CONTACTIN ASSOCIATED PROTEIN 1 (Caspr1) REGULATES THE TRAFFIC AND SYNAPTIC CONTENT OF α-AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLEPROPIONIC ACID (AMPA)-TYPE GLUTAMATE RECEPTORS
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Running Title: Caspr1 Regulates AMPA Receptors

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Keywords: AMPA receptors/Caspr1/glutamate/hippocampal neurons/synapse

Background: The strength of excitatory synapses is determined by the synaptic content of AMPA receptors.

Results: We found that Contactin associated protein 1 (Caspr1) binds to AMPA receptors and regulates their neuronal cell surface and synaptic expression.

Conclusion: Caspr1 is a binding partner for AMPA receptors, which regulates their traffic and synaptic targeting.

Significance: Caspr1 is a new player in plasticity mechanisms in excitatory synapses.

SUMMARY

Glutamate receptors of the AMPA-type mediate fast excitatory synaptic transmission in the CNS. Synaptic strength is modulated by AMPA receptor binding partners, which regulate receptor synaptic targeting and functional properties. We identify Contactin associated protein 1 (Caspr1) as an AMPA receptor interactor. Caspr1 is present in synapses, and interacts with AMPA receptors in brain synaptic fractions. Co-expression of Caspr1 with GluA1 increases the amplitude of glutamate-evoked currents. Caspr1 overexpression in hippocampal neurons increases the number and size of synaptic GluA1 clusters, whereas knockdown of Caspr1 decreases the intensity of synaptic GluA1 clusters. Hence, Caspr1 is a regulator of the trafficking of AMPA receptors to synapses.

Regulation of the function of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors is critical for long-lasting changes in synaptic strength, considered to underlie higher brain functions such as learning and memory (1). Protein–protein interactions and post-translational modification of AMPA receptor subunits regulate receptor function by modulating the localization of receptors at synapses, and/or modifying receptor channel properties (2). Recent studies have identified AMPA receptor auxiliary proteins, which influence various aspects of excitatory synapse function (3). The transmembrane AMPA receptor regulatory proteins (TARPs) regulate the trafficking and channel properties of AMPA receptors (3). Other transmembrane proteins affect AMPA receptor gating, such as the cornichons (4), and cysteine-knot AMPA receptor modulating protein of 44 kDa [CKAMP44 (5)]. In a recombinant system, the

1 The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CKAMP44, cysteine-knot AMPA receptor modulating protein of 44 kDa; Caspr1, Contactin associated protein 1; PSD, post-synaptic density; SynDIG1, Synapse Differentiation Induced Gene 1; TARP, transmembrane AMPA receptor regulatory protein.
cornichon protein CNHI-2 abrogates the γ8 TARP-mediated resensitization of AMPA receptors upon prolonged application of glutamate (6), suggesting different classes of auxiliary subunits can co-regulate AMPA receptors. Moreover, proteins such as TARPs and Synapse Differentiation Induced Gene 1 [SynDIG1 (7)] affect the cell surface and synaptic expression of AMPA receptors. Fine-tuning of the receptor gating kinetics and synaptic expression by auxiliary proteins may underlie the different kinetics of gating observed for native AMPA receptors, and may depend on the expression pattern of auxiliary subunits, those which have been described and additional ones, yet to be identified.

We searched for novel AMPA receptor binding partners using a pull-down assay in rat cerebellum extracts, with the intracellular C-terminus of GluA4 as bait. Mass spectrometry analysis of the purified proteins suggested an interaction of GluA4 with the transmembrane protein Contactin associated protein 1 (Casp1). Biochemical studies confirmed the interaction between Casp1 and AMPA receptors in the rat brain. Co-expression of Casp1 with GluA1 increases glutamate-evoked currents. Furthermore, Casp1 is localized to dendrites and excitatory synapses in hippocampal neurons, and regulates the cell surface and synaptic expression of GluA1-containing AMPA receptors.

EXPERIMENTAL PROCEDURES

Antibodies - The anti-GFP polyclonal antibody was purchased from MBL International Corporation (Woburn, USA), the anti-GFP monoclonal antibody was acquired from Roche Diagnostics (Carnaxide, Portugal), and the anti-actin antibody was purchased from Roche Molecular Biochemicals (Indianapolis, USA). The anti-GluA1 polyclonal antibody was from Tocris Bioscience (Missouri, USA) and the sheep anti-GluA1 N-terminal antibody was a kind gift from Dr. Andrew Irving (University of Dundee, Scotland). The anti-GluA2/3, the anti-GluN1 and the anti-PSD95 antibodies were from Millipore (Madrid, Spain), and the anti-MAP2 antibody was from Abcam (Cambridge, UK). The anti-synaptophysin antibody was purchased from Synaptic Systems (Germany).

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For plasmid-based RNA inhibition of Casp1, the complementary oligonucleotides that target nucleotides 2254-2273 (GAACAGCATTTCTACTGGG) of rat Casp1 (NM_032061) were annealed and ligated into the HpaI/XhoI sites of the U6 promoter-driven short hairpin RNA expression vector pLentiLox3.7(CMV)EGFP, which expresses EGFP under the CMV promoter. The construct for expressing Casp1* resistant against Casp1-shRNA was generated by making the following two point mutations, indicated by underlines, in the shRNA-targeting site: GAGCAGCATTTCTATGGG. All the constructs were verified by DNA sequencing.

Rat cerebellum total extracts - The rat cerebellum was homogenized in 10 volumes of ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 320 mM sucrose. The homogenate was centrifuged at 700xg, for 10 min, at 4°C. The pellet was homogenized again in the same Tris buffer and centrifuged at 700xg, for 10 min, at 4°C. Both supernatants were pooled, supplemented with protease inhibitors and submitted to protein quantification by the BCA
Production of recombinant proteins - The cDNA encoding the C-terminus of Caspr1 was amplified by RT-PCR from total RNA isolated from rat brain cerebellum, using the specific primers 5’-ccgctcgagtcattcagacctggactc-3’ and 5’-cgcggatcccaaaatcatcgatacaag-3’, with the restriction sites for BamHI and XhoI, respectively. The PCR product was subcloned in the pGEX-4T-2 vector and the construct was sequenced. The plasmid encoding the intracellular carboxy-terminal domain of GluA4 fused to GST has been previously described (8). Recombinant proteins were expressed in BL21 Escherichia coli as described before (8).

Pull-down assays - Rat cerebellum lysates were diluted with RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5), supplemented with 1 mM DTT and a cocktail of protease inhibitors, and sonicated. After centrifugation, the supernatant was incubated with 50 µl of Glutathione Sepharose, 30 min at 4°C. The supernatant was split in two tubes, one was incubated with 50 µg of GST and the other with 50 µg of the fusion protein of interest, at 4°C for 3 h. Fifty µl of RIPA-equilibrated Glutathione Sepharose were added to both samples and incubated at 4°C for 30 min. The samples were washed 4x with RIPA and the proteins were eluted by boiling at 95°C in 50 µl of sample buffer, separated by SDS-PAGE and stained with silver nitrate.

MALDI peptide mass fingerprinting and database searching - Protein bands were excised manually from the gel, and digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany), according to a previously described protocol (9). For peptide mass fingerprinting (10) spectra acquisition, an aliquot of α-cyano-4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid was mixed with an aliquot of the digestion solution and the mixture was deposited onto an AnchorChip MALDI probe (Bruker-Daltonics). MALDI Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics). Mass measurements were performed in positive ion reflector mode using 140 ns delayed extraction and a nitrogen laser (337 nm). The laser repetition rate was 50 Hz and the ion acceleration voltage was 25 kV. Mass measurements were performed automatically through fuzzy logic based software to accumulate 100 single laser shot spectra or manually to accumulate ca. 200 single laser shot spectra. Each spectrum was internally calibrated with the mass signals of two trypsin autolysis ions: [VATVSLPR+H]+ (m/z = 842.510) and [LGEHNIDVLEGNEQFINAAK+H]+ (m/z = 2211.105) to reach a typical mass measurement accuracy of ±30 ppm. Known trypsin and keratin mass signals, as well as potential sodium adducts (+21.982 Da) or signals arising from methionine oxidation (+15.995 Da) were removed from the peak list. The measured tryptic peptide masses were transferred through MS BioTools program (Bruker-Daltonics) as inputs to search the NCBInr database using Mascot software (Matrix Science, London, UK).

This analysis was performed at the Unidad de Proteómica, CNIC, Madrid.

Immunoprecipitation assays - For co-immunoprecipitation assays, lysates of COS 7 cells expressing the proteins of interest, or rat brain synaptosomes (1 mg), were solubilized in Immunoprecipitation Buffer [IPB: 10 mM Tris (pH 7.0), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1% Triton X-100, supplemented with protease inhibitors]. The samples were sonicated on ice, for 30 s, and the insoluble material was removed by centrifugation at 4°C. Soluble extracts were incubated with 100 µl of a 50% slurry of protein A Sepharose beads suspended in IPB, at 4°C, for 1 hour, to pre-absorb proteins that stick non-specifically to the protein A Sepharose beads. The supernatant was incubated either with the antibody of interest or with the same amount of non-immune IgGs at 4°C, for 3h, and was then incubated with 100 µl of a 50% slurry of protein A Sepharose beads (2h at 4°C). The beads were washed sequentially in IPB + 1% Triton (2x), in IPB + 1% Triton + 500 mM NaCl (3x) and in IPB (2x). The proteins were eluted by boiling in sample buffer, separated by SDS-PAGE and analysed by western-blot.
Gel electrophoresis and western-blot - Samples were resolved by SDS-PAGE in 7.5% polyacrylamide gels. For western blot analysis, proteins were transferred onto a PVDF membrane (Millipore, Madrid, Spain) by electroblotting. The membranes were blocked, incubated with primary and secondary antibodies, and immunostaining was visualized by the enhanced chemifluorescence method (ECF) on a Storm 860 Gel and Blot Imaging System (GE Healthcare, Carnaxide, Portugal).

Hippocampal and cortical cultures - Primary cultures of rat hippocampal and cortical neurons were prepared from the hippocampi or cortices of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, 37 °C; GIBCO Invitrogen), in Ca²⁺- and Mg²⁺-free Hank’s balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH₂PO₄, 137 mM NaCl, 4.16 mM NaHCO₃, 0.34 mM Na₂HPO₄·2H₂O, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampal cells were washed with 10% fetal bovine serum prepared in HBSS, to stop trypsin activity, and then washed with HBSS to remove serum and avoid glia growth. Cortical cells were washed with HBSS 6 times. Cells were transferred to Neurobasal medium (GIBCO Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin; the cells were mechanically dissociated and then plated in 6 well plates (1x10⁵ cells/cm² for cortical neurons), coated with poly-D-lysine (0.1 mg/ml). For imaging purposes, low density hippocampal cells were plated at a final density of 3 x 10⁵ cells/dish on poly-D-lysine-coated coverslips, in 60 mm culture dishes, in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid). After 2-4 h, coverslips were flipped over an astroglial feeder layer. These neurons grew face down over the feeder layer but were kept separate from the glia by wax dots on the neuronal side of the coverslips. To prevent the overgrowth of the glia, neuron cultures were treated with 5 μM cytosine arabinoside after 3 days in vitro (DIV). Cultures were maintained in Neurobasal medium supplemented with B27 supplement, in a humidified incubator of 5% CO₂/95% air, at 37°C.

Neuron transfection - Constructs were recombinantly expressed in primary cultures of hippocampal neurons using the calcium phosphate transfection protocol [adapted from (11)]. Briefly, a CaCl₂ solution (2.5 M in 10 mM HEPES) was added, drop-wise, to plasmid DNA to a final concentration of 250 mM CaCl₂. This was then added to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 11 mM dextrose, and 42 mM HEPES, pH 7.2). The mixture was vortexed gently for 2 to 3 s, and the precipitate was allowed to develop at room temperature for 30 min. The precipitated DNA was added drop-wise to the coverslips, and the cultures were incubated for 1 to 3 h in the presence of kynurenic acid (2 mM). Each coverslip was transferred to a fresh well of the 24-well plate containing 1 ml of culture medium with kynurenic acid (2 mM), slightly acidified with HCl (~5 mM final concentration), and the plate was incubated at 37°C in 5% CO₂ for 10 to 15 min. Coverslips were then transferred to a fresh well of the 24-well plate containing conditioned medium, and incubated at 37°C in 5% CO₂ to allow expression of the transfected constructs.

Subcellular fractionation - For purification of rat brain synaptosomes, two rat brains were removed, placed in 5 volumes of ice-cold solution A (5 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, 0.31 M sucrose, pH 7.4 supplemented with 0.2 mM PMSF, 1 mM DTT and a cocktail of protease inhibitors) and homogenized with a Potter Elvehjem (8 strokes). The homogenates were centrifuged at 14000xg for 10 min. The supernatant solution was saved, and the pellet was rehomogenized in 5 volumes (considering the initial weight) of solution A and centrifuged at 710xg for 10 min. Supernatants were pooled and centrifuged at 13800xg for 10 min. The pellet was resuspended in 24 ml/10g of Buffer B (6 mM Tris, pH 8.1, 0.32 M sucrose, supplemented with protease inhibitors). This fraction (P2) was used directly for immunoprecipitation studies.

The procedure for purification of synaptic fractions from cultured cortical neurons was adapted from (12), and modified according to the protocol described by (13) for high density cultured neurons. Post-synaptic densities (PSDs) were isolated from 15 DIV high-density
cortical rat neurons. Briefly, $38 \times 10^6$ cortical cells were collected and homogenized in HEPES-buffered sucrose (HBS) solution (0.32 M sucrose, 4 mM HEPES, pH 7.4) containing protease and phosphatase inhibitors (0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM sodium orthovanadate [SO], 0.5 mM sodium fluoride [NaF], 1µg/ml chymostatin, 1µg/ml leupeptin, 1µg/ml antipain, 1µg/ml pepstatin [CLAP]). Culture homogenate was collected and centrifuged at 900×g for 15 min to obtain the non-nuclear fraction (S1). The resultant supernatant was centrifuged at 18,000×g for 15 min to yield the crude synaptosomal pellet (P2). P2 was resuspended in HBS and centrifuged at 18,000×g for 15 min to yield the washed crude synaptosomal fraction. This fraction was submitted to hypo-osmotic shock by resuspending the pellet in HEPES buffer (4 mM HEPES, pH 7.4, plus protease and phosphatase inhibitors) and incubated 1 to 2 hours with orbital rotation at 4°C. The lysate was centrifuged at 25,000×g for 20 min and the pellet (lysed synaptosomal membrane fraction) was resuspended in HBS (without SO), placed on top of a discontinuous sucrose gradient (0.8 M, 1 M, 1.2 M) and spun at 150,000×g for 2 h in a swinging bucket rotor. Synaptic plasma membranes (SPM) were recovered between the 1.0 M and 1.2 M layers, diluted to 0.32 M Sucrose, and centrifuged at 150,000×g for 30 min. SPMs were resuspended in HEPES/EDTA (HE) buffer (50 mM HEPES, 2 mM EDTA, pH 7.4) containing protease and phosphatase inhibitors, and solubilized in 0.5% Triton X-100 for 20 min by moving the theta tube laterally with a piezoelectric device, under computer control.

Electrophysiology - HEK293 cells were transfected using FUGENE 6 with GFP and GluA1, with or without Caspr1 at a cDNA ratio of 1:1. Two to three days after transfection, cells were bathed in Hepes-buffered solution (HBS) containing (in mM): 145 NaCl, 2 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 10 glucose, and 10 Hepes, adjusted to 320 mOsm per liter and pH 7.4 with NaOH, at room temperature. Whole-cell recordings were performed on green fluorescent cells lifted off the coverslip, placed under the flow of a theta tube and held at -80 to -40 mV. Recording pipettes (resistance 3-5 MΩ) were filled with a solution containing (in mM): 130 CsCH$_3$SO$_3$, 2 NaCl, 2 MgCl$_2$, 10 EGTA, 10 HEPES, 4 Na$_2$ATP, adjusted to 310 mOsm per liter, and pH 7.2 with CsOH. Currents were evoked by long application of 10 mM glutamate for 100 ms or 1 ms, every 20 s by moving the theta tube laterally with a piezoelectric device, under computer control.

Immunocytochemistry, culture imaging and quantitative fluorescence analysis - Neurons were fixed for 15 min in 4% sucrose/4%paraformaldehyde in PBS at room temperature, and permeabilized with PBS + 0.25% Triton X-100 for 5 min, at 4°C. The neurons were then incubated in 10% BSA in PBS for 30 min at 37°C to block nonspecific staining, and incubated with the indicated primary antibodies diluted in 3% BSA in PBS (2h, 37°C). After washing in PBS, cells were incubated with the secondary antibody diluted in 3% BSA in PBS (45 min, 37°C). The coverslips were mounted using fluorescent mounting medium from DAKO (Glostrup, Denmark). For labeling surface GluA1-containing receptors, live neurons were incubated for 10 min at room temperature with the GluA1 N-terminal antibody diluted in PBS, after which the cells were briefly rinsed in PBS, fixed and probed as described above. Imaging was performed on a Zeiss Axiovert 200 M microscope, using a 63× 1.4 NA oil objective. Images were quantified using image analysis software (ImageJ). For quantitation, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The region of interest was randomly selected and the dendritic length was measured using the MAP2 staining. For Caspr1 measurements, the PSD95 and VGLUT signals were thresholded, dilated, and their colocalization was determined. The Caspr1...
signal was measured after thresholds were set, such that recognizable clusters were included in the analysis, and the Caspr1 signal present in glutamatergic synapses was obtained by measuring the Caspr1 puncta positive for both PSD95 and VGLUT. In order to calculate the fraction of glutamatergic synapses containing Caspr1, the number of synapses per dendritic length was determined by colocalizing the thresholded PSD95 and VGLUT signals; the number of glutamatergic synapses containing Caspr1, per dendritic length, was determined by identifying PSD95- and VGLUT-positive clusters that are also labeled for Caspr1. For quantifying the GluA1 signal, fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons. The cell surface GluA1 digital images were subjected to a user defined intensity threshold to select clusters and measured for cluster intensity, number, and area for the selected region. The synaptic GluA1 clusters were selected by their overlap with thresholded and dilated PSD95 signal. Measurements were performed in a minimum of three independent preparations, and at least 9 cells per condition were analysed for each preparation.

RESULTS

Contactin associated protein 1 (Caspr1) interacts with AMPA receptors

To identify proteins that specifically interact with the C-terminus of the long splice isoform of GluA4, pull-down experiments were performed using GST fused to the C-terminus of rat GluA4 (GST-CTA4) and adult rat cerebellum lysate, since GluA4 is particularly abundant in this brain region (14). MALDI Peptide mass fingerprint analysis identified Contactin associated protein 1 (Caspr1) and its binding partner Contactin1 (Fig. 1A, Supp. Figs. 1, 2) among the proteins that were exclusive to the GST-CTA4 condition. Contactin1 is a glycosylphosphatidyl inositol (GPI)-anchored member of the immunoglobulin gene superfamily, which has been previously implicated in synaptic plasticity (15). Caspr1, also known as paranodin, is a 190 kDa type I transmembrane protein, highly expressed in the brain. It has an extracellular architecture similar to neurexins and an intracellular region containing a domain that binds to 4.1 band proteins and a proline rich sequence (16,17). Mutations in the Caspr1 family member Caspr2 are implicated in autism spectrum disorders (18,19).

We used additional biochemical assays to confirm the interaction between Caspr1 and GluA4, and tested whether the interaction is specific to GluA4. Caspr1 coprecipitated with Flag-tagged GluA4 from co-transfected heterologous cells (Fig. 1B), whereas precipitation of Caspr1 was not observed when using non-immune immunoglobulins. Notably, Caspr1 also coimmunoprecipitated with co-transfected AMPA receptor subunits GluA1, GluA2 or the longer splice isoform of GluA2, GluA2L, but not with GFP-TrkB (Fig. 1B). To determine whether these interactions also occur in the brain, we used synaptosomes isolated from adult rat brain to immunoprecipitate GluA4, GluA1 or the NMDA receptor subunit GluN1 (Fig. 1C). Caspr1 coimmunoprecipitated with both GluA1 and GluA4 isolated from synaptosomes, but not with GluN1, and immunoprecipitation of Caspr1 resulted in co-immunoprecipitation of GluA1 (Fig. 1C). These results confirm that Caspr1 interacts with AMPA receptor subunits in the rat brain, and that synaptically localized AMPA receptors interact with Caspr1.

Since we identified Caspr1 as a potential GluA4 interactor using the intracellular C-terminus of GluA4, we tested whether the interaction between Caspr1 and GluAs occurs between the intracellular regions of these proteins. The intracellular C-terminus of Caspr1 fused to GST (GST-CTCaspr1) pulled-down GluA1 and GluA2 from rat cerebellum lysates (Fig. 1D), confirming a role for this region in the interaction with AMPA receptors.

Caspr1 localizes to dendrites and synapses

Caspr1 plays important roles in the correct assembly of the paranodes (20), but there are evidences that it is also present in dendrites and enriched in postsynaptic density (PSD) fractions (15). Immunolabeling of cultured hippocampal neurons (15 DIV) for Caspr1 showed that the protein is distributed throughout dendrites, and forms clusters that partially colocalize with the glutamatergic synapse markers PSD95, a postsynaptic
scaffold, and VGLUT1, a presynaptic vesicular glutamate transporter (Fig. 2A). To evaluate the presence of Caspr1 at excitatory synapses, we identified regions of overlap between the PSD95 and the VGLUT signals, and measured the Caspr1 signal at these sites (Fig. 2B). We found that 48.7±4.4% of the clusters positive for both PSD95 and VGLUT contain Caspr1 (Fig. 2B). Furthermore, Caspr1 was present in the PSD95-enriched PSDs isolated from 15 DIV cultured cortical neurons (Fig. 2C). Accordingly, Caspr1 was previously detected in the PSD fraction isolated from adult mouse brain (15). Taken together these evidence point to a dendritic localization of Caspr1 in neurons, and to synaptic localization of a fraction of the protein.

Caspr1 regulates the synaptic expression of AMPA receptors

Caspr1 was initially identified as a binding partner for GluA4 in the cerebellum (Fig. 1A), but subsequent analysis found that Caspr1 also binds GluA1 and GluA2 AMPA receptor subunits (Figs. 1B-D). Since GluA1 is widely expressed in the central nervous system, namely in the hippocampus, where GluA4 has a more limited expression pattern (21), and given the evidences pointing to an important role for the GluA1-containing AMPA receptors in plasticity events (22), we focused on a possible regulation of synaptic GluA1-containing AMPA receptors by Caspr1 in the hippocampus. To explore the effects of the interaction between AMPA receptors and Caspr1 on the cell surface and synaptic expression of GluA1-containing AMPA receptors, we performed quantitative immunofluorescence analysis of the expression of synaptic cell surface GluA1 in hippocampal neurons overexpressing Caspr1. Hippocampal neurons cultured at low-density were transfected with EGFP alone or together with Caspr1, and live-stained with an antibody against the N-terminal extracellular region of GluA1. After fixation, neurons were stained with an antibody for PSD95, to visualize excitatory synapses. Compared with neurons transfected with EGFP alone (Fig. 3A), neurons overexpressing Caspr1 (Fig. 3B) showed a significant increase in the fluorescence intensity of total (Fig. 3D) and PSD95-colocalized (Fig. 3E) GluA1 clusters. The density of GluA1 synaptic clusters was also increased in Caspr1-transfected neurons, (Fig. 3F). Since the C-terminus of AMPA receptor subunits in involved in the interaction with Caspr1, we co-transfected hippocampal neurons with EGFP and a plasmid encoding the C-terminus of Caspr1 fused to the extracellular and transmembrane domains of the T-cell surface protein CD4, a transmembrane reporter (CD4-CTCaspr1, Fig. 3C). The CD4-CTCaspr1 construct has been previously used (23), and the encoded protein is targeted to the cell surface. Neurons overexpressing CD4-CTCaspr1 showed a significant increase in the fluorescence intensity of total GluA1 clusters (Fig. 3D), as well as an increase in the fluorescence intensity and density of GluA1 synaptic clusters, compared to EGFP-transfected cells (Figs. 3E, F). These observations suggest a role for Caspr1 in increasing the levels of AMPA receptors at the neuronal surface and at synapses, through a mechanism that can be mediated by its C-terminus.

Caspr1 increases glutamate-evoked currents mediated by GluA1

In order to test the functional consequences of the association of AMPA receptors with Caspr1, we expressed GluA1 with or without Caspr1 in HEK293 cells, and recorded whole-cell currents activated by fast application of glutamate at a saturating concentration (10 mM), with either a long (100 ms) pulse revealing desensitization properties, or a short (1 ms) pulse indicative of the deactivation kinetics (24). Although the amplitude of AMPA receptor-mediated currents was variable from cell to cell, co-expression of Caspr1 significantly increased the average amplitude of currents evoked by glutamate (527±124 pA, n=16 vs 2459±622 pA, n=16 with Caspr1, p<0.05) without any effect on the desensitization or deactivation kinetics (Fig. 4). These results indicate that Caspr1 increases the expression of recombinant GluA1-containing AMPA receptors at the cell surface without modifying their kinetics.

Knockdown of Caspr1 reduces synaptic GluA1, an effect rescued by the C-terminus of Caspr1
The association of Caspr1 with AMPA receptors suggests that Caspr1 might be required for the expression of AMPA receptors at synapses. To test this possibility, a short-hairpin RNA (shRNA) sequence against rat Caspr1 mRNA was introduced in the pLentiLox3.7(CMV)EGFP vector. The Caspr1-shRNA construct decreased the expression of synaptic Caspr1 to around 32% of the endogenous expression level in transfected hippocampal neurons identified by the expression of GFP, and compared to neurons transfected with the empty vector (Supp. Fig. 3). Neurons transfected with Caspr1-shRNA showed a 34.0 ± 3.6% decrease on the intensity of cell surface total GluA1 clusters, and a 38.4 ± 3.6% decrease of PSD95-colocalized cell surface GluA1 clusters, when compared to neurons transfected with the control plasmid (Figs. 5A,B,E,F). The decrease in the fluorescence intensity of GluA1 clusters was accompanied by a decrease in the area of clusters in neurons transfected with Caspr1-shRNA, whereas the number of GluA1 clusters was not changed (data not shown). In order to exclude the contribution of off-target effects of the Caspr1-shRNA, a rescue construct was generated with silent mutations in the Caspr1 region targeted by the Caspr1-shRNA (Caspr1*). Neurons were co-transfected with the Caspr1-shRNA plasmid and the Caspr1* construct refractory to Caspr1-shRNA-mediated knockdown, and analysed for the cell surface expression of GluA1 (Fig. 5C,E,F). Indeed, the expression of the Caspr1* construct rescued the Caspr1-shRNA-mediated decrease on the fluorescence intensity of the cell surface total and synaptic clusters of GluA1. These results indicate that the defects observed for synaptic GluA1 levels with Caspr1-shRNA are specifically due to the loss of Caspr1. The reduction in the intensity of cell surface GluA1 clusters was also fully rescued by expression of the C-terminus of Caspr1 (CD4-CTCaspr1, Fig. 5D-F), indicating that endogenous Caspr1 is required for maintaining the cell surface and synaptic expression of GluA1, through a mechanism dependent on its C-terminal domain.

DISCUSSION

Alterations in the functional properties and in the number of AMPA receptors at synapses are central to the processes of synaptic plasticity that underlie learning and memory formation (1). The number of AMPA receptors at synapses is regulated by receptor interaction with intracellular scaffold proteins (2) and transmembrane proteins such as TARPs, cornichons, SynDIG1 and CKAMP44 (3). TARPs affect the export of AMPA receptors from the ER and their synaptic traffic, as well as receptor gating properties [reviewed in (3)]. Similarly, the cornichon proteins enhance the surface expression of AMPA receptors, and slow down receptor deactivation and desensitization kinetics (4), whereas CKAMP44 does not affect the cell surface expression of AMPA receptors, or the amplitude of mEPSCs, but leads to stronger and faster AMPA receptor desensitization (5). SynDIG1 increases the frequency and amplitude of mEPSCs, and increases GluA1 synapse density and area in hippocampal neurons (7). In this work we identify Caspr1 as a novel interactor for AMPA receptor subunits which, akin to SynDIG1, increases the synaptic content of GluA1.

Immunocytochemical studies in mature cultures of rat hippocampal neurons confirmed that Caspr1 distributes throughout dendrites, and >45% of glutamatergic synapses, identified by coincident staining for PSD95 and VGLUT, contain Caspr1 (Figs. 2A, B). These observations are in agreement with previous immunohistochemical studies in mouse hippocampal slices (15) which showed extensive colocalization of Caspr1 with MAP-2, as well as localization to membrane structures apposed to synaptophysin-positive axon terminals. Analysis of biochemically purified postsynaptic densities from cultured cortical neurons showed an enrichment of Caspr1 in the PSD (Figure 2C), in conformity with the studies by Murai and co-authors (15), and with the proteomic analysis of the PSD (25). At the paranodes of myelinated axons, Caspr1 regulates the segregation of Na⁺ and K⁺ channels to the nodes of Ranvier and to the juxtaparanodes, respectively (20). The presence of Caspr1 in glutamatergic synapses (Fig. 2) and its interaction with AMPA receptor subunits, both in the brain and in heterologous system (Fig. 1), are compatible with a novel
postsynaptic function for Caspr1 in regulating AMPA receptors.

In fact we demonstrated that co-expression of Caspr1 with GluA1-homomeric AMPA receptors increases the amplitude of glutamate-evoked currents (Fig. 4), and that overexpression of Caspr1 in hippocampal neurons increases the synaptic localization of GluA1-containing AMPA receptors (Fig. 3). On the other hand, endogenous Caspr1 is required for the synaptic localization of AMPA receptors, since knockdown of Caspr1 specifically reduces synaptic GluA1 (Fig. 5).

How does Caspr1 regulate the localization of AMPA receptors at the cell surface and at synapses? One possibility is that Caspr1, through its interaction with 4.1 band proteins (17), may reinforce the link between AMPA receptors and 4.1 band proteins, interactors for GluA1 and GluA4 subunits (26,27). In fact it has been found that the synaptic targeting of SAP97, another molecule involved in AMPA receptor traffic, depends on its interaction with the protein 4.1 (28). At the paranodal junction, Caspr1 connects through protein 4.1B to cytoskeletal components within the axon (29), including molecules such as ankyrinB, αII spectrin and βII spectrin (30). Interestingly, the interaction of Caspr1 with 4.1B is necessary for the generation of an efficient membrane barrier at the paranodal junction (31). Similarly, the association of Caspr1 with postsynaptic cytoskeleton proteins may allow it to modulate the interaction of AMPA receptors with specialized components of the cytoskeleton at the postsynaptic scaffold. Alternatively, the proline-rich region at the intracellular C-terminus of Caspr1 may provide anchoring to other binding partners at the PSD.

In summary, our data support a model in which Caspr1 regulates the content of AMPA receptors at synapses in hippocampal neurons in culture. Caspr1 may constitute a new member of the growing list of AMPA receptor auxiliary proteins.

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REFERENCES


FIGURE LEGENDS

Figure 1. Caspr1 associates with AMPA receptors. (A) Caspr 1 was pulled-down from rat cerebellum by the GluA4 C-terminus. Rat cerebellum lysate was incubated with GST fused to the intracellular C-terminal region of GluA4 (GST-CTA4) or with GST alone. The GST fusion proteins and associated proteins were purified, separated by SDS-PAGE and silver-stained. The protein bands exclusive to the pull-down assay with the GluA4 C-terminus were excised from the gel and the proteins were identified by MALDI Peptide mass fingerprint. (B) Caspr1 interacts with AMPA receptor subunits in heterologous cells. AMPA receptor subunits (GluA4-Flag, GluA1, GluA2L-myc or GluA2) or TrkB-GFP were co-transfected with Caspr1 in COS 7 cells. AMPA receptor subunits were immunoprecipitated using specific antibodies, as indicated, and Western blot analysis revealed that Caspr1 co-immunoprecipitated with AMPA receptor subunits. Immunoprecipitation of TrkB-GFP from cells co-transfected with Caspr1 and TrkB-GFP did not co-immunoprecipitate Caspr1. (C) Caspr1 and AMPA receptors associate in brain. Caspr1 co-immunoprecipitated with GluA4 and GluA1, but not with GluN1, from rat brain synaptosomes. Conversely, GluA1 was co-immunoprecipitated with Caspr1 from rat brain synaptosomes. (D) Caspr1 associates with AMPA receptors through its C-terminus. GluA1 and GluA2 subunits were pulled-down from rat cerebellum lysates when the C-terminus of Caspr1 was used as bait. Lower panel, Coomassie staining of GST fusion proteins.

Figure 2. Caspr1 localizes to synapses in hippocampal neurons and to cortical postsynaptic density fractions. (A, B) Caspr1 is present in excitatory synapses. Caspr1 is expressed in dendrites in hippocampal neurons (15 DIV), and is significantly colocalized with PSD95 and with VGLUT1 (A; Scale bars: 2 \( \mu \)m). Arrow heads point to synapses that contain Caspr1. Full arrow indicates a PSD95/VGLUT-positive synapse that lacks Caspr1. (B) Quantification of the number of dendritic clusters per dendritic length which are positive for both PSD95 and VGLUT (“glutamatergic synapses”), and of the number of glutamatergic synapses that contain Caspr1 (PSD95’/VGLUT’/Caspr1’). (C) Caspr1 localizes to postsynaptic density fractions. PSD fractions isolated from 15 DIV rat cortical neurons were analysed for the presence of Caspr1, GluA1, PSD95, synaptophysin and actin, as indicated.

Figure 3. Caspr1 overexpression promotes the synaptic expression of GluA1. Hippocampal neurons were transfected at 7 DIV with (A) EGFP, (B) Caspr1 and EGFP, or (C) CD4-CTCaspr1 and EGFP, and live stained at 15 DIV for cell surface GluA1. After fixation, neurons were stained for PSD95. Synaptic GluA1 is defined as GluA1 signal that overlaps with PSD95. Transfected neurons, identified by GFP fluorescence, were analysed for the total GluA1 cell surface fluorescence intensity (D), the GluA1 synaptic cluster fluorescence intensity (E) and number (F) per dendritic length. Results are presented as \% of GFP-transfected cells, and are averaged from three independent experiments (n\geq28 cells). Error bars, \pm S.E.M. Significance, ANOVA followed by Bonferroni’s Multiple Comparison Test (*p<0.05, **p<0.01), relative to GFP-transfected neurons. Scalebars: 10 \( \mu \)m, 2 \( \mu \)m for enlarged images.
Figure 4. (A-C) Caspr1 enhances GluA1 receptor currents. (A) Effect of sustained (100 ms, left traces) or brief (1 ms, right traces) applications of saturating glutamate concentration (10 mM) on GluA1-mediated currents, with or without Caspr1. (B) Plot of the amplitude of GluA1 currents (n=16) and GluA1 co-expressed with Caspr1 (n=16). Currents are activated by a long (100 ms) application of 10 mM glutamate. The asterisk indicates significant (*t* test, **p** < 0.005) difference between GluA1 and GluA1 co-expressed with Caspr1. (C) Effect of Caspr1 on GluA1 desensitization and deactivation, for long or short glutamate (10 mM) applications, respectively. Statistical analysis reveals non significant differences (ns) (*t* test, p>0.05). Error bars indicate SEM.

Figure 5. Loss of Caspr1 decreases the synaptic content of GluA1. Hippocampal neurons in culture were transfected at 7 DIV with pLentiLox3.7 (A), with the Caspr1-shRNA construct (B), or co-transfected with the Caspr1-shRNA and (C) the mutant Caspr1* construct resistant to the Caspr1-shRNA or (D) CD4-CTCaspr1. Neurons were live-stained at 15 DIV for GluA1, fixed and immunostained for PSD95. Transfected neurons, identified by GFP fluorescence, were analysed for the fluorescence intensity of the total (E) and PSD95-colocalized (F) GluA1 clusters. Results are presented as % of pLentiLox3.7-transfected cells (3 independent experiments, n≥30 cells). Error bars, ± S.E.M. Significance, ANOVA followed by Bonferroni’s Multiple Comparison Test (***(p<0.001), relative to neurons transfected with pLentiLox3.7. Scalebars: 10 µm, 2 µm for enlarged images.
Figure 1
Figure 2
Figure 3

A. Control  B. Caspr1  C. CD4-CTCaspr1

D. Intensity of total surface GluA1 clusters/Dendritic length (% of control)

E. Intensity of GluA1 clusters colocalized with PSD95/Dendritic length (% of control)

F. Number of GluA1 clusters colocalized with PSD95/Dendritic length (% of control)
Figure 4
Figure 5

A. Control  B. Caspr1-shRNA  C. Caspr1-shRNA + Caspr1*  D. Caspr1-shRNA + CT-Caspr1

GFP

Surface GluA1

PSD95

Surface GluA1 + PSD95

E
F

Intensity of total surface GluA1 clusters/ Dendritic length (% control)

Intensity of GluA1 clusters colocalized with PSD95/ Dendritic length (% of control)

Empty vector  Caspr1-shRNA  ShRNA+Caspr1*  ShRNA+CT-Caspr1
Supplemental Information

CONTACTIN ASSOCIATED PROTEIN 1 (Caspr1) REGULATES THE TRAFFIC AND SYNAPTIC CONTENT OF α-AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLEPROPIONIC ACID (AMPA)-TYPE GLUTAMATE RECEPTORS

Sandra D. Santos, Olga Iuliano, Luís Ribeiro, Julien Veran, Joana S. Ferreira, Pedro Rio, Christophe Mulle, Carlos B. Duarte and Ana Luísa Carvalho

Supplementary Figure 1. (A) MALDI-TOF spectrum of the trypsin-digested 1D gel band identified as *Rattus norvegicus* contactin associated protein 1 (Caspr1, gi|14091742). Mascot score: 99 (significant when greater than 76, p<0.05; Expect: 0.0003; 8% sequence coverage). (B) Sequence coverage for Caspr1, with underlined matched peptides. (C) List of identified peptides for Caspr1.

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Supplementary Figure 2. (A) MALDI-TOF spectrum of the trypsin-digested 1D gel band identified as Rattus norvegicus contactin 1 (gi|16923964). Mascot score: 107 (significant when greater than 76, p<0.05; Expect: 1.5e-07; 10% sequence coverage). (B) Sequence coverage for Contactin1, with underlined matched peptides. (C) List of identified peptides for Contactin1.
Supplementary Figure 3. Efficiency of the shRNA construct for Caspr1 knockdown in cultured hippocampal neurons. (A) Cultured hippocampal neurons at 7 DIV were transfected with the indicated shRNA vectors. We used pLL(CMV)EGFP as the shRNA vector, driving shRNA from the U6 promoter and EGFP from the CMV promoter. Neurons were fixed at 15 DIV and immunostained for endogenous Caspr1 and for PSD95. (B, C) Quantification of knockdown efficiency. (B) Caspr1-shRNA significantly knocks down the expression of dendritic Caspr1 to around 48% of the endogenous expression level. (C) The expression of Caspr1 clusters colocalized with PSD95 is decreased to around 33%. Data are presented as % of control cells, transfected with pLL(CMV)EGFP; Error bars, ± S.E.M. Significance, p<0.001, Student’s t-test; n>15 cells each. Scalebar: 10 μm (inserts: 2μm).
Contactin associated protein 1 (Caspr1) regulates the traffic and synaptic content of \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors

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