Elia et al. Supplemental Data

Supplemental Experimental Procedures

Construction of the targeting vector

BAC clones containing *p120ctn* genomic DNA from mouse strain 129 were isolated by Genome Systems, Inc. (St. Louis, Missouri). A 7.3kb genomic fragment containing exons 5-9 was amplified by PCR using the Herculase polymerase (Stratogene), cloned into pCR-XL-TOPO (Invitrogen), sequenced to confirm that PCR amplification did not introduce sequence changes, and used to construct the *p120ctn* targeting vector (**Figure 1A**). A 46 bp fragment containing a 34 bp *loxP* site and linker sequences was inserted into a *BamHI* site in the intron 5' of exon 6. A plasmid, pSL1180neoX, was constructed that contains the *PGKneo* expression cassette flanked by two *FRT* sites (5'-GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC-3'), and containing a *loxP* site that was inserted upstream of the *FRT* site at the 5' end of the *neo* cassette. The DNA fragment containing the flrted *neo* cassette and *loxP* site was inserted into a *PacI* site 79 bp into the intron DNA 3' of *p120ctn* exon 7. The targeting vector contained 3.5 kb of homologous DNA (left arm) upstream of the first *loxP* site and 2.6 kb of homologous DNA (right arm) downstream of the *PGKneo* cassette.

Generation of the p120ctn floxed allele

The targeting vector was linearized with *NotI* and electroporated into 129SVJ mouse embryonic stem cells. G418-resistant colonies were picked, expanded, and screened for homologous recombination by Southern blot analysis of *StuI* digested ES cell DNA using 5' and 3'probes containing sequences outside of those in the targeting vector. Homologous recombinant ES cells were injected into C57Bl/6 blastocysts and chimeric male mice were obtained which were mated to C57Bl/6 females to establish a mouse line carrying the $p120ctn^{flox\text{-}neo}$ allele. The *PGKneo* cassette was removed by mating F1 heterozygous mice to mice expressing the FlpE recombinase. *p120ctn* conditional knockout mice and their control littermates were obtained by breeding $p120ctn^{flox}$ mice

to homozygosity and then mating male *p120ctn$^{flox/flox}$* mice to female *p120ctn$^{flox/flox}$* mice expressing *emx1^{IREScre}*.

PCR genotyping

Conditional *p120ctn$^{flox/flox}$* mice were genotyped routinely by isolating genomic tail DNA and performing PCR with the following primers: P1(LE5lox1): 5'-AGGGAGAGAGTTCAGTTGGTGAAATG-3' and P2(LE13): 5'-CCTCTTCACCAATCATGTCTTCATAGCT-3'. The 400 bp wild-type and 440 bp *p120ctn^{flox}* bands were resolved on 1.5% agarose gels.

Western blotting, immunocytochemistry, and Nissl staining

Protein extracts were prepared from the hippocampi of *p120ctn$^{flox/flox}$* and *p120ctn$^{flox/flox}$;emx1^{IREScre}* mice (n=3 pairs). 20 μg of protein per lane were run on SDS-PAGE, followed by blotting with indicated antibodies.

For immunohistochemistry, anaesthetized animals were perfused with 0.9% saline followed by 4% paraformaldehyde in PBS and a series of 15-30% sucrose-PBS solutions. 50 μm serial coronal sections were cut in a sliding microtome. Littermates were processed in parallel in each experimental group (n=3). Free-floating sections were quenched with 10% methanol/3% H_2O_2, incubated in 5% BSA, 0.3% Triton X-100 in PBS for 2 h at room temperature, and incubated for 36 h at 4^{o}C with a monoclonal antibody against p120ctn (Transduction labs) diluted in 1% BSA, 0.3% Triton X-100 in PBS. Sections were rinsed in PBS and incubated with the appropriate biotinylated secondary antibody (Vector) diluted in 1% BSA and 0.3% Triton X-100 in PBS. Sections were processed for diaminobenzidine detection following the manufacturer's protocol (Vector). Following primary antibody incubations, sections were rinsed as above and incubated with the appropriate secondary antibodies: mouse or rabbit Alexa 488 or Texas Red goat antibody (Molecular Probes) diluted in 1% BSA and 0.3% Triton X-100 in PBS. Nissl-stained sections were dehydrated overnight in 70% ethanol prior to staining with 0.1% Cresyl Violet/0.5% acetic acid. Sections were then rinsed in dH_2O

followed by 70% and 95% ethanol, and chloroform, and differentiated with 1.7% acetic acid in 95% ethanol.

Hippocampal cultures and transfection

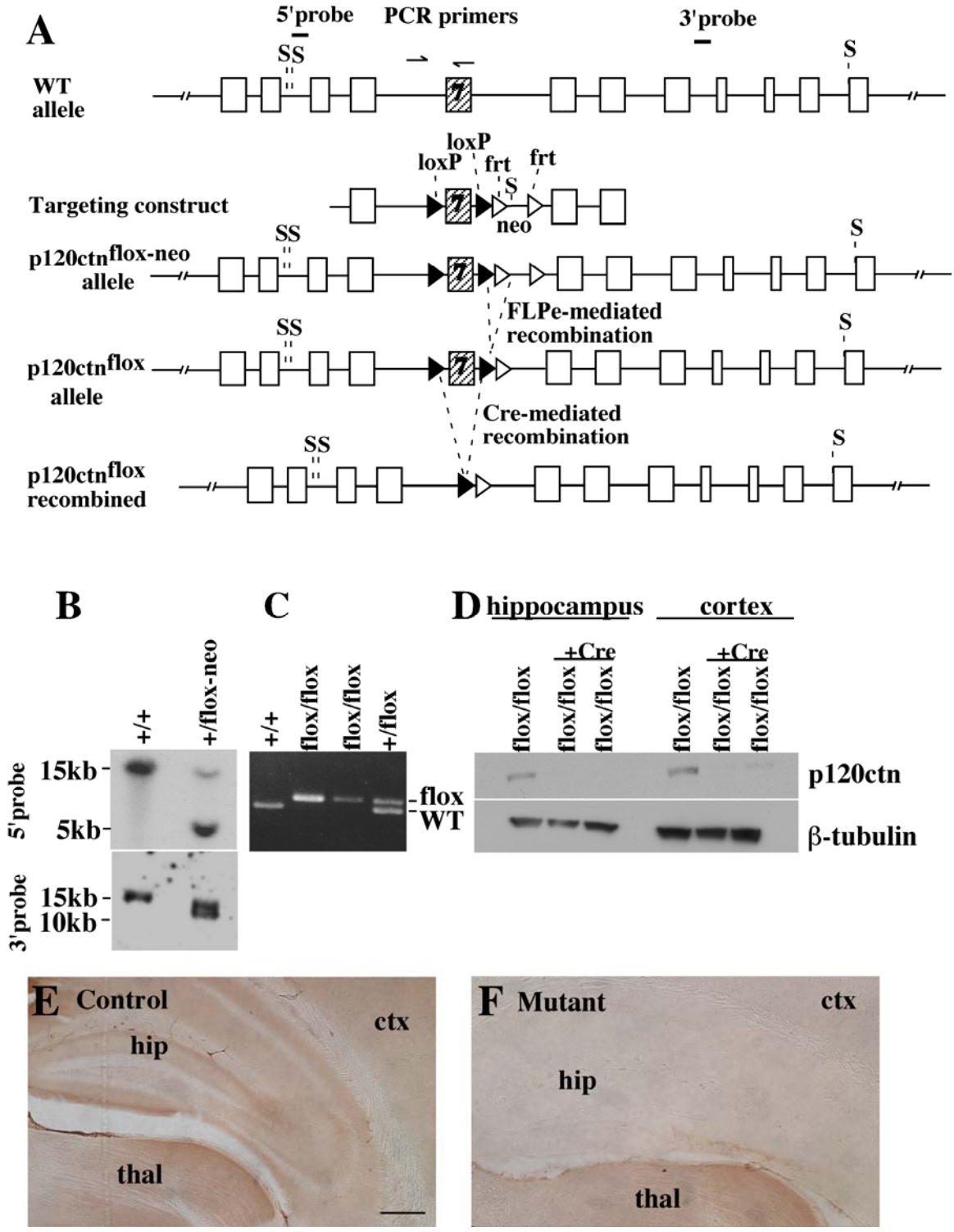
Hippocampal neurons were prepared from E18 fetal rats or E17 fetal mice and plated on poly-L-lysine-coated cover glasses at the density of 130 cells/mm^2 and maintained in NeuroBasal medium (Invitrogen) with B27 supplements (Gibco). cDNA transfection was performed using Effectine (QIAGEN) as indicated. For protrusion quantification, serial confocal images (Z steps of 0.5 μm) were taken with a Zeiss 40X plan-neofluar objective (n.a.=1.3, oil) with a digital zoom factor of one for low magnification or of four for high magnification images from a proximal 50-70 μm region in two or three of the largest dendrites in each neuron. Sections were merged using NIH Image, and the number of spines and filopodia counted. Spines were defined as headed dendritic protrusions up to 2 μm and filopodia as headless protrusions > 2 μm. Dendrite length was measured using NIH Image. Protrusion length was manually measured from base to tip; head width as the maximum width at the enlarged head portion or tip of each protrusion. Measured data were exported to Excel software and compared by using Student's t test.

For immunofluorescence, neurons were fixed in 4% paraformaldehyde, 4% sucrose in PBS, washed, permeabilized with 0.1% Triton X-100, and blocked with 10% normal goat serum and 1% BSA in PBS for 1h at room temperature. Neurons were incubated overnight at 4^{o}C with primary antibodies to rabbit synaptophysin (Zymed), mouse PSD-95 (D. Bredt), or N-cadherin (D. Coleman). Goat anti-mouse or anti-rabbit Alexa488 or Texas Red goat antibodies (Molecular Probes) were used for visualization.

Constructs were from the following sources: p120ctn1A, A. Reynolds; p120ctnΔ622-628 and p120ctnΔarm3, P. Anastasiadis; C3 transferase, R. Yuste; Rac1-G12V and RhoA-G14V, H. Bourne; PSD95-GFP, D. Bredt; N-cadherin, D. Coleman; pEGFP-N1, Clontech.

RhoA and Rac1 activity assays

GTP-bound RhoA and GTP-bound Rac1 were affinity purified from hippocampal lysates using glutathione beads coated with GST fusions of the RhoA binding domain of Rhotekin and the Rac1 binding domain of PAK, respectively. Bound proteins were resolved on 15% SDS-PAGE, after which anti-RhoA (1:250; Santa Cruz) and anti-Rac1 (1:1000; BD Transduction Laboratories) were used to quantitate active protein quantities. Aliquots of the hippocampal lysates were fractionated by SDS-PAGE and blotted with the same antibodies to determine total RhoA and Rac1. For visualization immunoblots were probed with the ECF Western blotting kit according to the manufacturer's protocol (Amersham Pharmacia Biotech). Samples were quantified using a phosphoimager and values of active RhoA and Rac1 were normalized to the total amount of each protein.


A
5'probe
PCR primers
3'probe
SS
S
WT allele
loxP
loxP
frt
frt
S
neo
Targeting construct
p120ctnflox-neo allele
FLPe-mediated recombination
p120ctnflox allele
Cre-mediated recombination
p120ctnflox recombined
B
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3'probe
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+/+
flox/flox
flox/flox
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cortex
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flox/flox
flox/flox
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p120ctn
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Supplemental Figure 1. Targeting strategy for conditional inactivation of the *p120ctn* gene and loss of p120ctn protein following forebrain-specific deletion of *p120ctn*

(A) Schematic diagram of targeting strategy depicting construction of a series of *p120ctn* mutant alleles. The mouse genomic locus (WT allele) is shown as a horizontal line with exons indicated by boxes. The positions of the 5'and 3' flanking probes and the *Stu1*(S) restriction sites used for target selection by Southern blot analysis and the positions of the primers (P1 and P2) used for PCR genotyping are indicated. The targeting construct contains exon 7 flanked by *loxP* sites and a *neo* selection cassette (placed in the same orientation relative to *p120ctn* transcription) flanked by *FRT* sites. Gene replacement at the *p120ctn* locus in ES cells produced the *p120ctn* $^{flox\text{-}neo}$ allele. Mice that had incorporated the targeting construct into the germline were mated to mice expressing *FlpE* under the ubiquitous *β-actin* promoter; the resulting removal of the *neo* selection cassette produced mice containing the *p120ctn* ^{flox} allele. Mating of mice homozygous for the *p120ctn*^{flox} allele (*p120ctn* $^{flox/flox}$) to *emx1*^{IREScre} expressing mice resulted in the dorsal forebrain-specific deletion of *p120ctn*. Deletion of exon 7 introduces a frameshift into the p120ctn coding sequence resulting in premature translation termination, which is expected to result in mRNA degradation.

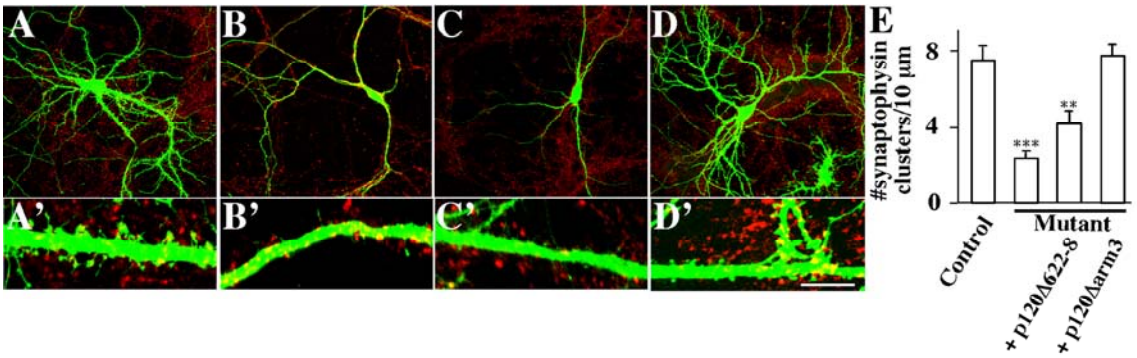
(B) Southern blot analysis of *p120ctn* $^{flox\text{-}neo}$ heterozygous mice was performed on tail DNA digested with Stu1 and detected using the 5' and 3' probes shown in (A). The WT allele (15 kb) and the mutant alleles [5 kb (5'probe) and 10 kb (3'probe)] are indicated.

(C) Genotype analysis by PCR performed on tail DNA isolated from mice containing the *p120ctn*^{flox} allele. Wild-type (WT), 400 bp; mutant (*p120ctn*^{flox}), 440 bp. Note that the same results are obtained with tail DNA isolated from mice containing the *p120ctn* $^{flox\text{-}neo}$ allele (data not shown).

(D) Immunoblot analysis of p120ctn protein in extracts prepared from the hippocampus or cortex of control (*p120ctn* $^{flox/flox}$) or mutant (*p120ctn* $^{flox/flox}$*;emx1*^{IREScre}) six week old male littermate mice.

(E and F) Immunohistochemical analysis of the loss of p120ctn protein in the hippocampus and cortex. Coronal sections prepared from six week-old male littermate

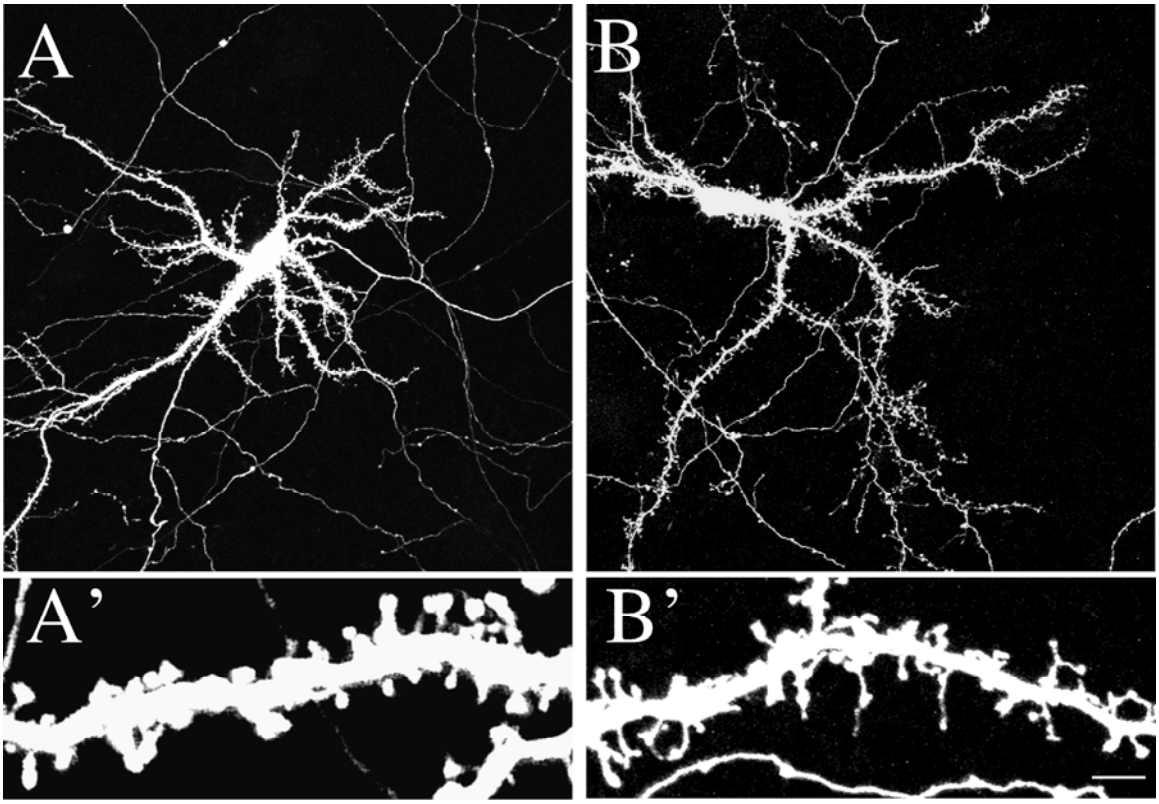
(E) control (WT*) p120ctn* $^{flox/flox}$ or **(F)** mutant *p120ctn* $^{flox/flox}$*;emx1*^{IREScre} mice (n= 3 pairs). Scale bar: 250 μm. Abbreviations: hpc, hippocampus; ctx, cortex. Expression of 120ctn persists in regions not targeted by *emx1*^{IREScre}, including thalamus, vasculature and other tissues.



Supplemental Figure 2. Synapse distribution in cultured hippocampal neurons from *p120ctn* conditional mutant mice

(A-D') Immunostaining for synaptophysin in dendrites of *p120ctn* $^{flox/flox}$ or *p120ctn* $^{flox/flox}$*;emx1*^{IREScre} neurons transfected at DIV10 with EGFP or EGFP and the indicated expression vectors and analyzed at DIV14. Lower magnification **(A-D)** and higher magnification **(A'-D')** images are shown. Scale bar= 10 μm. **(A, A')** A control (*p120ctn* $^{flox/flox}$) neuron transfected with a vector expressing EGFP (n= 6 cells). **(B, B')** A mutant (*p120ctn* $^{flox/flox}$*;emx1*^{IREScre}) neuron transfected with a vector expressing EGFP (n=7 cells). **(C, C')** A mutant (*p120ctn* $^{flox/flox}$*;emx1*^{IREScre}) neuron transfected with vectors expressing EGFP and p120ctnΔ622-628 (n= 6 cells). **(D, D')** A mutant (*p120ctn* $^{flox/flox}$*;emx1*^{IREScre}) neuron transfected with vectors expressing EGFP and p120ctnΔarm3 (n= 6 cells).

(E) Quantitation of the density of synaptophysin-positive puncta colocalized along dendrites.



Supplemental Figure 3. Profiles of dendritic spines when *p120ctn* is deleted in mature neurons

(A-B') Representative examples of hippocampal neurons cultured from *p120ctn* $^{flox/flox}$ mice transfected at DIV14 with the indicated expression vectors and analyzed at DIV17. Low magnification **(A, B)** and high magnification **(A', B')** images are shown. **(A, A')** A *p120ctn* $^{flox/flox}$ neuron transfected with a vector expressing EGFP (n= 11 cells). **(B, B')** A *p120ctn* $^{flox/flox}$ neuron co-transfected with vectors expressing EGFP and cre (n= 13 cells). Three independent experiments were analyzed. Scale bar= 10 μm.

Supplemental Table I. Quantitation of protrusion distribution and morphology following *p120ctn* deletion in mature neurons

	Control	+Cre Plasmid
Mean overall number of protrusions per 10 μm of dendrite	6.77±0.18	5.70±0.13
Mean number of mature spines per 10 μm of dendrite	6.59±0.16	3.89±0.16***
Mean overall protrusion length (μm)	1.16±0.03	2.08±0.01****
Mean overall protrusion head width (μm)	0.72±0.02	0.63±0.02*
Number of protrusions >2 μm long per 100 μm of dendrite	1.53±0.10	18.24±1.50**

p120ctn$^{flox/flox}$ neurons were transfected at DIV14 and analyzed at DIV17.
All values are expressed as the means ± s.e.m.
(****$p < 0.0001$; ***$p < 0.002$; **$p < 0.004$; *$p = 0.01$)