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The Homer-1 protein Ania-3 interacts with the plasma membrane calcium pump

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Abstract

The Homer family of scaffold proteins couples NMDA receptors to metabotropic glutamate receptors, and links extracellular signals to calcium release from intracellular stores. Ania-3 is a member of the Homer family and is rapidly inducible in brain in response to diverse stimuli. Here we report the identification of the plasma membrane Ca^{2+} ATPase (PMCA) as a novel Ania-3/Homer-associated protein. Ania-3/Homer interacts with the b-splice forms of all PMCAs (PMCA1b, 2b, 3b, and 4b) via their PDZ domain-binding COOH-terminal tail. Ectopically expressed Ania-3 colocalized with the PMCA at the plasma membrane of polarized MDCK epithelial cells, and endogenous Ania-3/Homer and PMCA2 are co-expressed in the soma and dendrites of primary rat hippocampal neurons. The interaction between Ania-3/Homer and PMCAs may represent a novel mechanism by which local calcium signaling and hence synaptic function can be modulated in neurons.

Keywords

Ania-3; calcium signaling; Homer; microdomain; PDZ domain; plasma membrane; PMCA; synaptic function

Introduction

Intracellular Ca^{2+} signals control multiple neuronal functions including excitability, neurotoxicity, neurotransmitter release and gene expression [1]. Homer proteins have gained attention because they are a component of the post-synaptic density and are involved in coupling of NMDA-binding ionotropic glutamate receptors to metabotropic glutamatergic receptors (mGluR) [2, 3]. The Homer family is encoded by three distinct genes, *homer 1*, *2*, and *3*. The *homer 1* gene is predominantly expressed in the brain and gives rise to the constitutively expressed homer 1b and 1c splice forms, the muscle-specific homer 1d variant [4], and the immediate early gene products homer 1a and Ania-3 [5–8]. Homer proteins

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share a conserved N-terminal segment that contains a PDZ-domain consensus sequence (RX_5GLGF) [5] within an Ena/vasodilator-stimulated phosphoprotein homology 1 (EVH-1) domain [6]. This N-terminal domain allows the binding of Homer proteins to ligands with proline-rich motifs, such as metabotropic glutamatergic receptors [9], IP_3 receptors [10], TrpC channels [11], and Shank/ProSAP scaffolding proteins [12, 13]. Constitutively expressed Homer proteins (e.g., Homer 1b, 1c) also have a carboxy-terminal region that promotes multimerization [6, 9, 14].

Homer proteins are involved in both the localization and function of type I mGluRs (for review, see [15–18]). The multimerization of the long forms of Homer proteins can serve to link mGluRs and TrpC channels to IP_3 receptors, and may thus link extracellular signals to the release of Ca^{2+} from intracellular stores and the emptying of stores to extracellular Ca^{2+} influx, respectively [18, 19]. The shorter Homer 1a and Ania-3 are induced in the brain in response to stimuli including long-term potentiation, electroconvulsive shocks and drugs of abuse [5, 7, 8]. These forms lack the C-terminal dimerization region and competitively interfere with the normal linkage between Homer-binding proteins. Indeed, expression of Homer 1a has physiological effects that include disruption of mGluR5-Homer 1b/1c-IP3 complexes [9], alteration of mGluR-induced Ca^{2+} release [10], and extracellular calcium influx [20].

To gain further insight into the possible role of Ania-3 in the formation of multi-protein signaling complexes, we used Ania-3 as bait in a yeast two-hybrid screen and identified the plasma membrane Ca^{2+} ATPase (PMCA) as a new partner for the Homer family proteins. We found that all PMCA b-splice forms interact with Ania-3 via their C-terminal PDZ domain-binding tail. This novel interaction expands the repertoire of Ania-3/Homer binding proteins to members of the Ca^{2+} extrusion pathway operating at the plasma membrane, suggesting tight coupling of the local regulation of neuronal Ca^{2+} signaling and protein scaffolding at the excitatory synapse.

Materials and methods

Plasmid constructs

The Ania-3:FLAG expression plasmid was obtained after PCR amplification using primers Ania-3-forward (5′-CGG GAT CCC GAA ATG GGG GAA CAA CCT ATC TTC AGC ACT CGA-3′) and Ania-3-reverse (5′-GGA ATT CCT CAC TTG TCA TCG TCG TCC TTG TAG TCA GGG GTC ATT TGT ATC-3′) and a rat brain cDNA library (OriGene Technologies Inc., Rockville, MD, USA) as template. The open reading frame of mGLuR5 was amplified by PCR using primers mGluR5-forward (5′-CGC GGA TCC GCG ATG GTC CTT CTG TTG ATC CTG TCA-3′) and mGluR5 reverse (5′-GGA ATT CCT CAC AAC GAT GAA GAA CTC TGC GTGT-3′). The PCR products were cloned into pcDNA3.1+ (Invitrogen, Carlsbad, CA, USA) using BamHI and EcoRI restriction sites included in the primer sequences. Mammalian expression plasmids for PMCA isoforms 1b, 2b, 3b, 4b and the C-terminally truncated PMCA4ct120 have been described [21–24]. The construct for PMCA2ct121 was made by an analogous strategy to that employed for PMCA4ct121 [22] and was a gift from William Ba-Thein and John T. Penniston.

Northern blot analysis

^{32}P-UTP-labelled antisense RNA for *ania-3* was generated by in vitro transcription. The template was a plasmid containing a 443bp cDNA sequence for a unique portion of *ania-3* 3′UTR [7]. The cDNA sequence is in GenBank, accession number **AF030088** (gi: 2613079). Hybridization of a rat multiple-tissue northern blot (OriGene Technologies Inc.) was

performed overnight at 65°C in NorthernMax hybridization buffer (Ambion, Austin, TX, USA).

Yeast two-hybrid screen

Two-hybrid screening was performed in yeast strain AH109 (Clontech laboratories, Palo Alto, CA, USA). The bait was full-length Ania-3 obtained after PCR amplification and cloning into pGBKT7 (Clontech). The bait was co-transformed with a rat inducible striatal MATCHMAKER cDNA library (Clontech) into yeast strain AH109 harboring *HIS3*, *ADE2* and *lacZ* reporter genes. 3.6×10^6 cDNAs were screened and positive clones were selected on plates lacking leucine, tryptophan and histidine. They were streaked on plates lacking leucine, tryptophan, histidine and adenine and were also tested for β-galactosidase activity by filter–lift assay. Interacting clones were rescued and sequenced. A mating test was performed according to the manufacturer's instructions (Clontech) to confirm the interaction between Ania-3 and the selected plasmids.

Western blot

Adult rat tissues from brain, heart, liver, lung, kidney, muscle, small intestine and spleen were homogenized in lysis buffer (25 mM Hepes pH 7.4, 137 mM NaCl, 1% NP-40, 0.5% DOC, 10 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin A) and spun for 15 min at 13,000 rpm in a microcentrifuge. The protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). 30 μg protein samples were heated in SDS sample buffer, the proteins resolved by SDS-PAGE using 4–15% Ready Gels (BioRad, Hercules, CA, USA) and transferred to nitrocellulose membranes following standard western blotting procedures. The membranes were incubated in TBST blocking buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) plus 5% milk for 1 hour at room temperature before exposure overnight at 4°C to primary rat anti-Homer antibody (AB5875, Chemicon International Inc., Temecula, CA, USA) diluted 1:1000 in the above blocking buffer. The membranes were washed in TBST, incubated in HRP-conjugated goat anti-rat IgG (Chemicon) for 1 hour at room temperature, washed again, and immunoreactive bands were detected by the ECL-plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA).

Cell cultures and transfections

COS-7 cells (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 10% fetal bovine serum, 1% fungizone, 100U/ml penicillin and 100mg/ml streptomycin (all from Life Technologies Inc, Rockville, MD, USA). COS-7 cells were plated on 35mm or 100mm tissue culture dishes and transfected at 60–80% confluency with the indicated plasmids using LIPOFECTAMINE™ 2000 (Invitrogen). Two days after transfection, cells were lysed on ice in lysis buffer (25 mM HEPES pH 7.4, 137 mM NaCl, 0.1 mM PMSF, 1% NP40, 0.5% deoxycholate, and protease inhibitor cocktail), centrifuged for 10 min at 13,000 x g, and the supernatants kept for protein determination and immunoprecipitations. Type I MDCK epithelial cells (ATCC number CCL-34) were grown to confluence on glass coverslips in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were transfected with a total of 2 μg of plasmid DNA per 35mm dish, using LIPOFECTAMINE™ 2000. Two days after transfection, the cells were fixed for 15 min at room temperature in 4% paraformaldehyde (Tousimis, Rockville, MD, USA) diluted in DPBS + Ca^{2+}/Mg^{2+} (DPBS+CM, Invitrogen) and processed for immunofluorescence staining. Rat hippocampal neuron cultures were obtained from 18-day embryos as described [25]. Briefly, freshly dissected hippocampi were placed in 2 ml of a 0.25% trypsin solution. After 20 min at room temperature, typsin was removed by three washes in HBSS plus 20% horse serum. After addition of 2 ml Neural Culture Medium (Invitrogen) the cells were

dispersed by trituration (10 times) using a pasteur pipette. Neuronal cells were plated at a density of 5,000 cells/cm^2 and cultured for 2–3 weeks prior to processing for immunostaining.

Immunoprecipitation

Cells were scraped on ice in lysis buffer and cellular debris removed by centrifugation. Cell lysates (about 500 μl) were then incubated at 4°C from 2 hours to overnight with antibody (2 μl antisera or 1–2 μg purified antibody) as indicated in the figure legends. 50 μl protein A/G-agarose beads (Sigma, St. Louis, MO, USA) were then added and the samples rocked end-over-end for 2 hours at 4°C. After multiple washes, beads were resuspended in 2X SDS loading buffer and proteins eluted by boiling before being electrophoresed on 8–16% SDS polyacrylamide gels (Invitrogen). After transfer to nitrocellulose membranes, proteins were detected using the following primary antibodies at the indicated dilutions: anti-FLAG (1:1000), anti-mGluR5 (1:5000), pan-anti-PMCA (5F10, 1:2000), anti-PMCA1 (NR-1, 1:1000), anti-PMCA2 (NR-2, 1:2000), anti-PMCA3 (NR-3, 1:1000), anti-PMCA4 (JA9, 1:2000) (all from Upstate Biotechnology, Lake Placid, NY, USA). After multiple washes in TBST, membranes were incubated with appropriate HRP-conjugated secondary antibodies (1:10,000) and washed again prior to visualization using SuperSignal (Pierce) and film exposure (X-OMAT, Kodak, Rochester, NY, USA).

Immunolocalization

Transfected MDCK cells or cultured hippocampal neurons were fixed for 15 min at room temperature in 4% paraformaldehyde and then further fixed and permeabilized in pre-chilled methanol for 15 min at −20 °C. After blocking in DPBS containing 10% normal donkey serum and 0.5% Triton, the cells were incubated for 1 hour at room temperature with affinity-purified polyclonal rabbit anti-PMCA2 antibody NR-2 (1:1000), rat anti-Homer antibody (1:1000), or anti-Flag antibody M2 (Sigma, St. Louis, MO, USA, 1:800). After washing 3 times for 5 min in DPBS+CM, the cells were incubated for 1 hour at room temperature with the appropriate secondary antibodies coupled to FITC, Cy3 or Alexa-594 (Molecular Probes, Eugene, OR, USA). The secondary antibodies were used at a dilution of 1:800, and all antibodies for immunofluorescence were diluted in blocking buffer. DAPI (4′, 6′-diamidino-2-phenylindole dihydrochloride) (Molecular Probes) was also added to the secondary antibody application at a dilution of 1:500 (final concentration 20 μg/ml) to stain nuclei. After final washing, coverslips were mounted in Prolong mounting media (Molecular Probes). Confocal micrographs were taken on a Zeiss LSM510 microscope using an Apochromat 63X oil immersion objective and captured using LSM510 software version 2.8 (Carl Zeiss Inc., Thornwood, NY, USA). Images were imported and edited using Adobe Photoshop 5.0.

Results

Ania-3 is a Homer 1 splice variant closely related to Homer 1a

An expressed sequence tag for *ania-3* was previously isolated through differential display PCR [7]. We screened a rat brain library by PCR to obtain a full-length cDNA for *ania-3*. The selected clone (~2.6kb) contained a 657 bp open reading frame that encoded a predicted protein of 219 amino-acids that is highly homologous to Homer 1 family members (Fig. 1). The amino-terminal 175 residues of Ania-3 are identical to those of Homer 1a, 1b, and 1c. Ania-3 contains the RX_5GLGF sequence characteristic of PDZ domains as well as the EVH-1 domain (spanning amino-acids 1 to 114) shared by other Homer family members [10]. Ania-3 differs from other Homer 1 family members in its C-terminal region; it is 33 residues longer than Homer 1a but lacks the coiled-coil dimerization domain present in the C-terminus of the longer Homers such as Homer 1b or 1c (Fig. 1). Ania-3 is generated by

alternative splicing of the large and complex *homer 1* gene; its unique C-terminal sequence is generated by the insertion of an exon that is excluded from all other *homer 1* transcripts as part of the large intron 5 [8].

A tissue northern blot showed that *ania-3* mRNA is expressed in the heart, kidney, liver and muscle, as well as in the brain (Fig. 2A). *Ania-3* mRNA accumulation correlated well with expression of Ania-3 protein, as shown by western blotting of multiple rat tissue extracts (Fig. 2B). Due to the lack of a specific antibody against the native Ania-3 protein, we used an anti-Homer antibody. This antibody recognized Ania-3 with good specificity (as shown by the molecular weight at which Ania-3 was detected and its regional pattern of expression) and high sensitivity, as demonstrated by its strong reaction with exogenously expressed Ania-3 in transiently transfected COS cells (Fig. 2C).

Ania-3 interacts with the plasma membrane calcium pump (PMCA)

As a member of the Homer 1 family of synaptic regulatory proteins, Ania-3 is expected to interact with class I metabotropic glutamate receptors [5]. Indeed, when COS-7 cells were co-transfected with expression plasmids encoding mGluR5 and Ania-3, both proteins could be co-immunoprecipitated in reciprocal pull-down experiments (Fig. 3). To better understand possible roles for Ania-3 in regulating glutamate receptor-dependent signaling events, we used yeast two-hybrid screening to search for additional partners of Ania-3 in a cDNA expression library prepared from dopamine D1 receptor–stimulated rat striatum. The isolated candidate clones included the known Homer 1a-interacting proteins IP_3R and Shank3A. In addition, however, we isolated a clone encoding the plasma membrane calcium ATPase isoform 4b (GenBank accession number **M25874**).

The interaction between Ania-3 and PMCA4b was confirmed in a yeast mating test (data not shown) and by co-immunoprecipitation (Fig. 4D). When COS-7 cells were co-transfected with plasmids encoding Ania-3:Flag and PMCA4b and the cell lysates immunoprecipitated with an antibody against the Flag-tag or against PMCA4, the calcium pump (detected as a band of about 130 kDa) and Ania-3 (migrating at about 35 kDa) were co-precipitated, respectively (Fig. 4D). In agreement with this result, PMCA4b could also be co-precipitated after co-transfection with Homer 1a and Homer 1c (data not shown). To determine if the interaction between Ania-3 and PMCA4b was isoform-specific, we performed similar co-immunoprecipitation experiments using PMCA1b, 2b, and 3b. As shown in Figure 4A–C, Ania-3 co-precipitated with each of these PMCA isoforms. The co-immunoprecipitation was reciprocal in each case, i.e., the antibody against Flag (specific for Ania-3:Flag) co-precipitated the respective PMCA isoform, and the antibody against the PMCA isoform co-precipitated Ania-3.

PMCAs interact with Ania-3 via their PDZ domain-binding C-terminal tail

The above data show that Ania-3 binds to the b-splice forms of all four PMCA isoforms. The C-terminal residues of the PMCA b-splice forms have been shown to be responsible for the interaction of these pumps with PDZ domains. Because Homer members contain a PDZ-like RX_5GLGF signature sequence in their N-terminal EVH-1 domain, we reasoned that the PMCAs interact via their C-terminal PDZ-binding motif with Ania-3/Homer. To test this hypothesis we co-transfected COS-7 cells with Ania-3:Flag and C-terminally truncated PMCA2b or PMCA4b constructs and performed co-immunoprecipitations as before. As shown in Figures 4B and 4D (last lane, second and bottom panel), PMCA2ct121 and PMCA4ct120 lacking 121 and 120 C-terminal residues, respectively, were unable to interact with Ania-3, although both proteins were abundantly expressed in the transfected cells (Fig. 4B and D, last two lanes on the top panel). These results demonstrate that the C-terminal residues of the PMCA b-splice forms play a key role in the interaction with Ania-3.

Ania-3 and PMCA2b colocalize in transfected MDCK cells and hippocampal neurons

Some Homer 1 family members such as Homer 1b inhibit cell surface expression of mGluR5 receptors by retaining them in the endoplasmic reticulum whereas the short, inducible Homer 1a splice form does not alter the subcellular localization of these receptors [26]. To test if Ania-3 expression influences the distribution of a co-expressed PMCA partner protein, we co-transfected MDCK epithelial cells with Ania-3:Flag and PMCA2b, and determined the localization of these recombinant proteins by confocal immunofluorescence microscopy. Figure 5A shows that most of the Ania-3 staining was concentrated at the cell membrane where it co-localized with PMCA2b, consistent with the co-immunoprecipitation data. The data also show that expression of Ania-3 does not prevent the pump from being targeted to the plasma membrane.

Homer 1/Ania-3 is highly expressed in the brain where it is enriched in the dendrites and spines of hippocampal neurons [13, 27]. PMCA2b is also abundant in the brain and present in the soma and dendrites of hippocampal pyramidal neurons [23, 28, 29]. To confirm the co-expression of endogenous Homer 1/Ania-3 and PMCA2b, we double-labeled mature (2 to 3 week-old) rat hippocampal neurons with antibodies specific for Homer and PMCA2. Figure 5B shows that both proteins are abundantly expressed and co-localized in the soma and dendrites of these cells. Although some Homer staining was also observed in the perinuclear cytoplasmic region, both Homer and PMCA2 were frequently co-stained in punctuate structures in the dendrites, indicating that they form clusters at specific membrane sites (inset in Fig. 5B).

Discussion

This study shows that the Homer 1 family member Ania-3 is a binding partner for the b-splice variants of the PMCAs. The PMCAs are calmodulin-regulated enzymes that are essential for maintaining low intracellular calcium concentrations in the living cell [30, 31]. Recent work has shown that PMCA isoforms and splice variants are active participants in localized Ca^{2+} signaling, and that their targeting to and abundance at defined membrane sites must therefore be closely regulated [32–35]. All PMCA isoforms are expressed in the brain, with PMCA2 being abundant in several regions including the cerebellum, forebrain and hippocampus [28, 36]. PMCAs can be regulated by a variety of mechanisms, and are stimulated by neurotransmitters and neuroactive peptides. By modulating local calcium efflux, PMCAs play a particularly important role in neuronal function [37, 38].

Much of the regulation of the PMCAs is mediated via their C-terminal cytosolic tail. This tail is subject to alternative splicing, which leads to the major a- and b-splice variants that confer different functional properties on the pump [31]. All PMCA b-splice variants display a PDZ-domain binding sequence at the extreme C-terminus. This enables them to interact with specific PDZ proteins such as PSD95, SAP97, and nitric oxide synthase [23, 32, 39]. These proteins are enriched at synaptic sites where they are a part of large multi-protein complexes involved in local signaling and signaling cross-talk. Homer family members, including Ania-3, contain a RX_5GLGF sequence characteristic for PDZ domains embedded in their N-terminal region. Our results show that the PDZ-binding C-terminal tail of PMCA2b and 4b is required for the interaction with Ania-3, thus providing the first demonstration that the N-terminal GX_5GLGF-containing EVH-1 domain of Homer proteins can interact with a bona-fide PDZ domain ligand. This is further supported by the fact that the PMCA2b and PMCA4b tail sequences are poorly conserved except for the extreme C-terminal sequences involved in PDZ domain binding [23]. Because Ania-3 shares its entire N-terminal portion including the EVH-1/PDZ-like domain with all other Homer 1 isoforms, the PMCA b-splice variants likely interact with these other Homers as well. Indeed, we found that PMCA4b co-immunoprecipitated with Homer 1c when the two proteins were co-

expressed in COS-7 cells (data not shown). Similar to mGluR5, the PMCAs thus interact with both the short, inducible Homers (Homer 1a, Ania-3) as well as with the constitutively expressed long forms (Homer 1b/c/d). Whether the interaction between inducible forms of Homer and PMCA limits or enhances Ca^{2+} pumping out of the neurons remains to be studied.

Clustering or retention of PMCA b-splice variants in specific membrane domains may be mediated by PDZ protein interactions. In this regard, our data showing an interaction between Ania-3/Homer 1 and the PMCAs suggest that Homer family members could play such a role, since they are expressed in close proximity to the post-synaptic density where they are involved in targeting/clustering channels and receptors at the synapse (for review, see [15, 17, 18]). The interaction with Homer 1 may recruit the PMCA to the immediate vicinity of Ca^{2+} influx systems at specialized sites in neurons, facilitating precise spatial control of an incoming Ca^{2+} signal. In cerebellar Purkinje neurons, Homer 1b/c is present in a complex junctional Ca^{2+} signaling network that links metabotropic glutamate receptors (mGluR1) to IP_3R-mediated Ca2+ influx from the ER [40]. Intriguingly, the PMCA was detected by confocal immunofluorescence microscopy as part of this network [40]. The rapidly inducible expression of short “dominant-negative” forms of Homer (such as Ania-3 and Homer 1a) modulates synaptic signaling by altering metabotropic glutamate receptor trafficking and dissociating functional protein-protein interactions within the signaling complex [11, 17, 18, 20, 27]. Similarly, the different Homer splice variants may influence the amplitude and duration of local Ca^{2+} signals by reversibly recruiting the PMCAs into these signaling domains.

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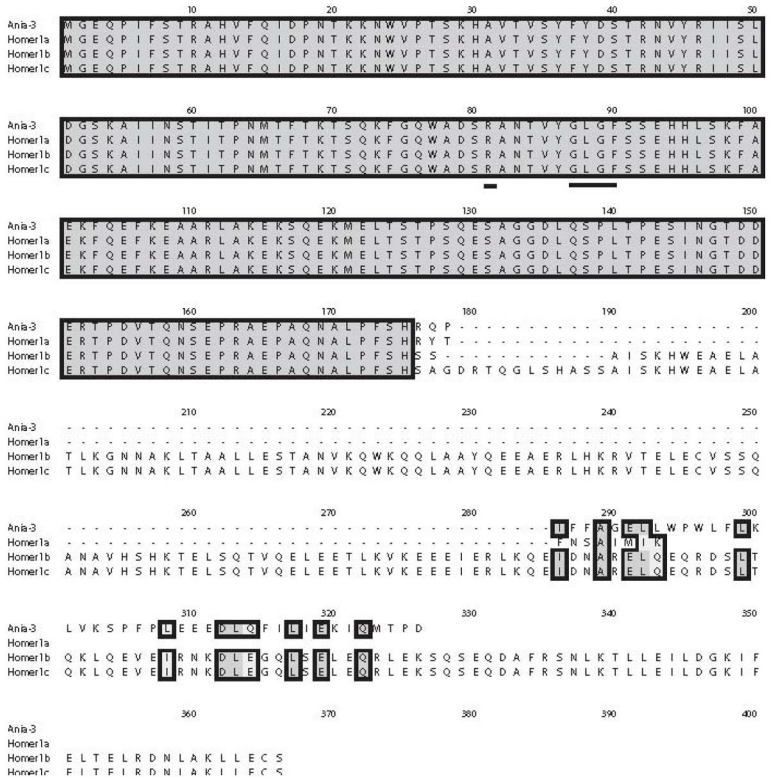

Figure 1. Predicted amino acid sequence of rat Ania-3 and comparison with Homer 1a, 1b, and 1c

Shaded sequences indicate areas of amino-acid similarity between Ania-3, Homer 1a, 1b and 1c. Note that the first 175 residues are identical in all four proteins whereas they diverge in the C-terminal portion due to alternative splicing. The RX_5GLGF motif in the PDZ-like N-terminal domain is underlined.

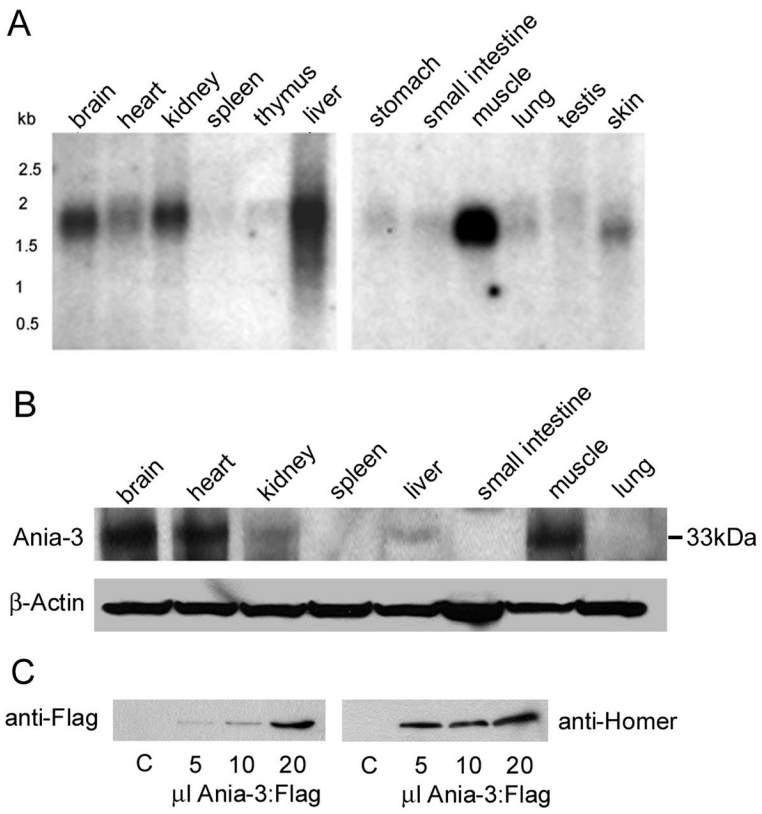

Figure 2. Tissue expression of Ania-3

A. Multiple-tissue northern blot showing baseline expression of ania-3 transcripts in various adult male rat organs as indicated on top of each lane. RNA size standards are indicated in kb on the left. **B**. Multiple-tissue western blot using an anti-Homer antibody to detect expression of Ania-3 in various adult rat tissues as indicated on top of each lane (top panel). A duplicate blot was probed with an antibody against β-actin to control for protein loading (bottom panel). **C**. A western blot of control lysate from untransfected COS-7 cells (20 μl, lane C) or of 5, 10, and 20 μl of lysate from COS-7 cells transiently transfected with Ania-3:Flag was probed with anti-Flag antibody (left panel) or anti-Homer antibody (right panel). Note that the anti-Homer antibody recognizes Ania-3 even at the lowest amount barely detected by the anti-Flag antibody.

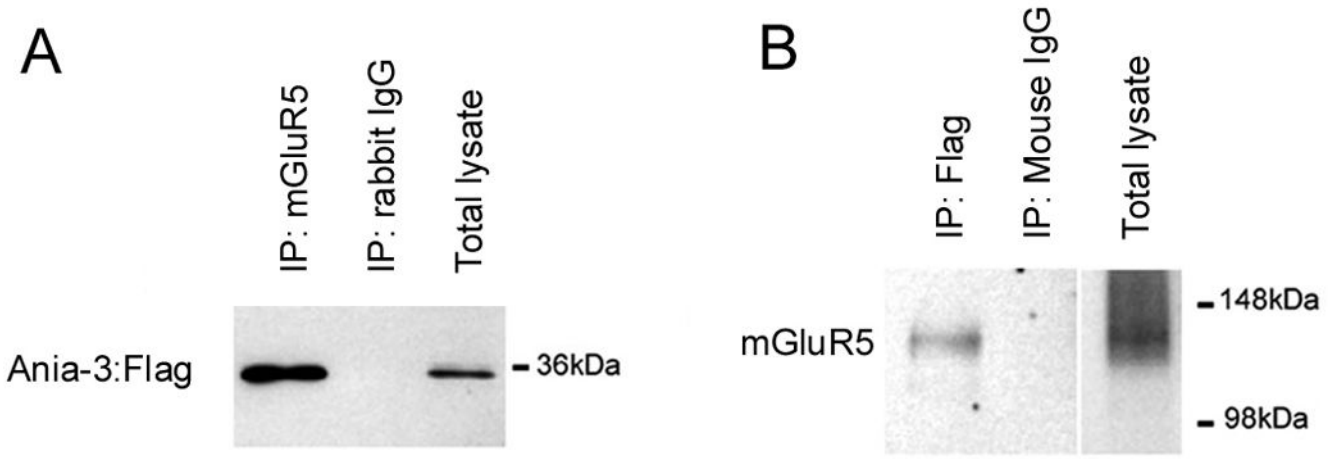

Figure 3. Ania-3 interacts with mGluR5

COS-7 cells were co-transfected with plasmids encoding Ania-3:Flag and mGluR5, and cell lysates were immunoprecipitated using specific antibodies for mGluR5, the Flag-tag on the Ania3:Flag fusion protein, or the rabbit or mouse control antibodies, respectively. Co-immunoprecipitated proteins were detected by western blot using anti-Flag (A) or anti-mGluR5 antibodies (B). An aliquot of the total lysate was also included on the westerns as a control. The position of molecular size markers is indicated on the right of each blot.

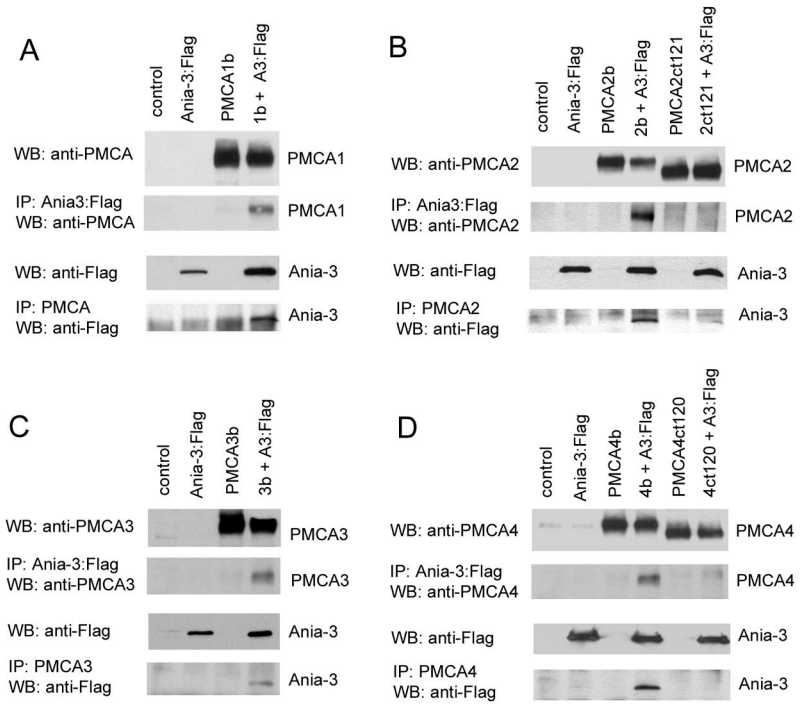

Figure 4. Ania-3 binds to the PMCA b-splice forms via their C-terminal tail

COS-7 cells were transfected with plasmids encoding Ania-3:Flag and different full-length or C-terminally truncated PMCA isoforms as indicated on top of each lane. Reciprocal immunoprecipitations (IP) were then performed on aliquots of the cell lysates with an antibody against Flag (recognizing Ania-3:Flag) or against the PMCAs, and the precipitated proteins were tested by western blotting (WB). Cell lysates from untransfected cells were treated in the same way and are included as controls (left lane). Aliquots (5% of input) of the cell lysates prior to immunoprecipitation were also included in the westerns (first and third panels) to check for protein expression levels. **A**. Cells were transfected with Ania-3:Flag, PMCA1b, or both together (1b + A3:Flag). PMCA1b co-precipitates with Ania-3:Flag when the latter is pulled down with an antibody against Flag (second panel, last lane); conversely, Ania-3:Flag co-precipitates when the PMCA is pulled down by an antibody (5F10) against the pump (bottom panel, last lane). B. Cells were transfected with DNA encoding Ania-3:Flag, PMCA2b, or PMCA2ct121 alone or in combination as indicated on top of the lanes. Full-length PMCA2b co-precipitates with Ania-3:Flag, whereas the C-terminally truncated PMCA2ct121 does not interact with Ania-3:Flag (compare lane *2b + A3:Flag* with lane *2ct121 + A3:Flag*). **C.** Cells were transfected with Ania-3:Flag, PMCA3b, or both (3b + A3:Flag). PMCA3b co-precipitates with Ania-3:Flag (second panel, last lane); conversely, Ania-3:Flag co-precipitates when the PMCA is pulled down by an antibody (NR-3) against the pump (bottom panel, last lane). **D.** Cells were transfected with DNA encoding Ania-3:Flag, PMCA4b, or PMCA4ct120 alone or in combination as indicated on top of the lanes. Full-length PMCA4b and Ania-3:Flag co-precipitate whereas the C-terminally truncated PMCA4ct120 does not interact with Ania-3:Flag (compare lane *4b + A3:Flag* with lane *4ct120 + A3:Flag*).

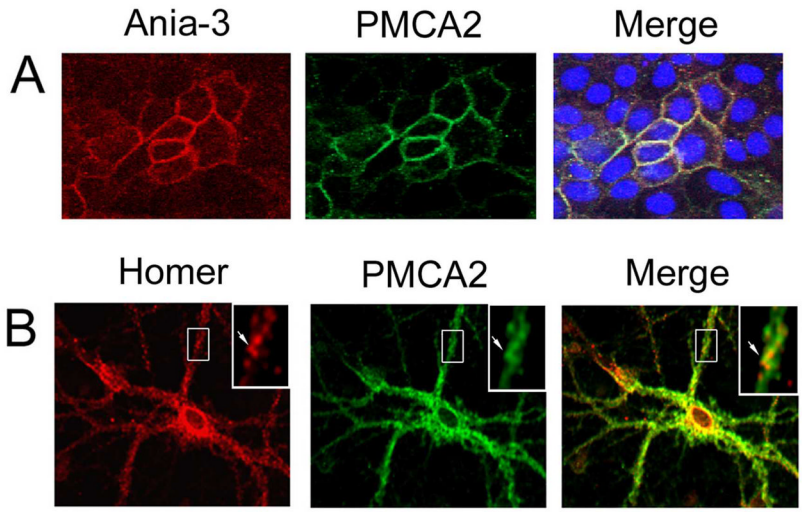

Figure 5. Co-localization of Ania-3 and PMCA2 in transfected MDCK cells and hippocampal neurons

A. Polarized MDCK cells were co-transfected with DNA encoding Ania-3:Flag and PMCA2b. 48 hours later, confocal fluorescence microscopy was performed to detect Ania-3 (using anti-Flag antibody, red channel) and PMCA2 (anti-PMCA, green channel). Merged images are shown on the right. Nuclei were stained with DAPI (blue). **B.** Mature (2–3 week-old) primary rat hippocampal neurons were fixed and stained for confocal fluorescence microscopy using a rat antibody against Homer/Ania-3 (left panel) and antibody NR-2 against PMCA2 (middle panel). The merged image is shown on the right. The inset shows an enlarged region of a dendrite with clusters of overlapping Homer and PMCA2 staining (arrows).