Supplementary Information

Materials and Methods

Antibodies

The following antibodies were used for Western blotting or immunoprecipitation: rat anti-radixin R21(Doi et al., 1999) (1:200, S. Tsukita); rabbit anti-phospho ERM (1:100, Cell signaling, Beverly, MA); mouse anti-myc, clone 9E10 (1:1000, Sigma, Taufkirchen, Germany); rabbit anti-$GABA_AR$ α5 (1:500, Acris, Hiddenhausen, Germany); rabbit anti-$GABA_AR$ α5 (1:500, Sigma, Taufkirchen, Germany); mouse anti-gephyrin (1:100; Synaptic Systems, Göttingen, Germany); peroxidase-conjugated goat-anti rat, goat-anti rabbit and goat-anti mouse (all 1:10.000, Dianova, Hamburg, Germany). The following antibodies were used for immunofluorescence: rat anti-radixin R21 (1:25, S. Tsukita); mouse anti-synaptic vesicle (SV2, 1:100, Hybridoma bank, University of Iowa, IA); rabbit anti-VIAAT (1:100; B. Gasnier); rabbit anti-$GABA_AR$ α5 (1:30, Acris, Hiddenhausen, Germany); rabbit anti-$GABA_AR$ α3 (1:100, Sigma, Taufkirchen, Germany); mouse anti-gephyrin (1:100; Synaptic Systems, Göttingen, Germany); rabbit anti-HA (1:100, Sigma, Taufkirchen, Germany); mouse anti-myc (1:100, Sigma, Taufkirchen, Germany); mouse anti-pan Cadherin (1:100, Abcam, Cambridge, UK); rabbit anti-phospho ERM (1:100, Cell signaling, Beverly, MA); CY3-, CY2- or CY5-conjugated donkey-anti rat, donkey-anti mouse or donkey-anti rabbit (all 1:500, Dianova, Hamburg, Germany). The specificity of radixin antibodies was controlled in Western blot and immunocytochemistry. The specificity of the $GABA_AR$ α5 antibody was controlled in Western blot, immunoprecipitation

and immunocytochemistry.

Cell culture/Transfection/Microinjection

Primary cultures of hippocampal neurons were prepared from mice at postnatal days 0-1, as previously described (Fuhrmann et al., 2002). Cells cultured between 7 and 9 days *in vitro* were used for transfection by a calcium phosphate coprecipitation protocol (Fuhrmann et al., 2002). Human embryonic kidney (HEK293) cells were cultured in 10 cm dishes. For heterologous expression, cells were transfected one day after plating at 40-50% confluency. During microinjection, neurons were maintained in prewarmed HEPES buffer (135mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 10mM HEPES/KOH, pH7.4, 15mM glucose). PTO-antisense oligonucleotides against radixin were synthesized as previously described (Takeuchi et al., 1994) (MWG Biotech, Ebersberg, Germany). A solution containing 0.1 mM oligonucleotides together with either 2.5% (w/v) lucifer yellow or 50ng/μl pEGFP-C2 vector (BD Biosciences, Heidelberg, Germany) was microinjected at 70-100 hPa for 0.1-0.3 s using a Transjector 5246 coupled with an Injectman system (Eppendorf, Hamburg, Germany). Visual control was obtained by the use of an inverted Axiovert 35 microscope equipped with a long distance phase contrast objective LD-Plan Neofluar 63x (Zeiss, Hallbergmoos, Germany). 24 hours after microinjection, cells were fixed and processed for immunocytochemistry.

Immunocytochemistry

At DIV 13-14, neurons were fixed in 4% paraformaldehyde (PFA) in PBS for 12 min and permeabilized with 0.25% TritonX-100 for 4 min. Alternatively, cells were fixed with PFA for 20 min and incubated in ice cold methanol on dry ice for 15 min, as

previously described (Fuhrmann et al., 2002). For live cell staining, coverslips were transferred to HEPES buffer, containing the antibody and incubated at 4°C for 30 min. Cells were then washed and fixed as described above. Fluorescence imaging was carried out with an inverted Leica TCS-SP2 laser scanning confocal microscope (Leica, Mannheim, Germany) using a 63x objective. For simultaneous multichannel fluorescence, images were taken in a sequential channel recording mode.

Images were saved as overlay pictures and printed on a FS-C5016N color laser printer (Kyocera Mita, Meerbusch, Germany). Puncta were quantified using Scion Image 1.63 (NIH, USA). All pictures were adjusted to equal photomultiplier values and then a cutoff was taken at intensity 100. Residual individual puncta were measured for pixel size and fluorescence intensity in all color channels. The threshold value for colocalization was determined once using various examples and then applied to all images analyzed. For quantification of radixin/$GABA_AR$ $\alpha5$ co-localization at synaptic and extrasynaptic sites, a total of 5,739 puncta were analyzed.

F-actin cosedimentation assay

To test for F-actin association, radixin (T564D)-GFP, radixin (1-468)-GFP and radixin (K(253, 254, 262, 236)N)-GFP were expressed in *E.coli* strain BL21 encoded by a modified pET28b expression vector. F-actin cosedimentation was tested following the manufacturer's instructions (Tebu-Tech, Offenbach, Germany). In brief, G-actin was polymerized at room temperature to form F-actin. Purified proteins were added to the mixture and then centrifuged at 150.000 g for 1h. Pellets and supernatants were separated and boiled in the appropriate volume of SDS sample buffer and analyzed by Western blotting.

Surface biotinylation

Neuronal culture medium was supplemented with 1 mM biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimid-ester sodium salt (Sigma, Taufkirchen, Germany) and incubated for 20 min at 37°C. Cells were then washed with icecold PBS and lysed in PBS containing 1% TritonX-100 (Merck, Darmstadt, Germany) and protease inhibitor cocktail (Roche, Mannheim, Germany). 20μl of prewashed magnetic MyOne streptavidin beads (Dynal, Olso, Norway) were added to the extract, followed by incubation at 4°C for 3 h. Beads were washed extensively, collected and boiled in SDS sample buffer.

Electrophysiology

Measurements were done with an EPC9 patch-clamp amplifier controlled by PULSE software (HEKA). Series-resistance, usually between 5 and 15 MOhm, was compensated up to 75 %. Signals were filtered at a cut-off frequency (-3 dB) of 2.9 kHz with 4-pole Bessel characteristics and digitised at 200 – 650 microsecond sample intervals. Cells were constantly superfused at a low rate (0.15 ml min^{-1}) using a pump-driven and valve-controlled local superfusion system (Auto Mate Scientific). Current traces were analysed with PULSEFIT (HEKA) and AxoGraph 3 (Axon Instruments). For the analysis of miniature inhibitory postsynaptic currents (mIPSCs) between 98 and 747 events (detection threshold = noise SD x 2.5) per neuron were averaged.

Figure Legends

Supplementary Figure 1 (**A**) C-terminally GFP-tagged radixin (A2) displays a similar distribution as the endogenous protein (A1) in neurons. Both form clusters along dendrites. Scale bars: 5μm. (**B**) Hippocampal neurons expressing radixin-GFP were stained with anti-radixin antibody (red). Both fluorescent signals highly colocalize and the C-terminal tag does not interfere with the antibody binding site. B2 represents a magnification of the boxed region in B1. Scale bar: 30μm. (**C**) Neurons expressing radixin-GFP were stained for the GluR2 subunit. No significant colocalization with GluR2 is seen for radixin. Scale bar: 5 μm. (**D**) Non-transfected hippocampal neurons were immunostained with antibodies against radixin (green) and the membrane marker pan-Cadherin (red) (D1), phospho-ERM (green) and the membrane marker pan-Cadherin (red) (D2), and phospho-ERM (green) and radixin (red) (D3). Clusters of endogenous radixin are in most cases positive for the phosphorylated C-terminus in a dendritic region. Also, these clusters colocalize with the cell membrane marker pan-Cadherin. Scale bars: 3 μm. (**E**) Tagged $GABA_AR$ α5 is recognized by the anti-$GABA_AR$ α5 antibody. Labelling of total endogenous $GABA_AR$ α5 (E1) and surface clusters with anti-HA-antibody in cells expressing HA-$GABA_AR$ α5 (E2). E3 represents the overlay of E1 and E2. Arrows depict examples of overlapping immunoreactivities. Scale bar: 2μm. (**F**) Colocalization of wildtype radixin-GFP clusters at F-actin positive sites in cultured hippocampal neurons. F1 represents a magnification of the boxed area. Arrows depict F-actin positive radixin clusters, the arrowhead shows an actin-rich synapse. Scale bar: 15μm.

Supplementary Figure 2 Immunocytochemical detection of surface HA-$GABA_A$R α5 and colocalization with either ezrin-myc (**A**) or moesin-myc (**B**) in cultured hippocampal neurons expressing the respective constructs. A2 and B2 represent magnifications of the boxed areas. Only minor fractions of HA-$GABA_A$R α5 colocalize with ezrin-myc (8%) or moesin-myc (4%). Scale bars: 20μm.

Supplementary Figure 3 (**A**) Radixin depletion upon antisense-oligonucleotide microinjection. Hippocampal neurons were microinjected with radixin anti-sense oligonucleotides together with lucifer yellow dye. A dendrite from an injected cell is shown in A1. A2 is the same image without the green channel. In A3, a comparable dendrite from a non-injected control cell within the same field of vision is shown. (**B**) Surface $GABA_A$R α1 clusters in radixin-depleted cells. Hippocampal neurons were microinjected with radixin antisense-oligonucleotides together with lucifer yellow dye and stained for surface $GABA_A$R α1. A dendrite from an antisense-microinjected cell is shown in B1. The same image without the green channel is represented in B2. A non-injected control cell from the same field of vision is shown in B3. Scale bars: 3μm.

Supplementary Figure 4 (A) Control stainings with anti-$GABA_A$R α5 subunit antibody on hippocampal slices of WT (+/+) animals. Pretreatment of the antibody with a GST-α5-loop fusion protein results in effective blocking of the immunoreactive signal. **(B)** Control stainings for radixin on hippocampal slices of WT (+/+) and radixin knockout (-/-) mice. **(C)** Control stainings for the α3 subunit of the $GABA_A$ receptor on hippocampal slices of WT (+/+) and radixin knockout (-/-) mice. Scale bars: 80μm.

Supplementary Figure 5 (**A**) Differential centrifugation of lysates from HEK293 cells expressing different radixin constructs. Both the active and truncated forms of radixin associate with the plasma membrane (P2 pellet), whereas the non-activatable mutant is found in the supernatnant frations (S1, S2, S3) and finally in the cytoplasm (S4). **(B)** Hippocampal neurons were transfected with radixin-GFP K(253, 254, 262, 263)N either alone or together with myc-tagged $GABA_AR$ α5. At DIV 13, cells were fixed and immunostained. Live cell staining was performed in the case of myc-$GABA_AR$ α5 to ensure surface labeling. Both myc-tagged $GABA_AR$ α5 (large image) and endogenous $GABA_AR$ α5 (inlay) display normal clustering. Scale bar: 30μm.

Supplementary Figure 6 Site-directed mutagenesis of the radixin binding site in $GABA_AR$ α5. (**A**) Two different group mutants in the $GABA_AR$ α5 TMIII-TMIV intracellular loop region were generated by alanine replacement of residues. Both mutants fail to activate reporter gene transcription in yeast together with the originally identified radixin clone (mutant 1: Y343A, F344A, T345A; mutant 2: W349A, W351A). (**B**) GST pulldown experiment with radixin-myc T564D, expressed in HEK293 cells. This active form of radixin is able to bind to the wildtype TMIII-TMIV intracellular loop of $GABA_AR$ α5. Binding is reduced to about 30% when the group mutant 1 (Y343A, F344A, T345A) or mutant 2 (W349A, W351A) are introduced to the GST-fusion construct of the receptor loop. (**C**) Coomassie staining of the inputs of GST and WT and mutant alpha5 loop fusion proteins (**D**) Expression of myc-tagged wild-type and quintuple mutant constructs in cultured hippocampal neurons. The mutant construct combines both group mutations shown in (**A**) (Y343A, F344A, T345A, W349A, W351A). Note that the wild-type receptor construct displays

clusters along dendrites (left), whereas immunoreactivity is reduced to background levels for the mutant construct. Scale bars: 5μm.

References

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