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The CNTNAP2-CASK complex modulates GluA1 subcellular distribution in interneurons

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Abstract

GABAergic interneurons are emerging as prominent substrates in the pathophysiology of multiple neurodevelopmental disorders, including autism spectrum disorders, schizophrenia, intellectual disability, and epilepsy. Interneuron excitatory activity is influenced by 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptors (AMPARs), which in turn affects excitatory transmission in the central nervous system. Yet how dysregulation of interneuronal AMPARs distinctly contributes to the molecular underpinning of neurobiological disease is drastically underexplored. Contactin-associated protein-like 2 (CNTNAP2) is a neurexin-related adhesion molecule shown to mediate AMPAR subcellular distribution while calcium/calmodulin-dependent serine protein kinase (CASK) is a multi-functional scaffold involved with glutamate receptor trafficking. Mutations in both genes have overlapping disease associations, including autism spectrum disorders, intellectual disability, and epilepsy, thus suggesting converging perturbations of excitatory/inhibitory balance. Our lab has previously shown that CNTNAP2 stabilizes interneuron dendritic arbors through CASK and that CNTNAP2 regulates AMPAR subunit GluA1 trafficking in excitatory neurons. The interaction between these three proteins, however, has not been studied in interneurons. Using biochemical techniques, structured illumination microscopy (SIM) and shRNA technology, we first confirm that these three proteins interact in mouse brain, and then examined relationship between CNTNAP2, CASK and GluA1 in mature interneurons.

[1]Contributed equally to this work.

Author contributions

R.G., M.D.M., L.E.D., and N.H.P. performed biochemistry experiments, R.G. and C.R.Z. performed all confocal and SIM imaging experiments. P.P. supervised the project. R.G., L.S., C.P.P., K.E.H, and P.P. wrote the manuscript.

Conflict of interest

The authors declare no competing financial conflict of interests.

Appendix A. Supplementary data

Four supplementary files are available for this article

Research data for this article

Research data for this article is available on Mendeley for download under the CC BY 4.0 license Data for: The CNTNAP2-CASK complex modulates GluA1 subcellular distribution in interneurons Raw data for Figures 1–4.

Using SIM, we ascertain that a large fraction of endogenous CNTNAP2, CASK, and GluA1 molecules collectively colocalize together in a tripartite manner. Finally, individual knockdown of either CNTNAP2 or CASK similarly alter GluA1 levels and localization. These findings offer insight to molecular mechanisms underlying GluA1 regulation in interneurons.

Keywords

Autism spectrum disorders; Schizophrenia; Language disorders; AMPA receptors; Neuropsychiatric disorders; Cntnap2; Excitatory-inhibitory balance; Structured illumination microscopy; Dendrites; Mechanism

1. Introduction

AMPARs mediate excitatory transmission in glutamatergic and inhibitory neurons, making their function critical for proper brain activity. Structurally, AMPARs are tetramers consisting of different GluA1-GluA4 subunit combinations, with each subunit possessing unique biological, structural, and physiological properties [3]. Equally as intricate, there exists a growing list of AMPAR-associated proteins that dynamically regulate the receptor's trafficking, composition, stability, and mobility in response to activity to ensure proper functional connectivity in synaptic circuits [13]. These regulatory mechanisms have been studied extensively in pyramidal cells, but are surprisingly understudied in inhibitory neurons, despite interneurons also being critical to proper circuit function [9].

Several recent studies have demonstrated that CNTNAP2 is involved in AMPAR-specific trafficking in pyramidal cells, as elimination of CNTNAP2 decreases synaptic GluA1, increases cytoplasmic GluA1 aggregates, and induces morphological changes in spiny synapses [2,42]. However, CNTNAP2′s role in AMPAR function within interneurons has not been explored despite the fact that *Cntnap2* knockout mice have spontaneous seizures, decreased GABAergic interneuron density, and inhibitory-specific electrophysiological defects [19,31,44]. Moreover, CNTNAP2 has been shown to be highly abundant in the embryonic ganglionic eminences [31], consistent with a role in interneuron development. Similarly, the literature demonstrates that CASK interacts with CNTNAP2 [9,38], maintains pyramidal dendritic spines [6], and traffics N-methyl-D-aspartate receptors (NMDARs) [18,22] and AMPAR subunits [14]. While CASK has not been exclusively linked to interneuron function and is itself widely distributed in the brain [16], it is a multi-domain, multi-functional adaptor that can become specialized through interactions with specific molecules [4,15,29]. Hence, the role of CNTNAP2 on CASK function within interneurons is a topic that should be investigated.

Genetic variation in the *CNTNAP2* gene, including copy number variations, exon deletions, truncations, single nucleotide variants, and polymorphisms have been associated with autism spectrum disorder, schizophrenia, intellectual disability, epilepsy, and language disorders[1,8,11,32,40,43]. Likewise, penetrant mutations within the *CASK* gene are causative for X-linked intellectual disability and have been associated with several cases of autism spectrum disorder [12,[25], [26], [27],35]. While both patient populations exhibit complex phenotypes, they share a common seizure comorbidity, which implicates a pathway

convergence onto inhibitory circuits. Taken altogether, these observations suggest that complex *CNTNAP2*/*CASK*mutations may disrupt interneuron function through AMPAR dysregulation, but this theory has not yet been explored.

Here, we first uncover the physiological existence of CNTNAP2-CASK, CNTNAP2-GluA, and GluA1-CASK protein complexes *in vivo*. We then use SIM to show that CNTNAP2, CASK, and GluA1 most frequently exist in a tripartite complex in interneurons. Finally, transiently decreasing CNTNAP2 via shRNA alters GluA1 levels and localization in interneuron dendrites and somas – effects that are generally phenocopied by CASK knockdown. Our findings reveal clues to a possible postsynaptic mechanismthrough which CNTNAP2-CASK regulates GluA1 trafficking in interneurons.

2. Materials and methods

2.1. Antibodies and plasmids

Antibodies and plasmids used for the experiments are listed in Supplementary tables 1 and 2 respectively.

The pEGFP-N2 plasmid was purchased from Clontech (Mountain View, CA, USA). shRNA plasmids for CNTNAP2 (TG510300), CASK (TG516864), and scrambled controls (TR30015) were purchased from Origene (Rockwall, MD, USA), and were modified by replacing turboGFP with eGFP (restriction sites BglII and NotI). All interneurons were identified with either GABA staining [10] or morphological characteristics (i.e. multipolar, aspiny) when GABA staining was not permitted (see ***SIM Imaging and Analysis*** and ***Confocal Microscopy*** **Imaging** sections for more specifics).

2.2. Immunoprecipitation

Mouse cortices (3–5 months of age) were homogenized in immunoprecipitation buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton X-100, with protease inhibitory cocktail from Roche, Basel, Switzerland) and solubilized for 1 h at 4 °C. Solubilized material was centrifuged at 20 000 *g* for 10 min at 4 °C and the supernatant was precleared with protein A/G (Thermo Fisher Scientific) for 30 min. Proteins in the precleared supernatant were then immunoprecipitated with 3 μg of antibody overnight at 4 °C, followed by a 1 h incubation with protein A/G the following day. Beads were then washed 3 times with IP buffer before adding 2x Laemelli buffer (Biorad, Hercules, CA, USA). Samples were analyzed by SDS-PAGE and western blotting using standard methods.

2.3. Neuronal culture and transfections

High density (300 000 cells/cm^2) cortical neuron cultures were prepared from Sprague-Dawley rat E18 embryos as described previously [39]. Cortical neurons were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations (between 3–5 μg of DNA per plasmid, 4 μL of Lipofectamine 2000 per reaction) and the neurons were maintained in the feeding media [39] for either 3 (GFP only, endogenous staining experiments; 24–27 DIV), or 5 days (knockdown experiments; 21–26 DIV).

2.4. Immunocytochemistry

For total protein staining, neurons were first washed in phosphate buffered saline (1 × PBS) and fixed in 4% formaldehyde-sucrose-PBS for 15 min. Fixed neurons were permeabilized and blocked with 5% normal goat serum (NGS) and 0.3% Triton X-100 in 1 × PBS (30 min at 4 °C), followed by incubation of primary antibodies with 5% NGS in 1 × PBS (O/N, 4 °C). Coverslips were washed three times with PBS and incubated with the corresponding fluorophore-secondary antibodies with 5% NGS in 1 × PBS (Alexa Fluro 405, 488, 568, and/or 647; Thermo Fisher Scientific) for 1 h at room temperature (RT). The coverslips were then washed (3×) and mounted onto slides using ProLong Antifade reagent (Invitrogen, Carlsbad, CA, USA).

2.5. SIM imaging and analysis

Neurons were fixed, and immunocytochemistry was performed as described above. All analyzed interneurons were identified by morphology (i.e. multi-polar configuration and largely aspiny processes) here due to antibody constraints. Four-channel images were taken using a Nikon SIM microscope with a 100 × 1.49 NA objective (Cell Imaging Facility & Nikon imaging center, Northwestern University, Chicago, IL, USA) that allows a resolution range of 110–130 nm. Images were reconstructed (reconstruction parameters of 0.96, 1.19, and 0.17 for Illumination Modulation Contrast, High Resolution Noise Suppression, and Out of Focus Blur Suppression respectively were kept constant for all reconstructions), background-subtracted, and analyzed with FIJI as described previously [36]. Analysis of CNTNAP2-CASK-GluA1 patterns was accomplished using FIJI on single plane SIM images, by first selecting a 5 μm section of the dendrite with little contaminating fluorescence from adjacent cells. Next, the background was subtracted using rolling ball radius (50 pixels). Each channel was thresholded to create ROIs for 488, 568, and 647 within the selected segment of dendrite. ROIs of puncta that overlapped by ≥1 pixel were considered colocalized. We assessed CNTNAP2, CASK, and GluA1 puncta colocalization alone or within a two-or three-protein complex.

2.6. Confocal microscopy imaging

Confocal images of neurons were obtained as described previously [42]. Briefly, images were acquired by a Nikon (Amsterdam, Netherlands) C2 confocal microscope using a 63X oil immersion objective with numerical aperture (NA) = 1.4 with 0.4 μm z-stacks. All cells analyzed were interneurons, as determined by GABA antibody staining; images were acquired in the linear range of fluorescence intensity within either primary or secondary interneuron dendrites. The acquisition parameters were kept the same for all conditions and were analyzed using FIJI software. For puncta analysis, the “Analyze Particle” function was employed on a background-subtracted GFP-defined region-of-interest (ROI); puncta with areas less than 0.025 μm^2 were excluded from the analysis. Puncta density was measured by number of puncta within a designated ROI divided by the ROI’s area. For immunofluorescence intensity, the mean pixel intensity of an ROI was measured; images were not background subtracted in this case.

2.7. Statistical analysis

All statistical tests were performed with GraphPad Prism (Version 7). Before analysis, data were first tested using D'Agostino's Omnibus Normality Test and Pearson correlation in order to determine whether parametric or nonparametric tests were to be used. P values < 0.05 were considered significant.

3. Results

3.1. CASK, CNTNAP2, and GluA1 complex *in vivo*

As *in vitro* studies show that CNTNAP2 and CASK interact in interneurons [10] and CNTNAP2 regulates GluA1 trafficking in excitatory neurons [42], we wanted to ascertain whether CNTNAP2 could physiologically interact with GluA1 and CASK *in vivo*. We first demonstrated that CASK could successfully pull down GluA1 and CNTNAP2 in adult mouse cortical homogenates (Fig. 1a). As supplementation, we also co-immunoprecipitated CNTNAP2 and CASK with GluA1 (Fig. 1b). These biochemical results support the literature and confirm that a fraction of CNTNAP2, CASK, and GluA1 participate in a biophysical multiprotein complex *in vivo*.

3.2. High resolution imaging reveals GluA1 in a complex with CNTNAP2 and CASK

Although immunoprecipitation studies indicated that CNTNAP2, CASK, and GluA1 interface in mouse cortical tissue, it is unclear whether this is through a single tripartite or multiple bipartite configurations. We therefore used SIM to examine the interaction patterns between the molecules in mature interneuronal dendrites (Fig. 2a). Endogenous CNTNAP2, CASK, and GluA1 were found either individually or complexed with one another in various combinations (Fig. 2b). Analytic studies of such compositions showed that the most common distribution was the tripartite configuration: 68.3% of CNTNAP2 puncta colocalized with GluA1-CASK molecules, 54.7% of CASK puncta colocalized with GluA1-CNTNAP2 molecules, and 49.6% of GluA1 puncta colocalized with CNTNAP2-CASK molecules (Fig. 2c). Interestingly, individual CNTNAP2 (13.2%), CASK (6.5%), and GluA1 (8.8%) molecules were rarely observed (Fig. 2c).

3.3. CNTNAP2 knockdown alters dendrite/soma total GluA1 patterns

Given that our data suggests CNTNAP2, CASK, and GluA1 colocalize, we sought to identify functional consequences of this interaction. We next assessed whether manipulation of CNTNAP2 would affect GluA1 expression patterns in cultured interneurons. We found that CNTNAP2 knockdown (21–26 DIV) in rat mature interneurons (Supplemental Fig. 1a) led to a surprising increase in average total GluA1 dendrite intensity (scrambled: 331.4 ± 12.2 AU vs. shCNTNAP2: 388.5 ± 12.1 AU; Fig. 3a–b) and puncta density (scrambled: 0.41 ± 0.02 puncta/μm^2 vs. shCNTNAP2: 0.48 ± 0.02 puncta/μm^2; Fig. 3a–b). Puncta size, however, was unaltered in either condition (scrambled: 0.15 ± 0.01 μm^2 vs. shCNTNAP2: 0.14 ± 0.006 μm^2; Fig. 3a–b).

Soma GluA1 patterns had distinct patterns compared to dendrites: soma total GluA1 intensity was unchanged between scrambled and shCNTNAP2 interneurons (scrambled: 545.7 ± 27.28 AU vs. shCNTNAP2: 594 ± 25.0 AU; Fig. 3c–d), while GluA1 puncta density

(scrambled: 0.08 ± 0.008 puncta/μm^2 vs. shCNTNAP2: 0.18 ± 0.01 puncta/μm^2; Fig. 3c–d) was increased and puncta size (scrambled: 0.95 ± 0.10 μm^2 vs. shCNTNAP2: 0.39 ± 0.04 μm^2; Fig. 3c–d) decreased in the knockdown.

3.4. CASK knockdown results in altered total GluA1 patterns in interneuron soma/dendrites

Next, we evaluated the effects of CASK knockdown (Supplemental Fig. 1b) on GluA1 levels and localization. We report that shCASK dendrites had increased total GluA1 intensity (scrambled: 430.9 ± 20.29 AU vs. shCASK 568.9 ± 37.57 AU; Fig. 4a–b) and puncta size (scrambled: 0.131 ± 0.01 μm^2 vs. shCASK: 0.186 ± 0.01 μm^2; Fig. 4a–b), but not puncta density (scrambled: 0.483 ± 0.05 puncta/μm^2 vs. shCASK: 0.482 ± 0.04 puncta/μm^2; Fig. 4a–b).

On the other hand, GluA1 clusters within the soma of shCASK interneurons appear much more widely dispersed but smaller compared to scrambled cells, as reflected in the quantifications: higher average total GluA1 intensity (scrambled: 643.5 ± 43.48 AU vs. shCASK: 930.3 ± 47.23 AU; Fig. 4c–d), smaller puncta size (scrambled: 0.643 ± 0.04 μm^{2}vs. shCASK: 0.376 ± 0.026 μm^2; Fig. 4c–d), and greater puncta density (scrambled: 0.118 ± 0.012 puncta/μm^2 vs. shCASK: 0.266 ± 0.016 puncta/μm^2; Fig. 4c–d). Thus, shCASK interneurons recapitulates some aspects of shCNTNAP2 interneurons, providing further evidence GluA1 is regulated by the CNTNAP2-CASK complex.

4. Discussion

Interneurons must possess a perfect balance of distinctive arborization morphology, unique electrophysiological properties, and precise positioning to successfully entrain pyramidal counterparts to fire at particular synchronous rhythms required for proper excitatory transmission [9,17]. Failure of such procedures can lead to devastating consequences for proper information transfer and ultimately cause behavior abnormalities. Consequently, inhibitory neurons have been implicated as one of the major substrates in the pathophysiology of complex psychiatric diseases such as schizophrenia, autism, and intellectual disability [23]. Indeed, multiple studies have uncovered molecules involved in the control of interneuronal glutamatergic synapses [5,30,41], suggesting the presence of cell-specific mechanisms of synapse plasticity, although the literature on this is relatively sparse.

Here we investigate how CNTNAP2, a molecule highly expressed in interneurons [24,44], may regulate GluA1 in inhibitory cell types. Utilizing multiple convergent methods – namely SIM, co-immunoprecipitation, and shRNA – we found that CNTNAP2 forms a complex with CASK and GluA1 *in vivo* and the three proteins colocalize frequently in tripartite patterns within interneuron dendrites. Transient knockdown of either CNTNAP2 or CASK leads to overall elevated GluA1, suggesting CNTNAP2 and CASK may synergistically regulate GluA1 abundance in interneurons. Since CNTNAP2 interacts directly with CASK at the membrane and CASK has been shown to traffic NMDARs/AMPARs, we speculate that CNTNAP2 binds GluA1 through CASK. Function-wise, both CASK or CNTNAP2 knockdown cause GluA1 to lose focalized distribution. This

observation, along with the finding that genetic ablation of *Cntnap2* causes GluA1 aggregates to form [42], provides strong evidence CASK and CNTNAP2 work in conjunction to traffic GluA1.

On the other hand, GluA1 dendrite patterns in CASK or CNTNAP2 knockdown interneurons are not entirely similar, suggesting different mechanisms in either scenario. For instance, CASK can interact with AMPAR subunits through other mediators like GRIP1 [14], while CNTNAP2 associates with protein 4.1, another CASK-interacting scaffold molecule involved with AMPAR trafficking [4,7,21]. Thus sudden loss of CNTNAP2 or CASK in interneuronal dendrites, while both affecting GluA1, may not have the same exact effects. Furthermore, we have previously shown that CASK and CNTNAP2 alter dendritic arborization morphology in inhibitory neurons [10]. Thus, it is also possible that changes in the CNTNAP2-CASK interactions destabilize neuronal structure which in turn alters synaptic GluA1 localization [20,28,45].

Patients with highly penetrant *CNTNAP2* or *CASK* mutations have overlapping symptomology, particularly in regards to seizures [12,33,37,40], thereby suggesting common pathways involving excitatory/inhibitory balance. From previous data, we speculate that CNTNAP2-CASK may serve as part of a cellular pathway that converges upon interneuron regulation. Here, we show initial evidence that GluA1 may be one of the downstream targets of CNTNAP2-CASK and that disruption of CNTNAP2 or CASK may contribute to neurodevelopmental diseases by interfering with GluA1 quantity and localization. Interestingly, we previously found reduced levels of GluA1 in the spines of Cntnap2 knockout pyramidal neurons, which directly conflicts with our current findings. This discrepancy may be due to differences in molecular abundance between different cell types, as CNTNAP2 is more highly expressed in interneurons [24]. Another explanation could be the lack of compensation in shRNA knockdown compared to knockout models [34]; for example, knockdown, but not knockout, of CNTNAP2 causes decreased pyramidal cell arborization [2,10]. While follow-up studies are critical to tease apart these subtle differences, our findings provide the first definite molecular link between CNTNAP2, CASK, and GluA1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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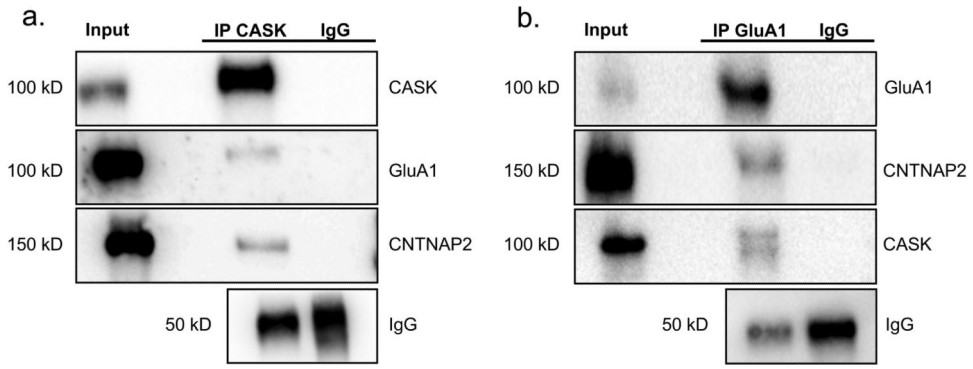

Fig. 1.

CNTNAP2 complexes with CASK and GluA1 in mouse cortical tissue. (a) Western blots of co-immunoprecipitation from mouse cortex using a CASK antibody. The CASK antibody successfully co-immunoprecipitated CNTNAP2 and GluA1. (b) Western blots of co-immunoprecipitation from mouse cortex using a GluA1 antibody. The GluA1 antibody successfully co-immunoprecipitated CNTNAP2 and CASK.

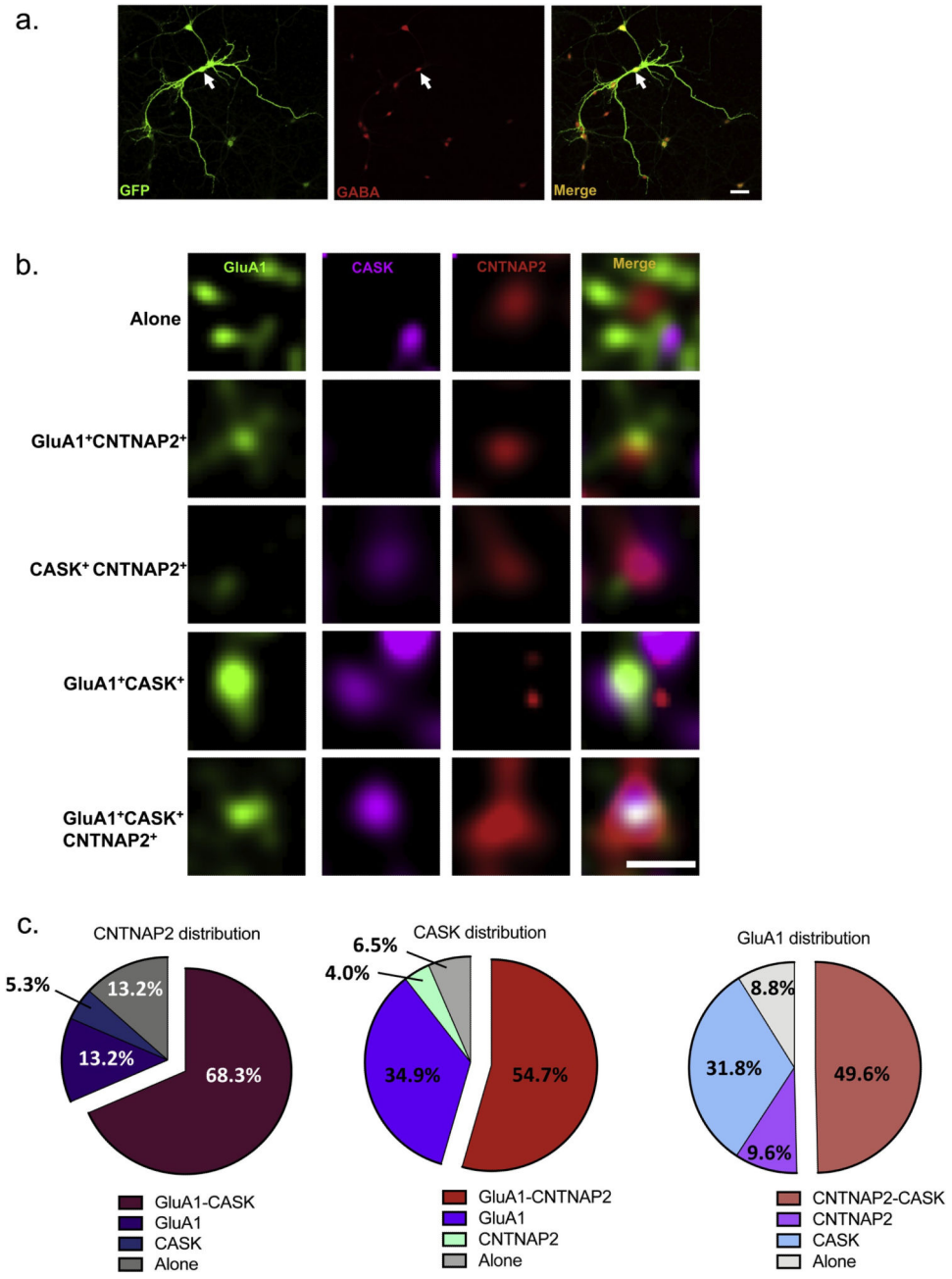

Fig. 2.

SIM reveals CNTNAP2, CASK, GluA1 most frequently form a tripartite complex. (a) Representative low-magnification confocal image of an interneuron, confirmed by co-staining with a GABA antibody (scale bar = 50 μ m). (b) Representative high-resolution SIM images displaying various distributions of endogenous CNTNAP2, CASK, and GluA in the dendrites of 27 DIV interneurons (scale bar = 0.5 μ m). (c) Categorization of the patterns seen in (b) (n = 98–135 total puncta from 12 branches and 3 independent experiments).

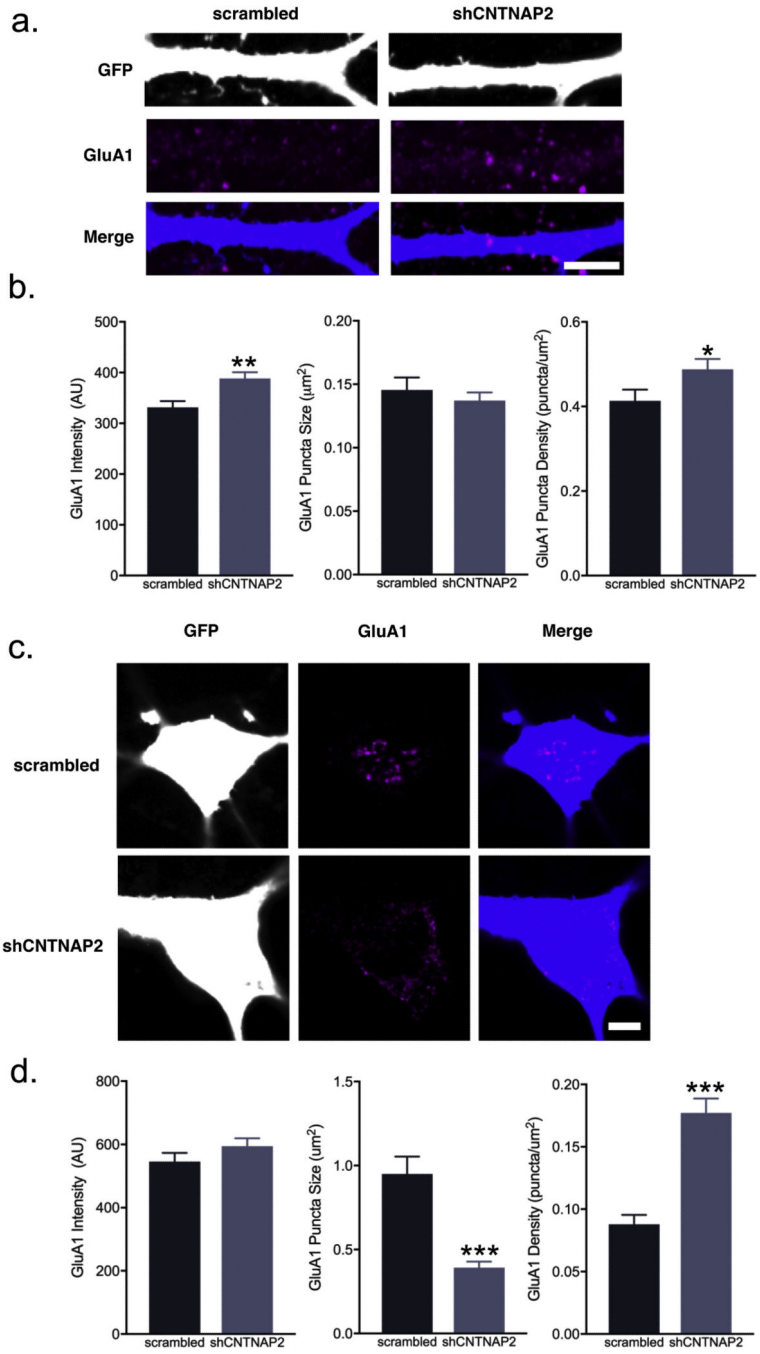

Fig. 3.

CNTNAP2 knockdown in interneurons leads to alterations of GluA1 content and clustering. (a) Representative confocal images and (b) quantification of total GluA1 average intensity, puncta size, and puncta area in scrambled or shCNTNAP2-treated (21–26 DIV) rat interneuron dendrites at 26 DIV (scale bar = 5 μ m; intensity: n = 72 branches; puncta size and density: n = 68–71 branches from 5 independent experiments). (c) Representative confocal images and (d) quantification of GluA1 average intensity, puncta size, and puncta area in scrambled or shCNTNAP2-treated (21–26 DIV) rat interneuron somas at 26 DIV

(scale bar = 5 μ m; intensity: n = 40 cells; puncta size and density: n = 39–40 cells from 5 independent experiments). Values are means ± SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; Student's *t*-test (d, intensity), Mann-Whitney test (b; d, puncta size and density).

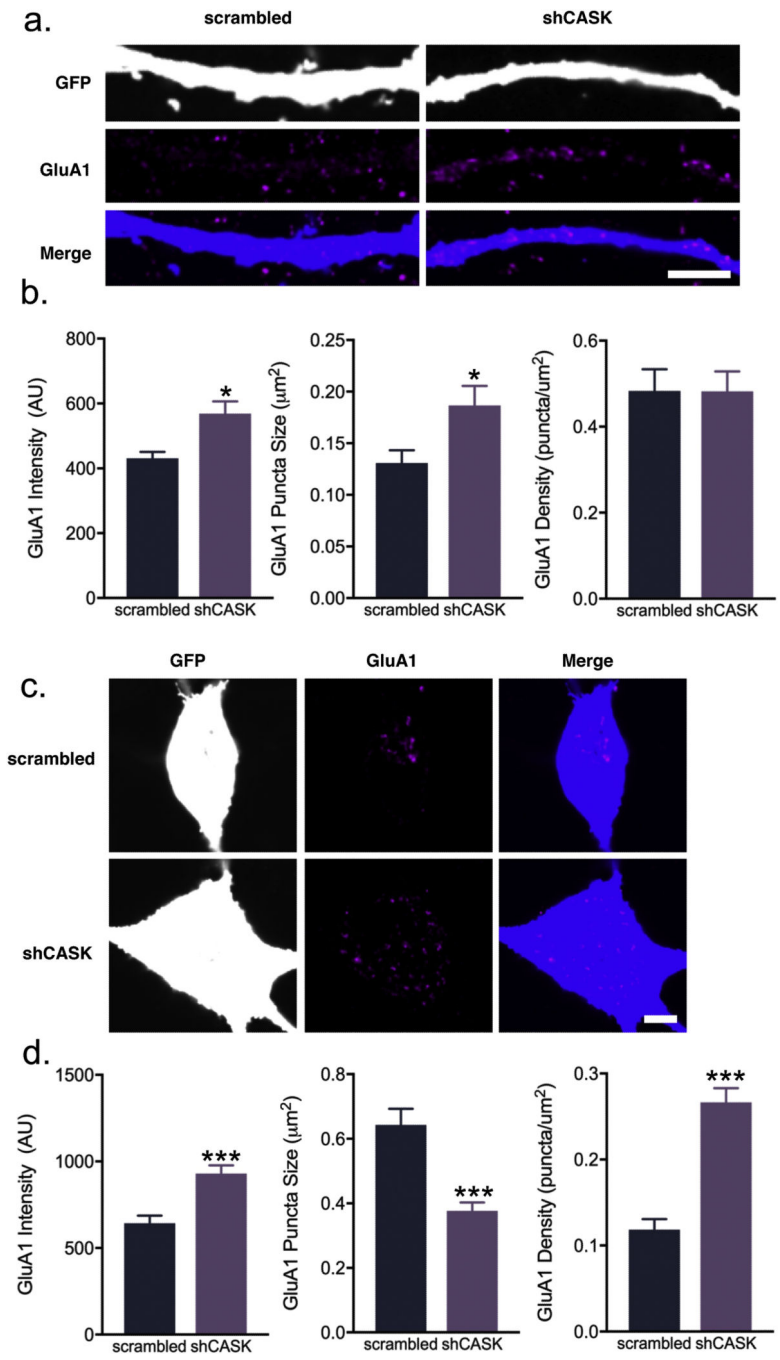

Fig. 4.

CASK knockdown in interneurons leads to alterations of GluA1 content and clustering. (a) Representative confocal images and (b) quantification of GluA1 average intensity, puncta size, and puncta area in scrambled or shCASK-treated (21–26 DIV) rat interneuron dendrites at 26 DIV (scale bar = 5 μ m; intensity: n = 35–36 branches; puncta size and density: n = 33 branches from 3 independent experiments). (c) Representative confocal images and (d) quantification of GluA1 average intensity, puncta size, and puncta area in scrambled or shCASK-treated (21–26 DIV) rat interneuron somas at 26 DIV (scale bar = 5 μ m; n = 20–21

cells from 3 independent experiments). Values are means ± SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; Student's *t*-test (b, intensity; d), Mann-Whitney test (b, puncta size and density).