Supporting Information

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SI Text

SI Methods and Materials. ***Animals.*** Timed-pregnant C57BL/6 mice were ordered from Jackson Laboratory, Inc. (Bar Harbor, ME) at gestation day 7 and used in all experiments according to a protocol described elsewhere (S1). The animals were kept in a temperature-controlled environment at 24 °C and exposed to a 12 h light and dark cycle. All the dams received AIN-76A pelleted diet with the standard 1.1 g/kg (7.8 mmol/kg) choline chloride (control diet; control (CT); Dyets, Inc., Bethlehem, PA) and water ad libitum until the end of embryonic day 11 (E11) when they were randomly assigned to one of three feeding groups: choline deficient (CD ; AIN-76A diet with 0 g/kg choline chloride), CT or choline supplemented (CS ; AIN-76A diet with 4.95 g/Kg choline chloride). The mice received the experimental diets from the night of gestational day 11 until they were killed on E17. On E15, a single intraperitoneal injection of 50 mg/kg body weight 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich, Inc., St. Louis, Mo) was administered to the pregnant dams.

Fetal brain collection. On E17 the fetal brains from two male pups from each litter were collected using methods we previously published (S2). The fetal brains were cut in 5 μm coronal sections, and the hippocampal sections were used for further immunohistochemical analysis. All the tissue sections were selected from anatomically equivalent areas according to a standard atlas of the developing brain (S3). This study was replicated. The remaining brains from the litter were collected individually, frozen in liquid nitrogen, and stored at −80 °C for gene expression determinations.

Fetal mouse neural progenitor cells (NPC) in culture. Neural Progenitor Cells (NPC) in culture maintain similar phenotypes to their corresponding progenitors from the hippocampal ventricular (VZ) and subventricular (SVZ) in terms of chromatin architecture, remodeling, histone modifications, and gene expression (S4–S8). NPC from embryonic day 14 C57BL/6 mice were obtained from Lonza (Walkersville, MD) and plated according to the manufacturer's protocol using Neurobasal medium (Invitrogen, Purchase, NY). The medium was supplemented with 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin—streptomycin (Invitrogen), 2% B27 supplement without vitamin A (Invitrogen), 20 ng/mL murine EGF (Invitrogen), 20 ng/mL human FGF (Invitrogen), and 2 mg/mL heparin (Invitrogen). Small tertiary neurospheres were generated as previously described (S9). The cells were assigned to two different treatment groups (up to $n = 7$ plates/group), passaged to new uncoated plates and suspended in custom Neurobasal medium (D700SA, Atlanta Biologicals, Lawrenceville, SA) containing 5 μM choline chloride (CD), 70 μM choline chloride (CT), or 280 μM choline chloride (CS) with the ingredients described above. These choline concentrations are similar to those measured in vivo in fetal brain after pregnant dams were fed the CD, CT, or CS diets (S9). The optimal exposure time, 72 h, was determined after the assessment of proliferation and apoptosis changes (S9) so as to avoid artifacts associated with cell death. This time frame allowed the NPC to complete several cell cycles at an expansion rate of 10–15 fold/7 d (S10). NPC colonies were pelleted by centrifugation and immediately processed for total protein extraction, genomic DNA extraction, total mRNA isolation, or chromatin precipitation.

Immunohistochemistry in paraffin-embedded sections. To assess EC proliferation, a double immunolabeling technique utilizing both BrdU (S-phase marker) and isolectin IB4 (vasculature marker) was used. Developmental angiogenesis occurs in waves (S11); therefore we evaluated EC proliferation over a period of 48 h (E15–E17). During S-phase, proliferating EC incorporate BrDU into newly synthesized DNA strands (S12). GSA-I lectins are markers of EC, selectively staining alpha-D-galactosyl residues expressed by the developing vascular tree (S13, S14). Paraffin-embedded hippocampal coronal sections were selected from anatomically identical regions in order to minimize differences due to the anterior- posterior gradient in neurogenesis (S15). The slides were deparaffinized with xylene and then rehydrated using ethanol in decreasing concentrations. Tissue auto-fluorescence was minimized by incubating the slides at room temperature for 1 h in 0.25% NH_4OH (Sigma) in ethanol 70%. Antigen retrieval for nuclear antigens was performed using citric acid buffer pH = 6 at 100 °C for 10 min, followed by incubation in Proteinase K 20 μg/mL (Sigma). The BrdU epitopes were further exposed by enzymatic digestion of genomic DNA with DNAse I 10 U/mL (Invitrogen Inc., Carlsbad, CA) for 40 min at room temperature. Reduction of nonspecific epitopes was accomplished by incubating all tissue sections in blocking buffer containing 10% normal goat serum (Sigma) in PBS + 0.1% Tween 20 (PBST) overnight at 4 °C, followed by endogenous biotin blocking (E-21390, Molecular Probes—Invitrogen) for 2 h at room temperature. The slides, except for the negative controls, were incubated for 3 h at room temperature in a cocktail of primary probes composed of: Isolectin GS-IB4—biotin conjugated antibody (5 μg/mL; I21414, Invitrogen) 1:100 dilution in PBST and rat antiBrdU monoclonal antibody (0.2 mg/mL; and MCA2060, AbDSerotec; Raleigh, NC) 1:100 dilution in PBST; 0.1 mM $CaCl_2$ was added to the labeling buffer, according to the manufacturer's protocol. For each staining, seven different fetal brains per group (CD and CT, respectively) were used, each from a different dam. An additional slide per group was placed into the blocking buffer, without the first antibody, and used as a negative control. After washing, all the sections were incubated for 2 h at room temperature in a mixture of secondary antibodies and probes: streptavidin-ALEXA 488 nm (Invitrogen) 1:100 dilution of 1 mg/mL stock solution and goat anti-rat—ALEXA 555 nm (Invitrogen) 1:500 of the 1 mg/mL stock solution in PBST.

Following a similar protocol, equivalent sections were probed for Von Willebrand factor (Factor VIII related antigen, VW factor)—Factor VIII complex using a cocktail of primary antibodies. Rabbit polyclonal antibodies against VW factor (18-0018, Zymed Laboratories, Invitrogen, Carlsbad, CA), and against factor VIII heavy (H-19) (sc-27647, Santa-Cruz Biotechnology Inc., Santa-Cruz, CA) and light chains (sc-33584, Santa-Cruz) targeting precursor and mature Factor VIII chains, were all diluted 1:200 from their initial concentration of 0.5 mg/mL in blocking buffer (10% goat serum in PBST). The slides were incubated for 48 h at 4 °C and rinsed in PBST. Finally, fluorescent labeling was performed with goat anti-rabbit IgG-HRP conjugated followed by ALEXA 546 nm-tyramide signal amplification (T20923, TSA Kit#13, Invitrogen-Molecular Probes). In all cases, 4′, 6-diamidino-2-phenylindole (DAPI, Sigma) 0.1 μg/mL for 20 min was used for staining the nuclear DNA.

Image analysis. Fluorescent images (magnifications 4×, 10×, and 20×) of VW Factor or BrdU-isolectin stained sections were collected with a Nikon FXA microscope (Nikon, Garden City, NY)

equipped with an Optronics TEC-470 CCD Video Camera System (Optronics Engineering). The images, acquired from the same field with different fluorescent probes, were then merged. The labeling level and background fluorescence level for selected areas were measured as OD using the ImageJ software version 1.37v (http://rsb.info.nih.gov/ij, NIH) and checked for variations across all samples. The number of blood vessels and the average cross-sectional area of isolectin positive vessels were counted using the ImageJ software with an integrated macro for analyzing particles. Dimensions were obtained by calibrating all images to a common reference image. The proliferation index of EC was calculated as the ratio of colabeled BrdU-isolectin positive cells to DAPI-isolectin positive cells in both hippocampi of each brain in six consecutive 5 μm sections. For analysis of capillary numbers and cross-sectional volume, we selected consecutive sections totaling 30 μm of thickness from medial-frontal region of the hippocampus from six fetuses from different dams. To assess the number of EC clusters that were VW Factor positive, the fluorescent structures were counted per entire hippocampus as well as for selected hippocampal regions: CA1 (Cornus Ammoni region 1), CA2, CA3, and DG (Dentate Gyrus).

Real-time RT-PCR. RNA was extracted from cultured NPC or from whole brain samples using the RNeasy Mini Kit (Qiagen, Valencia, CA). After determining the concentration and quality, the resulting total mRNA was stored at −80 °C. A two-step Real-Time PCR method was used for the assessment of gene expression. First, a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) containing random primers was used as described in the manufacturer's protocol to generate single stranded DNA template. After transcription, a volume of sample that did not exceed 2 ng cDNA/μL of the template per PCR was amplified in the second step. Amplification conditions were adjusted using the standard curve method for an optimal slope, 100% PCR efficiency, and no nonspecific products. Almost identical efficiencies were achieved for the gene of interest and the *TBP* housekeeping gene. *TBP* mature RNA levels do not vary with choline availability in the central nervous system, as shown in published microarray studies (S6). The following primers were designed for *Angpt2* (mus musculus angiopoietin 2): forward 5′-GTGTGGACTTCCAGAGGA-3′ and reverse 5′-GCTGGATCTTAAGCACGTA-3′, accession number NM_007426.3; and *Tbp* (TATA box binding protein): forward 5′-ACCCACACAGGGTGCCTCA-3′ and reverse 5′-TGGCAG GAGTGATAGGGGTCA-3′ (accession number NT_123456) genes. *Vegfc* (VEGF C) primer pair was designed and synthesized by SuperArray (PPM03061E, SuperArray, MD) sequence accession number NM_009506.2. The primers were designed using the Gene Fisher interactive PCR primers design software support (http://bibiserv.techfak.uni-bielefeld.de/genefisher/) (S16). The primer pairs were tested by RT-PCR for dimer formation. To ensure against nonspecific priming, nonredundant BLAST searches were performed for all primers (http://www.ncbi.nlm.nih.gov/BLAST/).

A 25 μL reaction mixture containing 450 nM primers, 2 ng cDNA, and the Real-Time PCR Master Mix IQ SYBR Green Supermix Biorad, was used for all reactions. The conditions for the amplification reactions were: 95 °C for 10 min; 40 cycles of 94 °C for 15 sec, 56 °C for 30 sec, and 72 °C for 50 sec; followed by a melting curve to ensure single amplicon generation. The relative quantification method ($2^{\Delta\Delta CT}$) was used to analyze gene expression changes as described elsewhere (S17).

Methylated CpG island recovery assay (MIRA). We used a method for analyzing CpG island methylation based on the high affinity of MBD protein complex for methylated cytosines(S18). CpG enrichment facilitates an accurate detection of methylated DNA even in low methylated sample (MethylCollector Ultra Kit; Active Motif, Carlsbad, CA). Genomic DNA was isolated from choline deficient and control NPC samples using Qiagen DNAeasy minicolumns (Qiagen) following manufacturer's protocol, quantified with a Nanodrop 8000 spectrophotometer (Nanodrop, Wilmington, DE) and stored in elution buffer at −80 °C. In preparation for the enrichment step, 4000 ng of gDNA from each sample were digested with *MseI* restriction enzyme for 2 h at 37° followed by heat-inactivation. Input DNA aliquots were purified by "QIAquick PCR purification Kit" (Qiagen), incubated with recombinant His-MBD2b/MBD3L1 protein complex followed by capture with protein G coated magnetic beads. The DNA isolated by this method as well as sample aliquots saved before the enrichment step were purified again by QIAquick columns in preparation for Real-Time PCR analysis.

Vegfc and *Angpt2* genomic sequences were retrieved from the NIH mouse genome database (http://www.ncbi.nlm.nih.gov/nuccore) including the gene features (CpG islands and exons/introns locations). For each CpG island several primer pairs were designed using the Gene Fisher interactive PCR primers design software support (http://bibiserv.techfak.uni-bielefeld.de/genefisher/) (S16) and synthesized (Operon Biotechnologies; Huntsville, AL). The oligos were tested for specificity and efficiency of Real-Time PCR amplification in a 20 μL volume comprising of Takara SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan) and a genomic DNA in serial dilution. The final PCR conditions for all primer pairs were: 95 °C for 15 sec., 60 °C for 15 sec., 72 °C for 20 sec. Selected primer sequences used in this assay for *Vegfc* were: CpG island 1 forward 5′- ACCCCCTCTTCTTTGCA -3′, reverse 5′- GAGCCTCGG ATCCTCA -3′; CpG island 2 forward 5′- AAACAGCTCAGGGTC-GAA -3′, reverse 5′- CCTGGGTGTCCTGGAA -3′; CpG island 3 forward 5′- GTCATTATCTGCCGGGAA -3′, reverse 5′- AAAAGC CTGTGAGCCTA -3′. For *Angpt2* gene we selected the following oligo pairs: CpG island 1 forward 5′- TTCTCCAGCACCTGCA -3′, reverse 5′- CTGTAGACTCAGGGGAGA -3′;. CpG island 2 forward 5′- CAGCCCTGAGACTCTGA -3′, reverse 5′- GGCTGATGAAG GTAGGAA -3′; CpG island 3 forward 5′- CGTGGGGACAAGCT-CA -3′, reverse 5′- CTGCAGCAAAGCAGTCA -3′. CpG islands were numbered from 1 to 3 starting from the TSS (transcription starting site). Positive and negative control primers were provided by the Activ Motif Kit and were able to amplify with great accuracy methylation free regions of *APC* gene (methylated only in adenocarcinoma cells) and *Xist* gene (fully methylated in males) (Fig. 5*B*—inset).

ELISA. NPC were cultured for 72 h in choline deficient, control and choline supplemented media as described above and total protein was extracted using RIPA buffer (Sigma) with 10% protease inhibitors (Sigma). Protein concentrations were determined by Lowry assay on a Synergy 2 microplate reader (Biotek, Vermont). The mouse ANGPT2 protein levels were evaluated using an ELISA kit (Cedarlane, Burlington, NC) as described by the manufacturer. The concentration of ANGPT2 was determined into 100 μg of total protein diluted into the sample buffer and the absorbance read at 450 nm using the Synergy2 system. A mouse VEGFC sandwich ELISA assay was developed using a polyclonal antibody rabbit anti-mouse VEGFC detecting the precursor VEGFC (sc-9047) and the secreted form as capture antibody (Fig. S1*A*, inset). We tested the specificity of binding by immunoprecipitation of VEGFC preprotein using Dynabeads M-280 Sheep anti-rabbit IgG (Invitrogen) followed by Western blot analysis of the elution. The VEGFC band was identified at 58 KDa. The detection antibody, goat polyclonal against mouse VEGFC (C terminus of the protein precursor) (Santa-Cruz), was biotinylated using BiotinTag Micro Biotinylation Kit (Sigma) as indicated by the manufacturer. 100 ng/mL of the capture antibody was bound to a coated Goat anti-rabbit 96-well microplate (cat. 15136, Pierce, Rockford, IL) for 1 h at room temperature and 50 μg of NPC protein samples were loaded onto the plate and incubated for 90 min at room temperature. The plate was

incubated with 100 ng/mL detection antibody for 1 h. 100 ng/mL streptavidin-HRP conjugated (Invitrogen) was bound to biotin for 30 min at room temperature and the signal development was performed using the SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific).

Maternal Hepatic Phosphocholine. Phosphocholine is the labile storage form for choline in liver and is an excellent indicator of dietary choline status (S19). Phosphocholine was analyzed by liquid chromatography—electrospray ionization—isotope dilution mass spectrometry as previously described (S20).

Bioinformatic Analysis. To analyze the distribution of cis—regulatory elements binding site (TFBS; transcription factor binding sites) within the CpG islands in the vicinity of TSS of *Vegfc* and *Angpt2* we used the public database NCBI NIH (http://www.ncbi.nlm.nih.gov/nuccore—mm8) to download the genomic sequence of both genes of interest and also gather information about their exons (Evidence Viewer) and CpG islands. Putative TSS were detected upstream of exon 1 for both genes using Promoter 2.0 Prediction Server (S21). Each CpG island sequence was uploaded to TFM Explorer (http://bioinfo.lifl.fr/TFME/) (S22) which uses position frequency matrixes from Transfac (S23) and Jaspar to extract cis-regulatory elements with the highest significance from all the predicted sites. The *P*-value is given by the probability of any TFBS to generate the same number of hits in the sequence of choice as in the Markovian background model (S22) (Fig. S2).

Statistical analysis. For gene expression the analysis was performed using the Relative Expression Software Tool—Multiple Condition Solver REST-MCS version 2 (free from the website http://www.gene-quantification.de/rest.html) which uses a pair wise fixed reallocation randomization test. All statistical analyses of epitope levels and vascular tree determinations were performed using JMP software (V 2; SAS Institute, Cary, NPC) with ANOVA and Tukey-Kramer tests. Data are presented as mean ± SE.

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Fig. S1. VEGFC and ANGPT2 protein levels in the mouse NPCs are influenced by choline concentration in vitro. E14 mouse brain neural progenitor cells were grown in low choline (5 μM; CD), (70 μM; CT), or (280 μM ; CS) medium for 72 h. *A*. VEGFC levels detected in 50 μg total protein. CD group has 33% more VEGFC relative to the CT group (Pair wise comparison by ANOVA raw *P* value = 0.0485 and Tukey—Kramer adjusted *P*=0.1103). The small inset represents a blot image of VEGFC protein (21 kDa) and precursor (58 KDa). Band identification was performed as described by Foster (2). *B*. ANGPT2 levels detected in 100 μg whole protein samples. CD group had 42.2% more ANGPT2 relative to the CT samples (**, $P < 0.01$ by Tukey-Kramer). Values are reported as mean ± SE for $n = 4$ samples per group.

2 Foster RR, Saleem MA, Mathieson PW, Bates DO, Harper SJ (2005) Vascular endothelial growth factor and nephrin interact and reduce apoptosis in human podocytes. *Am J Physiol Renal Physiol* 288:F48–57.

GENE	Transcription Factors	Motif analysis	GC content	Number of hits	Hits with >1 CpG	P Value
VEGFC	*AP-2*		0.61	13	13	3.23e-10
	Sp1		0.61	11	5	1.85e-08
	PAX 4		0.61	8	8	3.48e-06
	bHLH		0.61	6	6	1.78e-03
	NF-kappaB		0.68	2	1	2.17e-02
	NRSF		0.68	2	1	5.85e-02
ANGPT2	*HMG*		0.53	5	0	5.34e-05
	bHLH		0.53	5	5	1.11e-04
	ETS1		0.53	3	1	2.11e-02
	ARNT		0.53	5	4	1.43e-04
	PPAR		0.53	2	1	1.71e-03

Fig. S2. Bioinformatic analysis of putative transcription factors binding sites within CpG islands of *Vegfc* and *Angpt2*. Differentially methylated CpG sites were identified in *Vegfc* and *Angpt2* as described in Fig. legend 5. Using TFM Explorer (http://bioinfo.lifl.fr/TFME/) (1) we identified transcription factor binding sites within the genomic sequences of these CpGs. The parameter value set to 2.5 indicates the minimal average density of hits in the cluster relatively to the average number of hits in the reference model. We show data for transcription factor binding sites with the lowest *P* values (<0.01; *P* is the probability to observe the same number of hits in a given widow from a given power matrix (specific to each transcription factor binding site (TFBS) as compared to the background seqeunce model) and those binding sites known to influence *Vegfc* and *Angpt2* gene expression. Motif analysis results represent position-specific probability matrices that specify the probability of each possible nucleotide to appear at each possible position in an occurrence of a specific motif. The result is a stack of symbols (nucleotides of different colors), one stack for each position in the sequence. The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino or nucleic acid at that position. guanosine cytosine (GC) content indicates the percentage of CGs in all the hits for a given TFBS and in all cases it was higer than the one for the background matrix (data not shown). All putative TFBS had at least one CpG site for each individual hit found.

1 Defrance M, Touzet H (2006) Predicting transcription factor binding sites using local over-representation and comparative genomics. *BMC Bioinformatics* 7:396.

Table S1. Maternal dietary choline deficiency does not alter the number of blood vessels in fetal cortex.

Angiometric analysis	CD	CT	CS
Number of blood vessels per hippocampus	592 ± 17	617 ± 23	553 ± 50
Combined cross-sectional area (μm^2)	23,579 ± 1,475	23,378 ± 1,406	18,619 ± 2,150
Average size (μm^2)	19.8 ± 0.9	19 ± 1.4	16.6 ± 0.7

Maternal dietary choline influence on the fetal cortical vasculature. Pregnant mouse dams were fed a CD, CT, or CS diet from E12 to E17 when angiogenesis in fetal hippocampi was evaluated. Blood vasculature was fluorescent labeled using isolectin-ALEXA488 conjugated (green fluorescence) as described in the *SI Materials and Methods* section. The number, total surface area, and the average size of microvessels that were isolectin GS-IB4 positive were measured using the ImageJ Software in both hippocampi of one fetal brain and the average numbers were reported. Two consecutive sections (5 μm) of cingulate and midposterior neocortex from each sample (10 μm total) were analyzed. Statistical analysis was performed by ANOVA and Tukey-Kramer. Data is presented as mean ± SE, $n = 5$ pups from 5 different dams/group.