Menna et al., SUPPLEMENTARY INFORMATION

General health.
Each mouse (10 for genotype) was observed and any abnormalities in the general appearance of the fur (3-point scale), piloerection, body tone (3-point scale), skin color were recorded (Lee et al, 2008). In addition, mice were observed for the empty cage behaviour (nest-building behaviour) (Deacon 2006). Neurological reflexes were assessed, including righting reflex (4-point-scale) corneal (8-point scale), pinna (8-point scale), and tail pinch (8- point scale) (Irwin 1968). Motor function, coordination and balance, muscle strength were evaluated as previously described (Corradini et al., 2012). As for the sensory abilities, hearing was evaluated using Preyer's reflex testing through a handclap sound (Jero et al. 2001). Visual acuity was performed using the visible cliff test (Brandewiede 2005). Olfactory test was carried out as described in Moy et al. (2004). Pain sensitivity and aggression were tested as described in Corradini et al. (2012).

T maze
Mice were habituated to a black wooden T-maze (stem length 41 cm; arm length 91 cm). Each section was 11 cm wide with 19 cm high side walls. Mice were habituated to obtain food in the T-maze for 5 days (Moy et al. 2008). In the acquisition phase, one arm was designated as reinforce (Kellogg's cereal) for each of 10 daily trials. Each mouse was placed at the maze start and given a free choice to enter either arm. The number of days to reach the criterion - that is, showing 80% of correct choices for 3 days - was recorded. Each mouse that met the criterion for acquisition was then tested using a reversal procedure, in which the reinforcer was switched to the opposite arm, following the same method as described above.

Radial maze
The radial maze consisted of eight arms (each 30 cm long, 7.5 cm wide, and with the enclosing walls 10 cm high), that extended radially from a central 30 cm wide octagonal platform that served as a starting base. Small plastic cups mounted at the end of each arm held 15 mg food pellets as reinforcers. Access to the arms was controlled by eight pneumatically operated sheet-metal guillotine doors. The entire maze was painted black, elevated 50 cm from the floor, and placed in the center of a small room (2.5m × 2.5m) lit by fluorescent lights and fitted with several extramaze cues. Animal behavior was monitored by a video camera (Model CCD, Securit Alarmitalia) whose signals were digitized and interfaced by a PF6PLUSPAL apparatus 512×512 pixels (Imaging Technology, Woburn, MA), and sent to a video monitor (Trinitron KX-14CP1, Sony, Japan). Image analysis and pattern recognition were done by a Delta System computer (Addonics) using software provided by Biomedica Mangoni (Pisa, Italy). Starting 2 weeks before the experiment, body weights were reduced by 10% by means of a restricted feeding schedule of standard chow (Harlan- Italy).The animals were kept at 90% of their free-feeding bodyweight for the duration of the experiment. After 3 days of free exploration the animals were trained to complete the maze. During each daily session, working

memory was scored on the basis of the total number of errors (which corresponded to a re-entry into the arm just visited). Training continued, at the rate of one trial per day, until the mice reached the criterion of entering seven different arms in their first eight choices on 5 successive days, for a maximum of 30 days. The mean number of days taken to reach the criterion and the percentage of animals reaching the criterion were calculated.

Passive Avoidance

Briefly, the apparatus consisted of two compartments, one light and one dark, connected via a sliding door. In the acquisition trial, each mouse was placed in the light compartment and allowed to enter the dark compartment; the time (in s) taken to do so was recorded. Once the mouse was in the dark compartment, the sliding door was closed and an unavoidable electric shock (1mA for 1 s) delivered via the paws. The animal was then placed back in the home cage until the retention trial. The retention trial was carried out 24 h after the acquisition trial, by positioning the mouse in the light compartment and recording the time taken to enter the dark compartment (retention latency, cut-off 180 s). An increased retention latency indicates that the animal has learned the association between the shock and the dark compartment.

Novel Object Recognition test

Animals were habituated to the test arena for 10 min on the first day. After 1-day habituation, mice were subjected to familiarization (T1) and novel object recognition (T2). During the initial familiarization stage, two identical objects were placed in the centre of the arena equidistant from the walls and from each other. Each mouse was placed in the centre of the arena between the two objects for a maximum of 10 min or until it had completed 30 s of cumulative object exploration. Object recognition was scored when the animal was within 0.5 cm of an object with its nose toward the object. Exploration was not scored if a mouse reared above the object with its nose in the air or climbed on an object. Mice were returned to the home cage after familiarization and retested 120 min later, and in the arena a novel object (never seen before) took the place of one of the two familiar. Scoring of object recognition was performed in the same manner as during the familiarization phase. From mouse to mouse the role (familiar or new object) as well as the relative position of the two objects were counterbalanced and randomly permuted. The objects for mice to discriminate consisted of white plastic cylinders, colored plastic Lego stacks of different shape and a metallic miniature car. The arena was cleaned with 70% ethanol after each trial. The basic measure was the time (in s) taken by the mice to explore the objects in the two trials. The performance was evaluated by calculating a discrimination index (N-F/N+F), where n= time spent exploring the new object during T2, F= time spent exploring the familiar object during T2 (Pitsikas et al. 2001).

Sociability

The apparatus was a rectangular, three-chamber transparent polycarbonate box (width=42.5 cm; height=22.2 cm; centre chamber, length=17.8; side chambers, length=19.1 cm). The proband mouse was firstly placed in the middle compartment and

allowed to explore all three chambers for 10 min (habituation) (Moy et al., 2004, Sala et al., 2011). An unfamiliar adult DBA/2J male mouse was placed in one side compartment whereas the opposite contained an empty wire cage. The time spent in each chamber and the number of entries into each chamber were recorded for 10 min. Data are expressed as time spent in each chamber or the difference score between the time spent to explore the compartment containing the conspecific and that spent in the empty compartment (for sociability test), or containing the stranger animal (unfamiliar) and that for the familiar mouse (for social novelty test) (DeVito et al. 2009).

EEG

Mice were anesthetized with i.p. injection of 5% chloral hydrate dissolved in saline and given a volume of 10 ml/kg. Four screw electrodes (Bilaney Consultants GMBH, Dusseldorf, Germany) were inserted bilaterally through the skull over cortex (anteroposterior,+2.0–3.0 mm; left–right 2.0 mm from bregma) as previously described (Manfredi et al, 2009) according to brain atlas coordinates (Paxinos and Franklin, 2004); a further electrode was placed into the nasal bone as ground. The five electrodes were connected to a pedestal (Bilaney, Dusseldorf, Germany) and fixed with acrylic cement (Palavit, New Galetti and Rossi, Milan, Italy). The animals were allowed a week for recovery from surgery before the experiment.
EEG traces were analyzed as described (Manfredi et al. 2009) for spike activity. Basal cerebral activity was recorded continuously for 24 h in freely moving mice. For each 24-h EEG recording, the mean number of spikes was evaluated in both genotypes. After the recordings, the EEG and video (through a video camera placed inside the Faraday chamber) were analyzed for the incidence/amplitude of spontaneous cortical spike activity and the percentage of animals displaying spike activity, as previously described (Zhang et al. 2004; Manfredi et al. 2009).

Quantitative RT-PCR analysis

Total RNA was isolated with the RNeasy® Mini Kit (Qiagen) according to the manufacturer instructions. Two micrograms of RNA were used, with 100 ng of random examers, in a reverse-transcription reaction (SuperScript® VILO™ cDNA Synthesis Kit). One-tenth ng of cDNA was amplified, in triplicate, in a reaction volume of 25 µL with 10 pMol of each gene specific primer and the SYBRgreen PCR MasterMix (Applied Biosystems). Real-time PCR was carried out on the 14 ABI/Prism 7700 Sequence Detector System (Perkin-Elmer/Applied Biosystems), using a pre-PCR step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60s at 60°C. Specificity of the amplified products was confirmed by melting curve analysis (DISSOCIATION CURVE TM Perkin-Elmer/Applied Biosystems) and by 6% PAGE. Preparations with RNA template without reverse transcriptase were used as negative controls. Samples were amplified with primers for each genes (for details see Q-PCR primer list below) and rRNA GAPDH as a housekeeping gene. The Ct values were normalized to the GAPDH curve. The GAPDH gene was used as a control gene for normalization. Results were quantified using the 2- CT method (Livak and Schmittgen, 2001). PCR experiments were performed in triplicate, and standard deviations calculated and displayed as error bars.

Primer sequences used are listed below:
Assay ID Context Sequence Gene Symbol
Mm00519404_m1 ACCAGCTGGGCTGACTGGACAGGCA Eps8l3
Mm00509161_m1 GGTCAATGGTCAGCAAGATCCAGAA Eps8l1
Mm00519237_m1 ACATGCTAACAGGGGCTACCAGCCA Eps8l2

Determination of Rac activation.
The levels of Rac-GTP were measured using G-LISA™ Rac Activation Assay Biochem Kit™ (Cytoskeleton) according to the manufacturer's instructions. Results were normalized over the level of total Rac determined by western blot analysis. Monoclonal anti-Rac1 and anti-Eps8 antibodies (BD Transduction Laboratories, Franklin Lakes, NJ). Monoclonal anti-Vinculin (Sigma-Aldrich, St. Louis, MO).

Electron microscopy
Postnatal 90 days WT and Eps8KO mice were anesthetized with 4% chloralium hydrate and perfused with 4% paraformaldehyde, 1% glutaraldehyde in Phosphate Saline Buffer pH 7.4. Dissected brains were post-fixed in the same buffer for 2 hours before vibratomesectioning (Leica Microsystems, Germany) of coronal sections of 350 mm in thickness in the hippocampal region.. From these sections, regions containing the CA1 and CA3 of the hippocampi were dissected and they were further processed for electron microscopy sample preparation. Briefly the samples were fixed with glutaraldehyde (2% in cacodylate buffer 0.1M, pH 7.4), postfixed with 2% OsO_4 in the same buffer, en bloc stained with a saturated solution of uranyl acetate in 20% ethanol dehydrated and embedded in a mixture of Epon+Spurr epoxy resins. Ultra-thin sections were observed in a Philips CM10 microscope; images were collected at 28.500x and morphometric analysis was performed with Image J software.

Cell culture electrophysiology
During recordings cells were bathed in a standard external solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$, 6 glucose, and 25 HEPES-NaOH, pH 7.4. Recording pipettes were fabricated from borosilicate glass capillary using an horizontal puller (Sutter Instruments) inducing tip resistances of 3-5 MΩ and filled with a standard intracellular solution containing (in mM): 130 K-gluconate, 10 KCl,1 EGTA, 10 HEPES- NaOH, 2 $MgCl_2$, 4 MgATP, and 0.3 Tris-GTP. For miniature EPSC recordings 1μM tetrodotoxin, 20μM Bicuculline and 50μM AP5 (Tocris) were added to standard extracellular solution to block spontaneous action potentials propagation, GABA-A and NMDA receptors, respectively. Recordings were performed at room temperature in voltage clamp mode at holding potential of -70 mV using a Multiclamp 700B amplifier (Molecular Devices) and pClamp-10 software (Axon Instruments, Foster City, CA). Series resistance ranged from 10 to 20 MΩ and was monitored for consistency during recordings. Cells in culture with leak currents >100 pA were excluded from the analysis. Signals were amplified, sampled at 10 kHz, filtered to 2 or 3 KHz, and analyzed using pClamp 10 data acquisition and analysis program.

Live cell imaging and Fluorescence Recovery After Photobleaching (FRAP)
FRAP experiments were performed maintaining coverslips in a 37°C heated chamber with 5% CO_2 in their own growth medium. The construct FU(PSD95:EGFP)W was a kind gift from Prof. Noam Ziv, Israel Institute of Technology, Haifa, Israel. Live **cell** imaging was performed with a confocal **microscope** Leica SP5 using a HCX PL APO 63X/ 1.4 **oil immersion** objective **(Leica Microsystems, Germany)**. Photobleaching was **performed** using a 488nm laser light at 100%. Images were collected every 500ms. The **bleached** region of interest (ROI) was put on the spine and has been used for both the photobleaching and the fluorescence recovery analysis. **The fluorescence recovery was recorded and the analyses performed on the first 40 seconds after bleaching.** Each image at each time point was corrected for the background and for the not-intended bleaching and normalized according to this formula: $((F_t-F_b)/(F_r-F_b))/(F_a-F_b)$, where F_t is the fluorescence of a ROI at time t; F_b is the fluorescence of the background; F_r is the fluorescence of the reference ROI at time t and F_a is the fluorescence of the ROI immediately before photobleaching. The data obtained were fitted with a single exponential using the **LAS AF** software **(Leica Microsystems, Germany)**.

Golgi staining
Mice were deeply anesthetized with avertin (0.2ml/10g body weight, i.p.) and perfused intracardially with 0.9% saline solution. The brains were removed and stained by modified Golgi-Cox method described in Glaser and Van der Loos (1982). Coronal sections of 100μm thickness from the dorsal hippocampus were obtained using a vibratome (VT1000S, Leica, Wetzlar, Germany). These sections were collected on clean, gelatin-coated microscope slides and treated with ammonium hydroxide for 30 min, followed by 30 min in Kodak Film Fixer, and finally were rinsed with distilled water, dehydrated and mounted with a xilene-based medium.

Immunofluorescent staining on free-floating sections.
Immunofluorescent staining was carried out on free-floating sections as described in Frassoni et al., 2005. Free-floating sections were processed for PSD-95 (rabbit policlonal antibody kindly provided by C. Sala; 1:400) and VAMP2 (Vesicle Associated Membrane Protein, Synaptic System, Gottingen, Germany 1:800), followed by incubation with secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA), indocarbocyanine (Cy) 2-conjugated goat anti-mouse (1:200) and Cy3-conjugated goat anti-rabbit (1:600), mounted in Fluorsave (Calbiochem, San Diego, CA, USA). Sections were examined by means of a a Zeiss LSM 510 META confocal microscope (Leica Microsystems, Germany). To resolve individual synaptic puncta, the images (512x512 pixels) were acquired using the x40 oil immersion lens (numerical aperture 1.0) with additional electronic zoom factor of up to 4. The gain and the offset were lower to prevent saturation in the brightest signals. The pinhole size was kept at the minimum setting (1.0-1.8). Analysis was carried out on each mice (Eps8 wt and KO) and three different sample from CA1 area were taken at two hippocampal coronal levels.

In vitro binding assay

Standard procedures **for** in vitro binding, were as previously described (Disanza et al., 2006). The antibodies used were: monoclonal anti-Eps8 (Transduction Laboratories, Lexington, KY); rabbit polyclonal anti-GST (Santa Cruz Biotechnology, Santa Cruz, CA). Hs-Abi1 synthesized peptides (PPPPPVDYEDEE; AAAAAVAAEDEE) were from Mimotopes (Clayton Victoria, Australia). Recombinant purified His-Eps8 and GST-Abi1 were obtained as previously described (Disanza et al., 2004; Menna et al., 2009).

Supplemental figure legends

Supp. Fig. 1 - No gross alterations in brain architecture or in the ultrastructure of presynaptic terminals are detected in Eps8 KO mice.

A-L. Detailed brain morphological analysis. Sagittal sections through the entire CNS (**A, B**) or coronal sections of cortex (**C, D**), hippocampus (**E, F**) and cerebellum (**G, H**) of wt (A,C,E,G) and Eps8 KO mice (B,D,F,H) were analyzed by staining with thionine. The thickness and layering of the cerebral cortex, the architecture of the hippocampal formation and cerebellum are undistinguishable between wt and Eps8 KO mice. Immunostaining for the calcium binding protein Calbindin (**I** wt, **L** Eps8 KO) revealed no differences in the cytoarchitectural organization of the CA subfields and dentate gyrus of the hippocampus. **M**. Normalized expression of Eps8L family members in hippocampus from eps8 wt and KO adult mice. Expression profiles of Eps8L1, Eps8L2 and Eps8L3 mRNAs in the hippocampus were determined by quantitative RT-PCR. Data are expressed as the ratio of mRNA levels of each Eps8L family member relative to that of GAPDH mRNA levels, normalized on the mRNA levels in WT. Bars show the mean ± SD (n=3) of three independent experiments. **, $P < 0.01$ (tra L1 WT e KO). Eps8L3 mRNA was not detected either in wt or in KO samples (not shown). Scale bars, A,B=200 μm; C,D= 220 μm; E,F=450 μm; G,H= 320 μm; I,L= 400 μm. **N-O.** Electron micrographs of excitatory synapses of neurons from hippocampal sections of wt and Eps8KO mice. No difference in the size and organization of the synaptic vesicle pool (SV) can be observed In the presynaptic terminals. (E): endosome; PSD: post-synaptic density (Scale bar N,O=200 nm). **P**. Synaptic vesicles area (left graph) (wt, n=3732; Eps8KO, n=4003), SV abundance, evaluated with respect to the presynaptic bouton profiles (middle graph), and synaptic bouton area (right graph) (wt, n=53; Eps8KO, n=54) are unchanged in wt and KO animals.

Suppl. Fig. 2 – Excessive synaptic growth and altered PSD-95 mobility in Eps8 KO neurons

A. 21 DIV old Eps8 WT or KO cultures stained for beta III tubulin (green), vGlut1 (blue) and PSD-95 (red). Quantitation of the PSD-95 positive (top histogram) or PSD-95 and vGlut1 positive (bottom histogram) puncta per unit length reveals higher number of synaptic contacts in Eps8 KO neurons relative to wt. Scale bar, 20 μm. **B**. FRAP experiment in wt or Eps8 KO neurons transfected with PSD-95-GFP. Selected spines are chosen, bleached with a high-power laser and the fluorescence recovery is monitored during time. The recovery is expressed as mobile fraction percentage. A faster recovery of PSD-95 fluorescence occurs in Eps8 KO neurons. Mann-Whitney rank sum test $p<0.001$. Data are normalized and expressed as mean ± SEM. Scale bar, 2 μm. **C**. mEPSC recording in wt and Eps8 KO neurons reveals significantly lower mEPSC amplitude, but not frequency, in KO neurons. **Number of cell examined: 12 wt and 12 KO. Number of independent experiments: 3**. Scale bars depict 10pA and 250 ms.

Suppl. Fig. 3 – Reduced Eps8 expression impairs synaptic potentiation

A. Dendrites of rat hippocampal neurons transfected with RFP, Eps8 wt fused to GFP or the actin capping mutant Eps8H1 fused to GFP. Staining for PSD-95 and tridimensional reconstruction with Imaris. **A'.** Quantitation of PSD-95 reveals increased mean size upon transfection of Eps8 wt but not Eps8H1. **B.** Treatment of rat hippocampal cultures with Eps8 siRNA potently reduces the expression of the protein (red: tubulin; green: Eps8). **B'**. The mean size of PSD-95 after chemical LTP is not increased in neurons where the expression of Eps8 is acutely down regulated by siRNA, indicating lack of potentiation. Scale Bar, 5 μm.

Suppl. Fig. 4 – Saturation of actin capping by overexpression of Eps8, but not of its actin capping mutant, precludes LTP. A-C. 14-16 DIV rat hippocampal neurons transfected with RFP (A), cDNA coding for Eps8 wt fused to GFP (B) or cDNA coding for the actin capping mutant Eps8H1 fused to GFP (C). Cultures are subsequently exposed to chemical LTP treatment and stained for PSD-95 (blue). **Number of cell examined: 18, RFP; 25, Eps8 wt; 24, Eps8 H1. Number of spines analyzed: 179, RFP; 372, Eps8 wt; 286, Eps8 H1. Number of independent experiments: 5**. Scale bars, 8 μm. **D**. **Eps8 removal does not affect Rac-GTP levels.** The levels of Rac-GTP in hippocampal neurons derived from wt and Eps8 KO mice are similar (P=0,19). Equal amounts of cell

protein extract were processed to determine Rac activation (see Material and Methods section for details). The results obtained were normalized on the total level of Rac. Equal amounts of lysates from wt and Eps8 KO neurons were processed for western blotting with the indicated antibodies (right panel).

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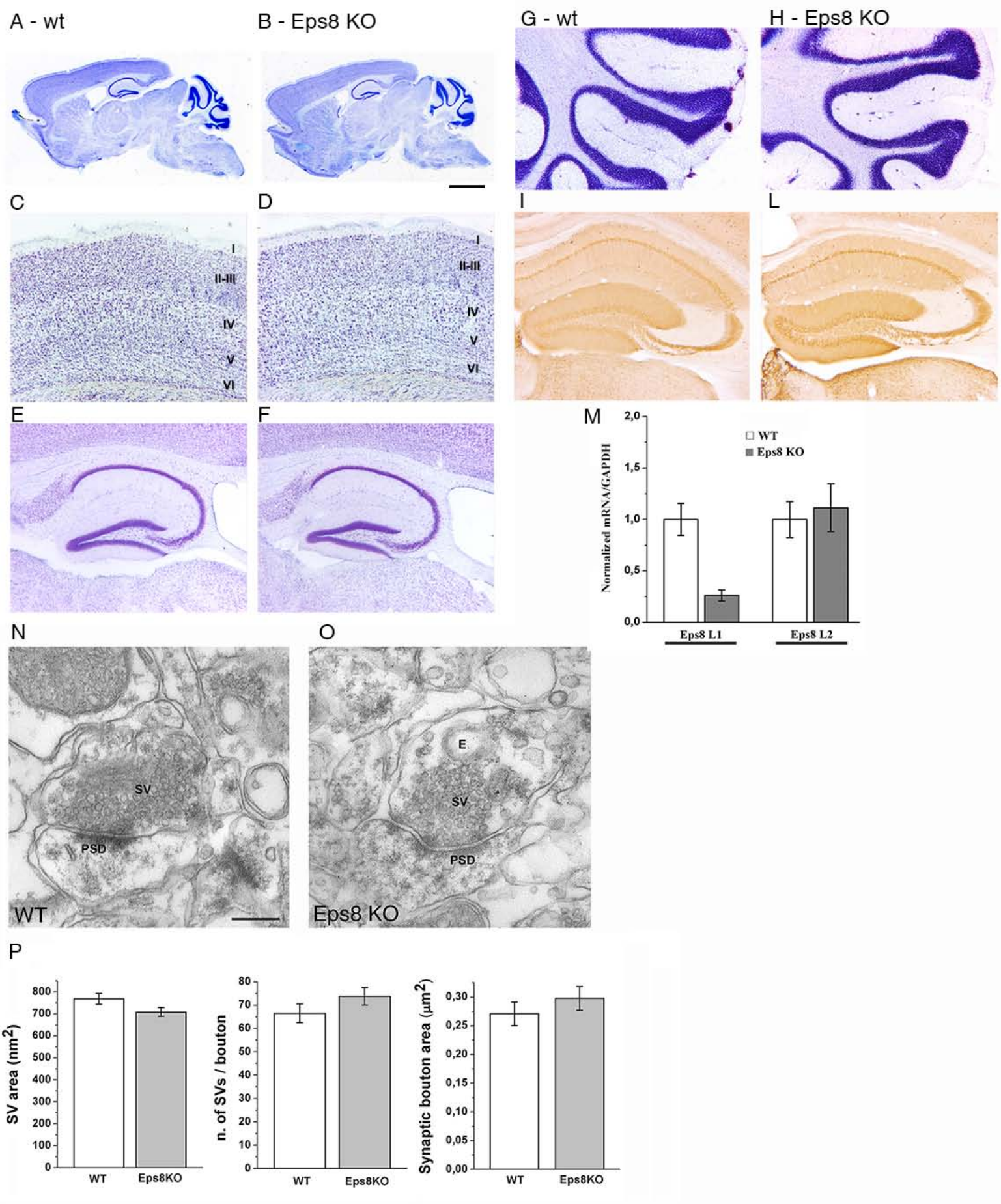
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B - Eps8 KO
G - wt
H - Eps8 KO
C
D
I
L
I
II-III
IV
V
VI
E
F
M
WT
Eps8 KO
Normalized mRNA/GAPDH
2,0
1,5
1,0
0,5
0,0
Eps8 L1
Eps8 L2
N
O
SV
PSD
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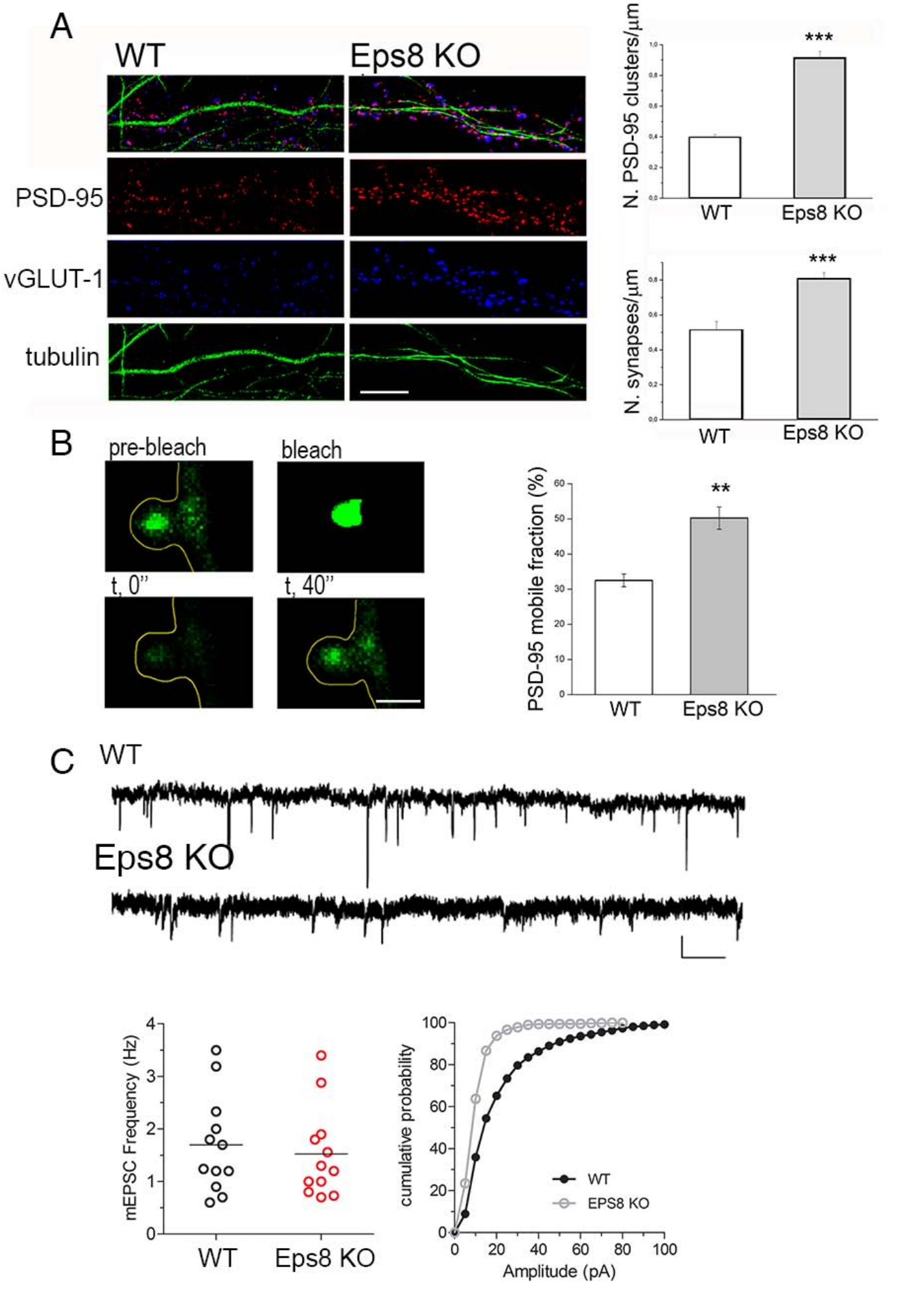
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vGLUT-1
tubulin
N. PSD-95 clusters/µm
***
WT
Eps8 KO
N. synapses/µm
***
WT
Eps8 KO
B
pre-bleach
bleach
t, 0"
t, 40"
PSD-95 mobile fraction (%)
**
WT
Eps8 KO
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WT
Eps8 KO
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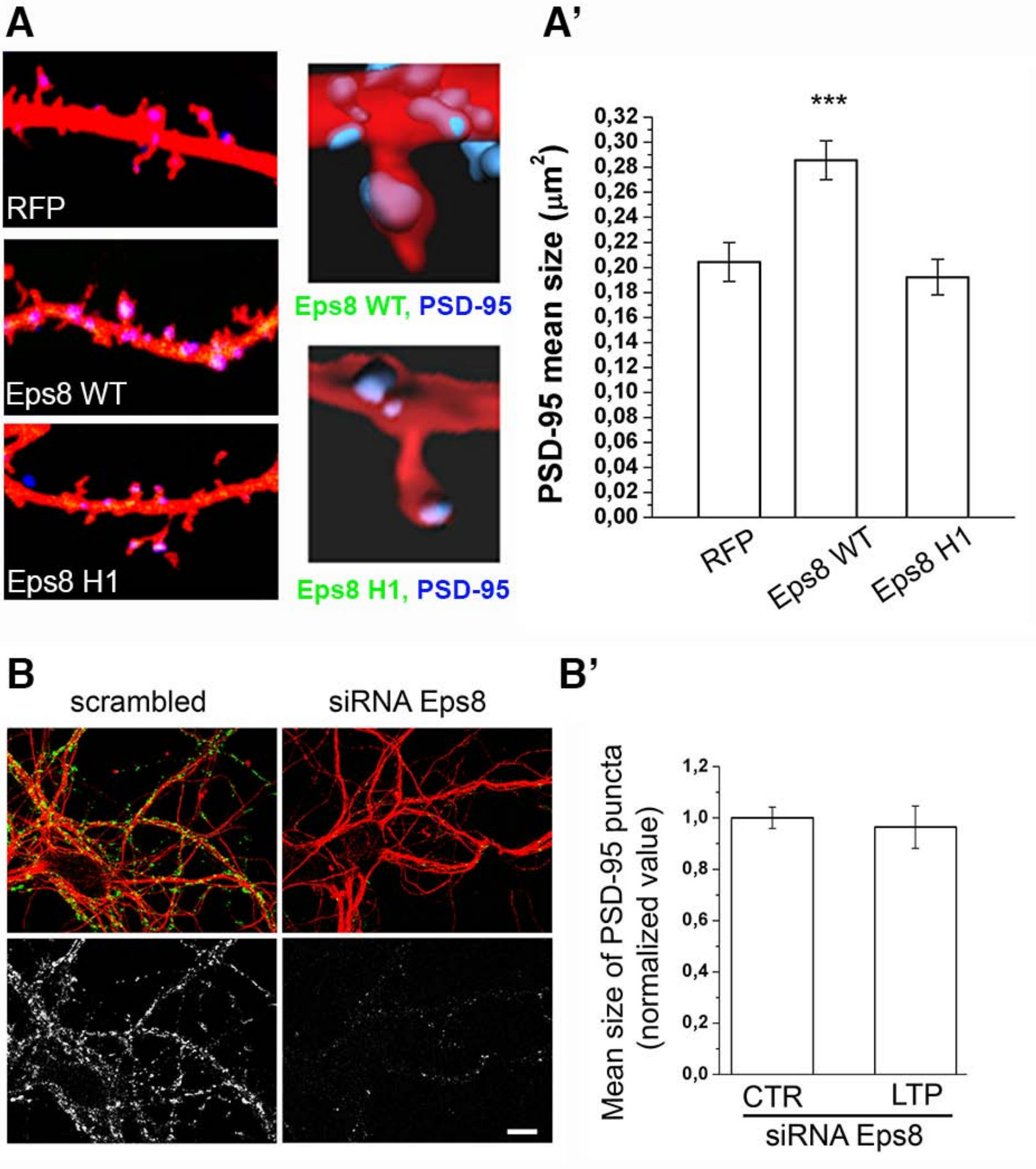
WT
Eps8 KO
cumulative probability
WT
EPS8 KO
Amplitude (pA)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RFP
Eps8 WT
Eps8 H1
Eps8 WT, PSD-95
Eps8 H1, PSD-95
A'
***
PSD-95 mean size (μm²)
0,32
0,30
0,28
0,26
0,24
0,22
0,20
0,18
0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
RFP
Eps8 WT
Eps8 H1
B
scrambled
siRNA Eps8
B'
Mean size of PSD-95 puncta (normalized value)
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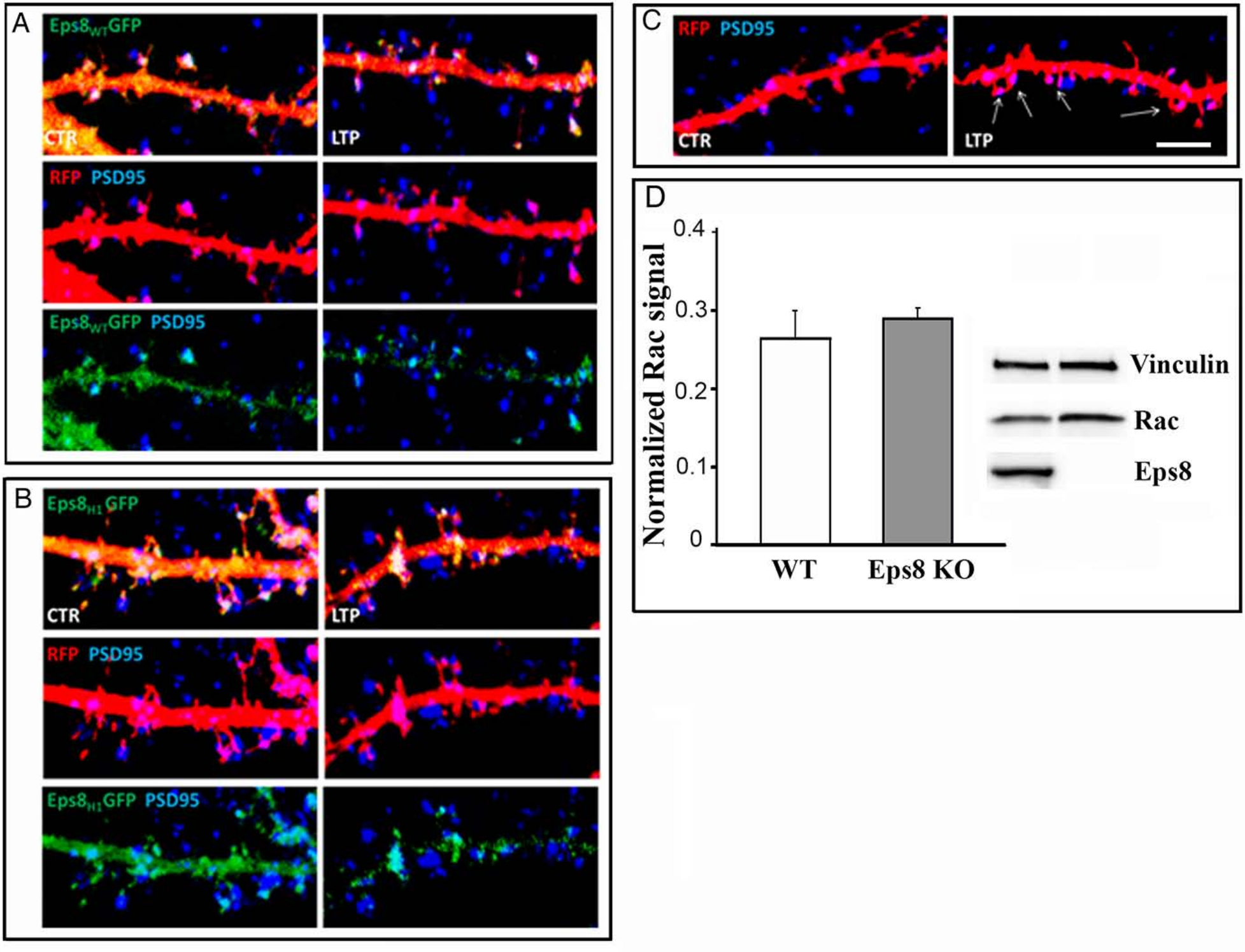
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LTP
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B
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CTR
LTP
RFP PSD95
Eps8H1GFP PSD95
C
RFP PSD95
CTR
LTP
D
Normalized Rac signal
0.4
0.3
0.2
0.1
0
WT
Eps8 KO
Vinculin
Rac
Eps8

Supplemental Table 1
Phenotypic characteristics of male WT and *Eps8* null mice

General Health			
Weight (g)	26.45±1.08	26.12±0.77	0.81
Fur condition (3-point scale)	3.0±0.0	3.0±0.0	-
Piloerection (%)	0	0	-
Body Tone (3-point scale)	3.0±0.0	3.0±0.0	-
Skin colour (%)	100	100	-
Physical abnormalities (%)	0	0	-
Empty cage behaviour			
Nesting building (5-point scale)	4.80±0.2	4.75±0.25	0.87
Reflexes			
Righting reflexes (4-point scale)	0	0.01±0.01	-
Corneal (8-point scale)	4±0.3	3.9±0.2	0.78
Pinna (8 point scale)	4±0	3.9±0.2	0.62
Tail pinch (8-point scale)	3.9±0.04	4±0.2	0.63
Motor function			
Locomotor activity			
Horizontal counts (n)	3869±141	3963±204	0.76
Vertical counts (n)	921.5 ±56.01	824.5±56.2	0.24
Motor coordination			
Rotarod (%)	60	50	0.89
Wire hanging (s)	260.2±10.8	277.2±15.4	0.37
Sensory abilities			
Hearing (%)	100	100	-
Visual acuity (%)	100	100	-
Emotional-like reactivity			
Anxiety:			
Elevated plus maze			
Open arm entries %	43.7±6.54	44.05±10.49	0.97
Open arm time %	26.18±6.75	37.08±16.82	0.53

Mice were assessed on general health, empty cage behaviour, reflexes, sensory abilities, motor coordination. N = 8-10 per group.