; Gapdh (F): GGCATGGCCTTCCGTGTTCCTAC, Gapdh (R): GCCAGCCCCAGCATCAAAGGTG. Reactions were carried out 4 separate times in duplicate and mRNA expression levels were calculated by the comparative C_T method.

SUPPLEMENTAL REFERENCES

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