Supplementary information

Supplementary Materials and methods

DNA constructs
The GFP-pCDNA3-CIT, GFP-CIT-ΔRR were previously described (Camera et al., 2003).
The mutant GFP-CIT-ΔCMG was obtained from the GFP-pCDNA3-CIT construct by deleting nucleotides 5620-6534 of the published cDNA sequence. RNAi constructs and controls were previously described (Berto et al., 2007).

Antibodies
Where not stated otherwise, the following antibodies were used: anti-Citron rabbit polyclonal antibody were previously described (Camera et al., 2003). The mouse monoclonal antibody anti-PSD95 was from ABCAM; mouse monoclonal antibodies against GM130, TGN38 and ROCK-Ⅱ were from BD Biosciences. Mouse monoclonal anti RhoA (clone 26C4), goat polyclonals anti-Ribophorin-Ⅱ and anti-Actin were from Santa Cruz Biotechnology; rabbit polyclonal anti PIIa was a kind gift of Dr. Walter Witke.

Brain fractionation
The protocol used to purify synaptosomal fractions from adult rat brain is based on well-established methods used by Cohen et al. (1997) and Carlin et al. (1980). For the experiments performed only on wild type animals, 3 g of adult mouse brains were homogenized in 4 vol/g of buffer A (0.32 mM sucrose, 1 mM MgCl2, 0.5 mM CaCl2, 1 mM NaHCO3, chymostatin, leupeptin, antipain, pepstatin, and 1 mM dithiothreitol) at 800 rpm/7 strokes in a Dounce glass homogenizer (total homogenate). After the addition of 10 vol/g of buffer A, the homogenate was centrifuged at 1,400 g for 10 min to recover the supernatant (S1) and the pellet (low speed pellet=P). P was resuspended in 4 vol/g of buffer A, homogenized at 800 rpm/3 strokes, and recentrifuged at 700 g for 10 min. The
obtained pellet constitutes the nuclear fraction, while the resulting supernatant was combined with S1 to obtain the low speed supernatant (S). Centrifugation of S at 13,800 g for 10 min resulted in a final pellet (crude synaptosomal fraction), while the supernatant (S2) was centrifuged at 100,000g for 1h to give the cytosolic fraction. For the experiments performed on mutant mice, the protocol was adapted to 1 g of tissue from P15 mouse brain.

**Synaptosome immunoprecipitation**

Synaptosomal preparations (1mg) were precleared with prewashed protein G–Sepharose beads and incubated with 3 µg anti-CIT-N antibody for 1 h at 4°C. Subsequently, protein G–Sepharose beads were added, and samples were incubated overnight at 4°C under gentle rotation. Samples were then washed twice (20 min each) with immunoprecipitation buffer (1% NP40, 0.5% deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and protease inhibitors), twice with high salt buffer (0.1% NP40, 0.05% deoxycholate, 500 mM NaCl, 50 mM Tris-HCl, pH 7.5, and protease inhibitors) and once with low salt buffer (same as high salt buffer but with no NaCl). Beads were pelleted in between washes by centrifugation at 1,600 g for 30 s. After the final wash, beads were pelleted down by high-speed centrifugation, and the supernatant was analyzed by Western blotting.

**KCl treatment**

Coverslips were placed into 55 mM KCl (high potassium) buffer (10 mM Hepes, 2.2 mM CaCl2, 0.33 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3, 5.6 mM glucose, 77 mM NaCl, and 55 mM KCl) or control buffer (same as high-potassium but containing 127 mM NaCl and 5 mM KCl) and incubated for 3 min at 37°C and 5% CO2 in a humid chamber. Coverslips were then processed for immunofluorescence.

**Generation of CITnull/null mice**

The CITnull targeting vector was obtained by replacing a Ncol–Stul restriction fragment with the corresponding sequence from the CIT-K cDNA. A floxed Neo cassette was then
inserted in a Sma I restriction site located in intron 9, 460 bp upstream of exon 10. The targeting vector was transfected in ES cells and two homologous recombinant clones were selected by southern blotting with DNA probes external to the construct. Upon injection in C57/bl6 blastocysts, we obtained germ-line transmission from each clone. The corresponding mouse lines produced identical phenotypes.

**In vivo spine analysis**

P17 mice were perfused intracardially with 4% paraformaldehyde. Brains were then processed with the rapid Golgi method as in Garey and Saini (Garey and Saini, 1981). Single branches were photographed with a Nikon 90i microscope for a qualitative description of the morphology of dendritic spines. For quantitative analysis, single pyramidal neurons (three for each animal) in layer V of cerebral cortex were fully reconstructed, dendritic spines included, at a Nikon Eclipse 600 light microscope equipped with a motorized stage interfaced to the computer using the program Neurolucida (Microbrightfield Inc., VT) at high magnification (100x oil immersion objective). The density of dendritic spines in basal dendrites, expressed as the ratio number of spines/length of dendritic segment, was obtained with the software program NeuroExplorer (Microbrightfield Inc.) for all dendritic branches, except first order ones.

**In vivo analysis of synaptic markers**

Vibratome-cut 50 µm-thick sections from P17 WT, CIT-K<sup>−/−</sup> and CITnull/null mice (three animals per genotype) were immunostained with antibodies against vGLUT1 (guinea pig polyclonal, 1:5000, Chemicon), PSD95 (1:200, monoclonal, Chemicon), NMDA receptor 2A/B (1:50, rabbit polyclonal, Chemicon) and AMPA receptors (1:50, rabbit polyclonal, Chemicon), followed by Cy3 (for vGLUT1, 1:200, Chemicon) or Cy2 (1:100, Chemicon) secondary antibodies. Sections were mounted onto gelatin-coated slides and observed at the Confocal Laser Scanning microscope (CLSM, Fluoview 300 Olympus, Hamburg, Germany). Stacks of images were taken at a section thickness of 0.5 µm, and viewed with
the program Photoshop. Positive profiles in the neuropil were counted in 5x5 µm squares in single 0.5 µm-thick sections. The density of profiles was expressed in number of profiles/100 µm². The percentage of double labeled profiles was then calculated for each kind of profiles.

**Supplementary references**


**Supplementary Figure legends**

**Supplementary Figure 1**

(A) 21-DIV PHN were co stained with anti CIT-N and PSD95 antibodies, as well as with phalloidin (green, red and blue in the merge, respectively). Arrows show the co localization of CIT-N/F-actin clusters with PSD95 protein. (B) High magnification of a 21DIV PHN dendrite co-stained for CIT-N (green), F-actin (blue) and GM130 (red). Arrows indicate some of the structures where all the analyzed proteins colocalize. (C) Quantitative analysis of the colocalization between CIT-N and GM130 or TGN38, respectively. Error bars = standard error.

**Supplementary Figure 2**
(A) Domain structure of the different constructs used for the overexpression experiments.

(B) 21 DIV neurons were transfected with the indicated GFP-CIT-N constructs and processed to reveal active RhoA and F-actin. Arrows indicate recombinant protein clusters.

Supplementary Figure 3: Production of the CITnull allele.

CIT-K and CIT-N are produced by a complex transcription unit, through an alternative transcriptional initiation mechanism (Di Cunto et al., 2000). The expression of the CIT-K transcript is driven by a proximal promoter, activated at the G2/M phase of the cell cycle in all proliferating cells. In contrast, CIT-N mRNA is produced from a second promoter, distal to the exons encoding the kinase domain and specifically activated in differentiating and differentiated nerve cells (Di Cunto et al., 2000).

To address the function of CIT-N in vivo, we devised a gene targeting experiment aimed at the specific inactivation of this isoform. To reach this objective we removed the intron 10, which comprises a 1000 bp conserved sequence block (CSB) spanning the CIT-N transcription start site (our unpublished observations). To this aim we generated, by homologous recombination in ES cells, an allele carrying the CSB deletion and a floxed Neomicin cassette.

Note: Removal of the Neo cassette by crossing with Cre balancer mice did not restore completely the expression of CIT-K, but resulted in a knockodown of both isoforms (data non shown). The phenotype of these mice will be described elsewhere.

Supplementary Figure 4: Quantitative analysis of synaptic proteins in CIT knockout mice.

Colocalization of a pre-synaptic marker (VGLUT1) and of post-synaptic proteins (PSD95, NMDA and AMPA glutamate receptors) was determined at P15 for the indicated genotypes. Error bars=standard error. The differences were not statistically significant.
Supplementary Figure 2

A

CIT-WT

CIT-ΔRR

CIT-ΔCMG

Coiled-coil

Pleckstrin Homology

Rho-binding

CMG

Zinc finger

PDZ-binding

B

GFP-CIT-WT

Rho A

merge

GFP-CIT-ΔRR-BG

Rho A

merge

GFP-CIT-ΔCMG

Rho A

merge