Supplementary Information

Supplementary Note 1

DNA Microarrays. Total RNA from the medial basal hypothalamus (MBH) of two Jv (341 and 351days), 2 EP (2 yr 83 days, 2 yr) and one MP (3 yr 70 days) female rhesus monkey was isolated using TriReagent solution (MRC, Cincinnati, OH). RNA concentration was determined spectrophotometrically and its integrity was assessed in 1.8% agarose-formaldehyde gels. Labeled cDNA was prepared according to the manufacturer's protocol. In brief, 0.5 µg of experimental RNA were added to 5.0 µl of control RNA provided with the Incyte Genomics Human LifeGrid™ 1.0 filters (Incyte Genomics, Palo Alto, CA) and the mixture was heated at 70°C for 10 min. Thereafter, the mixture was cooled on ice for 5 min and vacuum-dried. To each tube, 4 µl of [α^{33}-P] dCTP (2000-4000 Ci/mmol, 10 µCi/µl) and 3 µl of a 15 µl RT premix containing 7 µl 5 x M-MLV RT buffer, 2 µl 10 mM dA/dG/dT mix and 6 µl M-MLV RT (200 units/µl) were added before incubation at 42°C for 1-2 h. Unincorporated nucleotides were then removed with a spin column provided with the kit. Incorporation of radiolabeled dCTP was estimated by liquid scintillation counting. Just prior to hybridization the labeled samples were incubated at 95°C for 3 min and immediately placed on ice. Two Human LifeGrid™ 1.0 array filters containing 8,400 cloned, double spotted genes and 27 controls each were used for analysis of two samples. Filters were prehybridized at 42°C for 2 – 16 h. Each aliquot of fresh hybridization solution contained 50 µl of herring sperm DNA (10 mg/ml) and one labeled, heat denatured cDNA probe. Each filter was hybridized with this mixture at 42°C for 14 – 16 h with gentle shaking.

Filters were then rinsed in 2x SSC at room temperature for 5 min with gentle agitation, followed by two washing steps with pre-warmed 2x SSC, 1% SDS and pre-warmed 0.6x SSC, 1% SDS solution, respectively at 68°C for 30 min each. The filters were then placed into a PhosphorImager (BioRad, Hercules, CA) for approximately 16 h at a resolution of 50-100 µm. Images were sent to Incyte Genomics for initial analysis using the ArrayVision software

(Imaging Research Inc., St. Catharine, Ontario, Canada). Differentially expressed genes were identified according to the field and position in the filter specified by Incyte Genomics. Normalization of the signals was achieved using as a reference the house-keeping control genes provided on each filter. A change of >1.7-fold was considered significant (1). Filters were stripped according to the manufacturer's instructions, and the hybridization was repeated using labeled probes derived from other animals of the same developmental stage, but in a reverse arrangement.

Supplementary Note 2

Real-time PCR. Total RNA was prepared using TriReagent solution and reverse transcribed utilizing Superscript II reverse transcriptase and random hexamers (Invitrogen, Carlsbad, CA). Real-time PCR was performed essentially as described (2). In brief, after reverse transcribing 200 ng total RNA from the monkey hypothalamus, aliquots of each reaction (10 ng cDNA/μl) were diluted 1:10 before using 2 μl for PCR amplification. Each sample was run in triplicate along with a relative and an absolute standard curve. Relative standard curves, generated by diluting one sample (1:10-1:10,000), served to estimate the content of 18S ribosomal RNA of each sample. The primers used to detect 18s ribosomal RNA were purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit, Perkin Elmer Applied Biosystems, Foster City, CA). Absolute standard curves were constructed by using serial dilutions (1:10) of sense EAP1 mRNA (2 ng-2 ag) transcribed from a cloned monkey (mk) EAP1 cDNA fragment (see below). The threshold cycle number (C_T) from each sample was referred to this curve to estimate the corresponding RNA content, and each RNA value was then normalized for procedural losses using the 18S ribosomal RNA values estimated from the relative standard curve. C_T is the fractional cycle number at which the fluorescence accumulates to a level 10 times greater than 1 SD from basal values. EAP1 primers and the fluorescent probe used were designed to target a segment comprised within the cloned mkEAP1 cDNA described above. They were selected with the assistance of the program, Primer Express (Perkin Elmer Applied Biosystems). The primer sequences (Invitrogen) for mkEAP1 were: 5' forward (5'-

CAAACGGCTTCCCCAAA-3') corresponding to nt 605-621 in mkEAP1 mRNA (GenBank DQ323548) and 3' reverse (5'-GCTGAAGACGAGTTGGGACT-3'), complementary to nt 661-680. The internal fluorescent oligodeoxynucleotide probe (Applied Biosystems) had a sequence (5'-GGGGGTCCCTCCTCCGGTG-3') complementary to nt 626-644 in mkEAP1 and was covalently linked to the fluorescent dye, FAM, at the 5'-end, and to the quencher dye, TAMRA, at the 3'-end. Real-time PCR reactions were performed in a total volume of 10 µl, each reaction containing 2 µl of the diluted reverse transcribed sample or 2 µl of sense RNA standard, 5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM of each gene-specific and ribosomal fluorescent probes, 300 nM of each gene specific primer, and 10 nM of each ribosomal primer. The real-time PCR program used consisted of an initial annealing period of 2 min at 50°C, followed by 10 min of denaturing at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C.

To clone the mkEAP1 cDNA fragment that served as a template for the preparation of mkEAP1 sense mRNA standards, we used primers derived from the human sequence (GenBank NM_024496). The sense primer (5'- CCGCTGCGGTGGAACAGCGCA-3') corresponds to nucleotides (nt) 1384-1404 and the antisense primer (5'-ATACACCCGGGGTACCCCCGA-3') is complementary to nt 1795-1815. The PCR program used consisted of an initial activation step of 15 min at 95°C, and 36 cycles as follows: 1 min of denaturing at 94°C, 1 min of annealing at 52°C and 1 min of extension at 72°C, and a final extension of 10 min at 72°C. This procedure resulted in a single PCR product that was cloned into the plasmid pGEM-T (Promega, Madison, WI), and sequenced from both ends to verify its identity.

Semi-quantitative PCR

To quantify rat (r)EAP1 mRNA, we used a semi-quantitative PCR procedure described in detail elsewhere (3). Prior to carrying out the amplification procedure, optimal primer concentrations for amplification of EAP1 (1 µM) and cyclophilin (0.6 µM) cDNAs were determined, in addition to the linear range of the PCR reactions. Cyclophilin mRNA is constitutively expressed and thus serves as an internal standard. The sense EAP1 primer

used (5'-AGCCCCAACTCATCCTCAG-3') corresponds to nt 935-953 in rEAP1 mRNA (GenBank AY879229); the antisense primer (5'-ACGCTCCTGGTCTGTGCTC-3') is complementary to nt 1297-1315. The sense cyclophilin primer (5'-ACGCCGCTGTCTCTTTTC-3') corresponds to nt 9-26 in rat cyclophilin mRNA (GenBank M19533) and the antisense primer (5'-CTTGCCACCAGTGCCATTAT-3') is complementary to nt 251-270. The PCR program employed consisted of an initial activation step of 15 min at 95°C, and 34 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec and 1-min extensions at 72°C, followed by a final extension of 10 min at 72°C.

To quantify mouse (m) EAP1 mRNA, we used the same semi-quantitative PCR procedure indicated above. The sense EAP1 primer used (5'-AGCCCCAACTCATCCTCAG-3') corresponds to nt 1033-1051 in mEAP1 mRNA (XM_977351); the antisense primer (5'-ACGCTCCTGGTCTGTGCTC-3') is complementary to nt 1395-1413. The sense cyclophilin primer (5'-GGCGACTTCACCAACCACAA-3') corresponds to nt 378-397 in mouse cyclophilin mRNA (NM_134084) and the antisense primer (5'- AACATCCATGCCCTCTTTGAC-3') is complementary to nt 579-599. The PCR program employed consisted of an initial activation step of 5 min at 95°C, and 32 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and 1-min extensions at 72°C, followed by a final extension of 10 min at 72°C.

Supplementary Note 3

***In situ* hybridization and immunohistochemistry.** *In situ* hybridization. The monkey brain sections used were provided by Dr. Cynthia Bethea (ONPRC) from animals utilized in an unrelated study (4). The brains were fixed by intracardiac perfusion of 4% paraformaldehyde-borate buffer, pH 9.5 (3). Both solutions were made with 0.1% diethyl pyrocarbonate treated water to minimize RNAse contamination. After dissection of the brain, tissue blocks were postfixed in 4% paraformaldehyde for 3 h, and transferred to 0.02 M potassium phosphate-buffered saline (KPBS) containing 20% glycerol and 2% dimethyl sulfoxide (DMSO) at 4°C for 3 days to cryoprotect the tissue. After infiltration the blocks were frozen in isopentane cooled to -55°C, and stored at –80°C until sectioning. The sections (25 μm) were cut on a sliding

microtome, mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), dehydrated under vacuum overnight and then frozen at –80°C until processing for hybridization. Every tenth section (each 250 μm) was stained with hematoxylin for morphological reference and anatomical orientation (5). The hybridization procedure was performed as recommended by Simmons et al. (6), and described by us (3, 7), using an ^{35}S-UTP-mkEAP1 labeled cRNA probe transcribed from the mkEAP1 cDNA described in Supplementary Note 2. Control sections were incubated with a sense probe transcribed from the same plasmid, but linearized on the 3' end to transcribe the coding strand of the cDNA template.

Following an overnight hybridization at 55°C, the slides were washed and processed for cRNA detection (3, 7). After dehydration, the slides were dipped in NTB-2 emulsion, and were exposed to the emulsion for three weeks at 4°C. At this time the slides were developed, counterstained with 0.1% thionin, quickly dehydrated in ascending concentrations of alcohol, and coverslipped for microscopic examination.

Immunohistochemistry. To identify the cells and cellular sites containing EAP1 protein in the monkey and rat hypothalamus we developed rabbit polyclonal antibodies against EAP1 (Sigma Genosys, The Woodlands, TX). These antibodies recognize 14 unique amino acids (AA) at position AA 338 – AA 351 in the human EAP1 protein. Extensive search of GenBank databases did not identify any other peptide with a potentially cross-reacting sequence. Preadsorption of the affinity purified EAP1 antibodies used for immunohistochemistry with the antigenic peptide (10 μg/ml antiserum, overnight at 4°C) eliminated all staining from sections of the rat brain. Frozen sections (30 μm) derived from the brain of immature 30-day-old rats (perfusion-fixed with 4% paraformaldehyde- PBS, pH 7.4) and some of the 25 μm tissue sections from the monkey hypothalamus collected for *in situ* hybridization were incubated overnight at 4°C with EAP1 antibodies diluted 1:8,000. The next day, the sections were incubated with a biotinylated donkey antirabbit gamma globulin (1:250, Vector Laboratories, Burlingame, CA) for 1 h at room temperature, followed by incubation in AB complex (Vector Laboratories) for another hour. The immunohistochemical reaction was then developed to a brown color with 3,3'-diaminobenzydine-HCl (DAB) and 0.005% H_2O_2.

Combined immunohistochemistry/in situ hybridization. To determine if EAP1 is expressed in preproenkephalinergic neurons of the rat hypothalamus we used a combined IHC/ISH procedure (8, 9). After completing the EAP1 immunohistochemical procedure, the sections were mounted on glass slides and dried overnight under vacuum prior to hybridization with an ^{35}S-UTP-labeled preproenkephalin cRNA probe (10). All reagents used for the immunohistochemical procedure were prepared in DEPC-treated water.

Immunofluorescence-Confocal Microscopy. To detect the presence of EAP1 in GnRH neurons, we used 30 μm frozen sections obtained from 28-30 day-old female rats. The sections were incubated with EAP1 antibodies (1:2500) and mouse monoclonal antibodies against GnRH (11) (1:2000). The EAP1 reaction was developed with biotinylated donkey antirabbit immunoglobulins (1:250) followed by Alexa 488-Streptavidin (Invitrogen; 1:400); the GnRH reaction was developed with Texas Red-labeled goat antimouse IgG (1:250).

To verify the anatomical site of the intrahypothalamic injections carrying lentiviruses, we perfusion-fixed the brain of the injected rats 40-42 days after the initial infection with 4% paraformaldehyde- PBS pH 7.4, blocked a portion of the brain containing the POA and hypothalamus, and serially sectioned the blocks at 30 μm intervals. The sections were then incubated overnight at 4^{o} C with rabbit polyclonal antibodies against GFP (Invitrogen; 1: 800 dilution), and the reaction was developed the next day with Alexa 488 donkey antirabbit IgG (Invitrogen, 1:250). In some sections, GnRH neurons were also identified using mAb HFU 4H3 (11) diluted 1:3000, followed by Alexa 594 chicken antimouse gamma globulin (Invitrogen;1:250).

Supplementary Note 4

Functional promoter assays and plasmid constructs. *Promoter assays.* To determine GnRH promoter activity we used a construct (kindly provided by P. Mellon, University of California, San Diego) containing a hybrid fragment of the rat GnRH gene 5' flanking region (12) consisting of the enhancer region (-1863 to -1571) and the proximal promoter (-173 to +112) of the gene cloned into the luciferase reporter plasmid pGL-3 Basic (Promega). In the

case of the preproenkephalin promoter, we used a reporter construct prepared by subcloning a 2.7-kb *Hin*cII DNA fragment containing nt -2495 to +207 (including the first intron) of the human preproenkephalin gene (kindly provided by L. Kobierski, Harvard Medical School, and K. Van Koughnet, NINDS, NIH) cloned into pGL-2 Basic. The transregulatory effects of EAP1 (or EAP1 mutants) on preproenkephalin gene activity were examined in the rat hippocampal neuronal cell line HiB5 (13) and the mouse GnRH-secreting cell line GT1-7 (14). The latter was used to detect EAP1 effects on GnRH promoter activity. The procedure employed for these assays has been described in detail (15). In brief, 24 h after seeding 300,000 cells/well in six-well plates, each of the reporter plasmids (500 ng/ml for GnRH-Luc-pGL3 and 250 ng/ml for proEnk-Luc-pGL2) was transiently transfected for 5 h using Lipofectamine (Invitrogen) in conjunction with (500 ng/ml) of an expression vector (pcDNA3.1Zeo, Invitrogen) containing either the coding region of human EAP1 or a mutated EAP1 construct. One of these mutants lacks the RING finger domain; in the other, the highly conserved first cysteine residue of the RING finger domain (amino acid 715) was replaced with a alanine. Transfection efficiency was normalized by cotransfecting the plasmid CMV-Sport-β -gal (Invitrogen) at 20 ng/ml. The total amount of DNA transfected was kept constant at 1 μg by adding the appropriate amount of pcDNA3.1Zeo to each well. The cells were harvested 48 h after transfection and assayed for luciferase and β-gal, as reported (15).

Plasmid constructs. To prepare an expression vector containing the coding region of human EAP1 mRNA, we PCR-amplified this DNA segment from a BAC library clone (No.2289B16, generously provided by Dr. Lee Rowen, Institute for Systems Biology, Seattle, WA). The 5' sense primer used (5'-TATAGGATCCTACCTTCCCCAGGGCAGGCA-3') corresponds to nt 878-897 in the human EAP1 mRNA sequence (NM_024496), and contains a Bam HI site at its 5' end. The antisense primer (5'-TATACTCGAGGCAAAGGAGGTGGCTGCCCAGT-3') is complementary to nt 3294-3315 in EAP1 mRNA and contains a Xho I site at its 3' end. The PCR reaction was performed using the FailSafe PCR PreMix selection kit (Epicentre, Madison, WI) following the manufacturer's recommendations. The single PCR product obtained was first cloned into pGEM-T

(Promega), before excising it by Bam HI-Xho I digestion and subcloning it into the BamHI-Xho I sites of the expression vector pcDNA3.1Zeo (Invitrogen). The sequence of the EAP1 cDNA was verified by automatic sequencing.

To test the importance of the RING finger domain for EAP1 functions, we prepared two EAP1 mutants, one lacking the entire domain, and the other carrying an inactivating point mutation. The human EAP1-pGEM-T construct described above was used as the template. The RING finger domain was deleted by first introducing a NruI restriction site (QuikChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) immediately upstream from the 5' end of the domain .The forward mutagenesis primer used (5'-CCAACAGCGGACCCCTCTTCGCGAGCTGCACCATTTGCCACG-3'), corresponding to nt 3022-3057 in human EAP1 mRNA (NM_024496). The NruI restriction site (underlined) was introduced at nt 3039. The reverse mutagenesis primer had a sequence complementary to the forward primer. Since there is a NruI restriction site downstream from the 3' end of the domain in wild-type EAP1 mRNA (nt 3238-3243), digestion with NruI and religation of the NruI-NruI plasmid resulted in removal of a 201bp fragment containing the RING finger domain, but maintaining the EAP1 sequence in frame. The mutation was verified by sequencing, and the mutated EAP1 cDNA was removed from pGEM-T and subcloned into pcDNA3.1Zeo, as outlined above. To generate an inactivating point mutation, the bases TG at nt 3042-3043 were substituted by GC (underlined below) resulting in conversion of the first cysteine in the RING finger domain into an alanine. The forward mutagenesis primer used (5'-AACAGCGGACCCCTCTGCGCCACCATTTGCCACGAACGT-3) corresponds to nt 3024 to 3062 in human EAP1 mRNA. As before, the antisense mutagenesis primer is complementary to the forward primer.

Supplementary Note 5

siRNA preparation and testing of siRNA biological activity. We resuspended single-stranded siRNA oligonucleotides purchased from Ambion in RNase-free water to a final concentration of 200 μM. The sense and antisense siRNA strands were then heated 90°C for

1 min, and annealed for 60 min at 37°C in a volume that resulted in a 20 μM concentration of double stranded siRNA. The biological activity of three siRNAs was tested in rat hippocampal HiB5 cells, which express EAP1 endogenously (Supplementary Figure 1). The cells were seeded at 500,000 cells/well in 6-well plates in DMEM (Sigma, St. Louis, MO), plus 10% fetal calf serum (HyClone Laboratories, Logan, UT), without antibiotics. Twenty-four h after seeding, we transfected the cells with each of the siRNAs using Lipofectamine 2000 (Invitrogen) in Optimem (Invitrogen). For each well, 5 μl Lipofectamine 2000 were added to 250 μl Optimem; after 5-min incubation at room temperature, siRNA diluted in 250 μl Optimem was added and the mixture was incubated for additional 20 min at room temperature. At this time, the volume was increased to 1 ml with Optimem and the solution was applied to the cells, which had been washed first with PBS and Optimem. Final siRNA concentration in each well was 100nM per well. Total RNA was extracted 48 h later for measurement of EAP1 mRNA using semi-quantitative PCR. The PCR procedure employed is outlined in Supplementary Note 2.

Supplementary Note 6

Functional analysis of siRNA specificity. For siRNAs to inhibit the expression of a particular target gene, the antisense strand has to be completely complementary to the target mRNA (16). To determine the importance of this requirement, we performed a “rescue” experiment (www.nature.com/horizon/rna/highlights/s4_spec1.html) in which the sequence in EAP1 mRNA targeted by EAP1 siR1 and 3 was subjected to site-directed mutagenesis (Quick change XL-site directed mutagenesis kit, Stratagene) to change the third base of one or two codons. This procedure changes the nucleotide sequence of the mRNA, but maintains the protein sequence, i.e., it generates a silent substitution. The following mutations were introduced (with the location of the mutation referring to the rat EAP1 sequence AY879229 and using rat EAP1 pcDNA3.1Zeo as the template): In the mRNA region targeted by siR1 the first mutant consisted of exchanging a G to A at position 1318, resulting in no change of the corresponding amino acid glutamine. The second mutant contained two mutations, an

exchange of T to C at position 1315 and of A to T at position 1321,resulting in no change of the corresponding amino acids arginine and leucine, respectively. The region targeted by siR3 was mutated as follows: the first mutant contains an exchange of G to A at position 1624 and the second mutant an additional exchange of C to T at position 1630. As before, neither mutation changed the wild-type amino acid sequence in this region. All four mutations were verified by sequencing.

To construct a rat EAP1 expression vector for the above outlined rescue experiments, we PCR-amplified the coding region of rat EAP1 from RNA of HiB5 hippocampal cells using a sense primer (5'-TATAGGATCCTACCTTCCCCGAGGCTGGCA-3') corresponding to nt 307-326 in rat EAP1 mRNA (AY879229), and containing a Bam HI site at its 5' end, and a reverse primer (5'-TATACTCGAGGCAAAGGAGGTCGCTGCCCTGCG-3'), complementary to a segment starting five nt downstream from the EAP1 stop codon, which is complementary to nt 6883-6905 in Rattus norvegicus 6 WGS supercontig (NW_0477621.1). This primer contained a Xho I site at its 3' end. The PCR product was first subcloned into pGEM-T before insertion into the Bam HI-Xho I sites of pcDNA3.1Zeo. The sequence of the entire full-length EAP1 cDNA was verified by sequencing.

To test the effectiveness of siR1 and siR3 to decrease wild-type and mutated rat EAP1 mRNA levels we used monkey COS-7 cells, which do not express EAP1. The cells were grown at 37°C, 5% CO_2 in DMEM (Sigma) containing 10% fetal calf serum (HyClone), penicillin G (100 U/ml, Sigma) and streptomycin sulphate (100 µg/ml, Sigma). We seeded 150,000 cells/well into 6-well plates and transfected them 24 h later with 100 ng/ml of wild-type or mutant EAP1 plasmid DNA using Lipofectamine 2000. Four hours later, the incubation medium was removed and replaced with siRNA-Lipofectamine 2000 transfection solution, as described in Supplementary Note 4.

Total RNA was extracted 48 h after the siRNA transfection and rat EAP1 mRNA was PCR- amplified as described in Supplementary Note 2, but using 28 instead of 34 cycles. The same rat EAP1 primers described above were used, but in conjunction with monkey- specific cyclophilin primers. The sense cyclophilin primer (5'- CAGGGTTTATGTGTCAGGGTGGTG-

3') corresponds to nt 173-196 in mk cyclophilin mRNA (GenBank NM_001032809), and the reverse primer (5'-ATGGTGATCTTCTTGCTGGTCTTG-3') is complementary to nt 450-473. The PCR product is 301 bp in length.

Supplementary Note 7

Assessment of a lentivirus EAP1 shRNA-induced interferon response. To determine if the rat hypothalamus responds to infection with lentiviral constructs encoding rEAP1 shRNAs with an interferon response (17, 18), we quantified the mRNA content of the mRNA encoding 2'5'-oligoadenylate synthetase (OAS1), a major interferon target (18) in hypothalamic slices infected with EAP1 sh1. Horizontal slices (200 µm each) were prepared from the medial basal hypothalamus-POA area region of 23-day-old female rats using a vibrotome (Leica VT 1000 S, Nussloch, Germany). The slices (three per animal) were collected into chilled Hanks' balanced salt solution (Invitrogen) enriched with 4.5 g/l glucose and 20 mM HEPES. Thereafter, they were placed onto 12 mm diameter porous (3 µm pore) collagen-coated trans-wells (Fisher Scientific) that were inserted into 12-well plates containing 500 µl of Neurobasal-A medium (Invitrogen) plus 1X B27 (Invitrogen), used as serum replacement. Thereafter, the slices from each rat were assigned to one of three groups (three rats per group): non-infected control, infected with LV-GFP, and infected with EAP1 sh1. The concentration of virus added to each well was $4x10^7$ TU/ml. After a 72 h incubation at 37° C-5% CO_2, RNA was extracted, quantified using the Ribogreen quantitation system (Invitrogen) and its integrity was verified using the RNA 6000 nano-assay method (Agilent Technologies, Waldbronn, Germany). OAS1 mRNA levels were determined by semi-quantitative PCR, using cyclophilin mRNA as the normalization unit. The PCR products were separated in a 2% agarose gel stained with ethidium bromide and the relative optical density of each band was estimated using the Quantity-One software (BioRad, Hercules, CA) before calculating OAS/cyclophilin ratios. The sense OAS1 primer (5'-TACAGCAATCCTGATCCCAAGA-3') corresponds to nt 538-559 in rat OAS1 mRNA (NM_138913); the antisense primer (5'-GAGCTCCGTGAAGCAGGTAGA-3') is complementary to nt 613-633. The primers used for amplification of cyclophilin were those

shown under Supplementary Note 2. The PCR program used consisted of an initial activation step of 15 min at 95°C, followed by 36 cycles of 30 seconds of denaturing at 94°C, 30 seconds of annealing at 55°C and 1 min of extension at 72°C, followed by a final extension of 10 min at 72°C.

Supplementary Note 8

Intrahypothalamic injections of lentiviral particles. The procedure employed has been previously described (19). In brief, the animals were positioned in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA; incisor's bar at +5 mm), and a total volume of 1 µl of LV-eGFP or EAP1 sh1 was injected bilaterally into the POA using a 10 µl Hamilton microsyringe (parameters: +2.4 mm rostral from bregma, +/- 0.4 mm lateral from midline, -8 mm ventral from the surface of the skull). Each injection administered in two steps as follows: 0.5 µl of viral suspension was injected over a period of 30 seconds, then the syringe was withdrawn 0.2 mm and left there for 1 min. Thereafter, the syringe was raised 0.3 mm and an additional volume of 0.5 µl was injected over a period of 30 seconds. Again, the syringe was withdrawn 0.2 mm and left there for an additional 2 min before being slowly removed over a 1 min interval.

Supplementary Note 9

Imaging and quantitation of EAP1 immunoreactivity. Fluorescent images were acquired with either a Leica TCS SP confocal microscope as described (20, 21), or using a Marianas™ digital imaging workstation (Intelligent Imaging Innovations, Denver, CO) with a 40x C-apochromat NA1.2 objective. The Marianas workstation is equipped with a Zeiss Axiovert 200M microscope (Zeiss, Thornwood, NY) and a motorized stage (API, Eugene, OR).

To define the intracellular localization of EAP1, neurons were imaged using a 63x, PlApo NA1.4 objective and a Coolsnap HQ (Roper Scientific, Tucson AZ) camera. Two color, three-dimensional stacks of images 250 nm apart were deconvoluted using Slidebook 4.0 software (Intelligent Imaging Innovations, Denver, CO).

To quantify the changes in cellular EAP1 immunoreactivity after *in vivo* injections of lentiviral particles encoding EAP1 sh1, frozen sections (30 μm) from injected brains, fixed as indicated in Supplementary Note 3, were stained with EAP1 polyclonal antibodies (diluted 1:8,000). After an overnight incubation at 4^{o}C, the reaction was developed as indicated in Supplementary Note 3, and visualized by laser confocal microscopy as indicated above. eGFP-positive cells were identified by detecting the natural eGFP fluorescence using a standard fluorescein filter. Fluorescence images were acquired using the Marianas^{TM} digital imaging workstation with a 40xC-apochromat NA1.2 objective. Approximately 4 fields per section were imaged, covering the area where the injection site could be identified by the appearance of eGFP fluorescence. Ten-micron thick stacks of images, 0.5 μm apart, were sum projected in one plane and masks were drawn over neuronal nuclei labeled with Hoechst, that were completely contained within the stack. An area devoid of cells was selected for background measurement in each field. After background subtraction, the mean intensity of each channel was measured for each selected nucleus, using Slidebook 4.1 and transferred to Excel (Microsoft, Redmont, WA) for statistical analysis. Cells were grouped into eGFP positive and negative, depending on the intensity of the fluorescence in the green channel. The level of EAP1 expression was estimated as the mean red fluorescence intensity for nuclei in each group. The mean Hoechst fluorescence was also measured as a control. We used two sampling protocols with similar results: a) systematic uniform random sampling and b) cells within the injection site compared to cells in a region close to but excluding the injection site. The rationale behind the b) protocol was to ensure that cells that expressed eGFP, but at levels below detection, were not inadvertently included in the control group.

Supplementary Figures

Supplementary Figure 1. Selection of EAP1 siRNA sequences for subsequent construction of U6-promoter-directed EAP1 shRNA transcriptional cassettes. a, Of three siRNAs transfected into hippocampal neuronal HiB5 cells (Supplementary Note 5), siR1 and siR3 were similarly effective in decreasing EAP1 mRNA levels 48 h after transfection (n = 3/group).

b, The suppressive effect of these siRNAs, shown for siR3, requires absolute sequence identity to the intended target region in EAP1 mRNA. Plasmid constructs encoding wild-type or mutant EAP1 mRNAs (see below) were co-transfected with siR3 into COS-7 cells (Supplementary Note 6), and the levels of the resulting mRNA transcripts were detected 48 h later by semi-quantitative PCR. The cellular content of mutant EAP1 mRNAs carrying silent substitutions of the third base of either one (3M1) or two codons (3M2) within the region targeted by siR3 remained unaffected after siR3 treatment.

Supplementary Figure 2. Production of lentiviruses. Viral titer was determined based on the expression of eGFP as detected by flow cytometry. Naïve 293T cells were infected with viral stocks, and expression of eGFP was detected by fluorescence activation (excitation at 488 nm, reading at 530 nm) in a Becton-Dickinson FACSCalibur (BD Biosciences, San Jose, CA). **a,** Histogram of fluorescence reading from non-infected cells. Fluorescence of 10,000 cells was read over 4 orders of magnitude. The counting window M1 was set such that 0.18% of the naive cells were recorded as positive. **b,** Photomicrograph of naïve, non-infected cells. **c,** Histogram of eGFP fluorescence of cells infected with EAP1 sh1. Counting window M1 remained as in frame a, showing that 51.65% of the cells were eGFP-positive. **d,** Photomicrograph of eGFP-positive 293T cells. Serial dilutions of viral stocks were used to calculate titers. In this example the viral titer was determined to be $3.6x10^6$ TU/ml.

Supplementary Figure 3. The absence of leptin does not affect hypothalamic EAP1 mRNA levels in adult mice. **a,** Assessment of the number of PCR cycles required for accurate semi-quantitative determination of EAP1 mRNA levels in the mouse hypothalamus. Thirty two cycles were found to generate an optimal non-saturated signal for both EAP1 and the house-keeping gene cyclophilin. **b,** EAP1 mRNA levels are identical in the MBH of wild-type C57/B6 mice and that of *ob/ob* mice lacking leptin. Numbers on top of the gel image indicate number of animals per group. MM = molecular marker.

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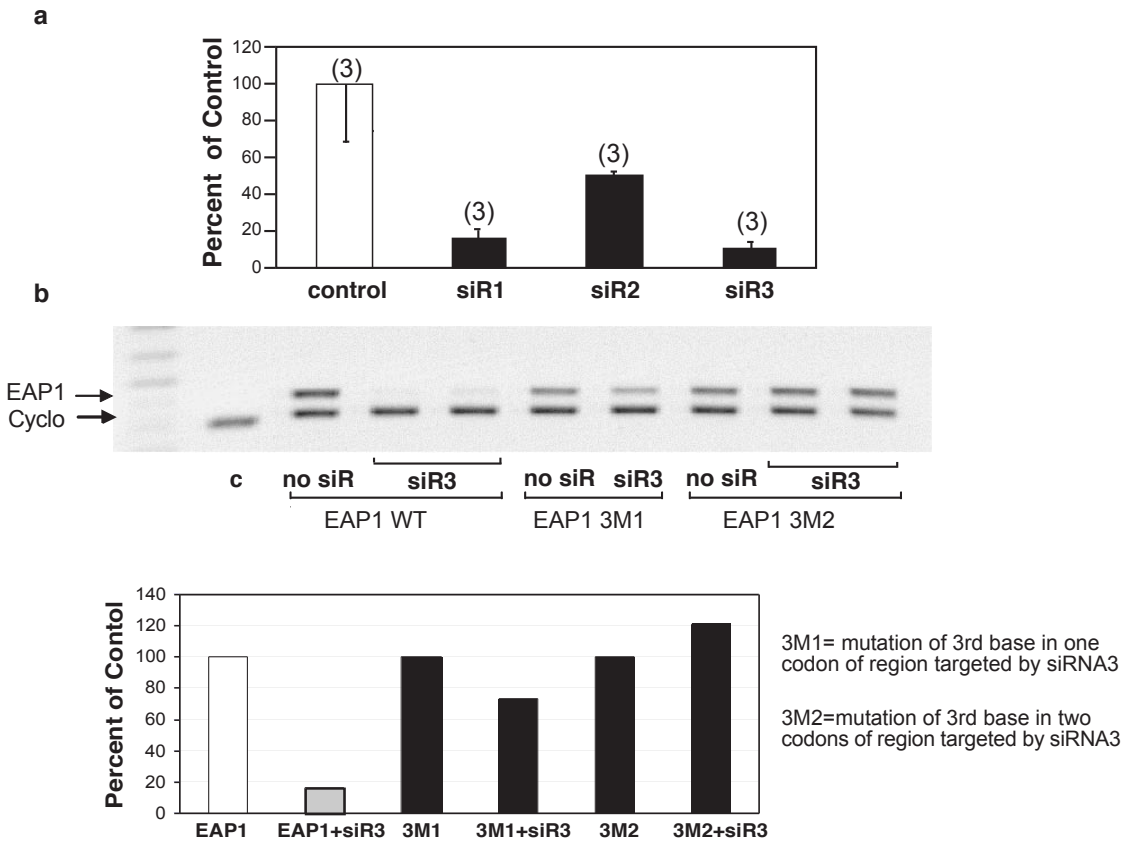
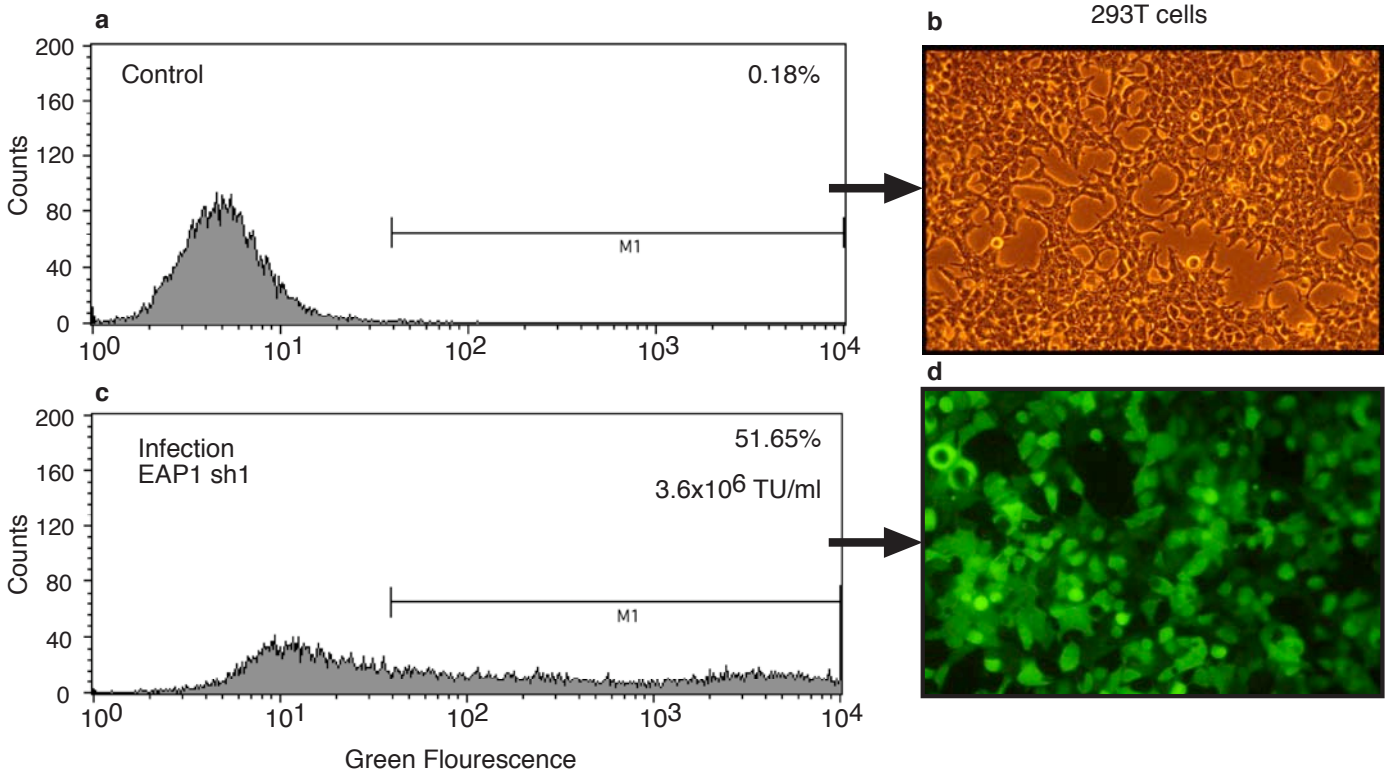
Suppl Fig. 1


a
Percent of Control
120
100
80
60
40
20
0
(3)
(3)
(3)
(3)
control
siR1
siR2
siR3
b
EAP1
Cyclo
c
no siR
siR3
no siR
siR3
no siR
siR3
EAP1 WT
EAP1 3M1
EAP1 3M2
Percent of Contol
140
120
100
80
60
40
20
0
EAP1
EAP1+siR3
3M1
3M1+siR3
3M2
3M2+siR3
3M1= mutation of 3rd base in one codon of region targeted by siRNA3
3M2=mutation of 3rd base in two codons of region targeted by siRNA3

Suppl. Fig. 2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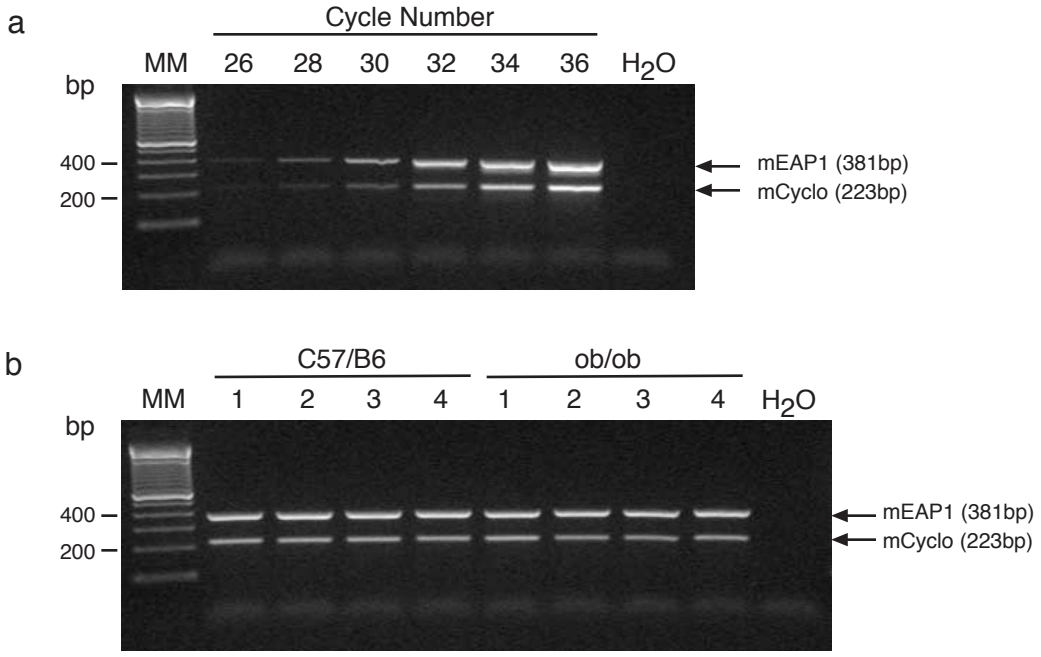
160
120
80
40
0
M1
10^0
10^1
10^2
10^3
10^4
b
293T cells
c
Infection
EAP1 sh1
51.65%
$3.6x10^6$ TU/ml
Counts
200
160
120
80
40
0
M1
10^0
10^1
10^2
10^3
10^4
Green Flourescence
d

Suppl Fig. 3


a
Cycle Number
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28
30
32
34
36
H2O
bp
400
200
mEAP1 (381bp)
mCyclo (223bp)
b
C57/B6
ob/ob
MM
1
2
3
4
1
2
3
4
H2O
bp
400
200
mEAP1 (381bp)
mCyclo (223bp)