Published in final edited form as:

Eur J Neurosci. 2020 February ; 51(4): 1074–1086. doi:10.1111/ejn.14620.

CNTNAP2 is targeted to endosomes by the polarity protein PAR3

Ruoqi Gao, Ph.D.¹, Christopher P. Pratt, Ph.D.¹, Sehyoun Yoon, Ph.D.¹, Maria Dolores Martin-de-Saavedra, Ph.D.¹, Marc P. Forrest, Ph.D.¹, Peter Penzes, Ph.D.^{1,2,3,*}

¹Department of Physiology, Northwestern University Feinberg School of Medicine, Chicago, 60611 IL, USA

²Department of Psychiatry and Behavioral Sciences, Northwestern University Feinberg School of Medicine, Chicago, 60611 IL, USA

³Northwestern University Center for Autism and Neurodevelopment, Chicago, 60611 IL, USA

Abstract

A decade of genetic studies has established Contactin-associated protein-like 2 (*CNTNAP2*) as a prominent susceptibility gene associated with multiple neurodevelopmental disorders. The development and characterization of *Cntnap2* knockout models in multiple species have bolstered this claim by establishing clear connections with certain endophenotypes. Despite these remarkable *in vivo* findings, *CNTNAP2*'s molecular functions are relatively unexplored, highlighting the need to identify novel protein partners. Here, we characterized an interaction between *CNTNAP2* and Partitioning-defective 3 (PAR3) – a polarity molecule isolated in a yeast-two hybrid screen with *CNTNAP2*'s C-terminus. We provide evidence that the two proteins interact via PDZ domain-mediated binding, that *CNTNAP2*⁺/PAR3⁺ complexes are largely associated with clathrin-coated endocytic vesicles in heterologous cells, and that PAR3 causes an enlargement of *CNTNAP2* puncta size. Live imaging and fluorescence recovery after photobleaching (FRAP) reveals that PAR3 limits the mobility of *CNTNAP2*. Finally, over-expression of PAR3 but not a PAR3 mutant lacking all PDZ domains (PAR3 PDZ_{all}) can cluster endogenous *CNTNAP2* in primary neurons. Collectively, we conclude that PAR3 regulates *CNTNAP2* spatial localization.

Graphical Abstract

*To whom correspondence should be addressed: Peter Penzes, 303 E. Chicago Ave, WARD 7-176, Chicago, IL 60611, Tel: +1 312 503 5379; p-penzes@northwestern.edu.

Author Contributions

R.G. led the project. R.G. and C.P.P. performed all confocal imaging experiments. R.G., M.D.M., and M.P.F. performed biochemistry experiments. S.Y. performed live imaging experiments. P.P. supervised the project. R.G., C.P.P., and P.P. wrote the manuscript.

Conflict of Interest

The authors declare no competing financial conflict of interests.

Data Accessibility

Primary data is available and can be accessed by contacting the corresponding author (see Materials and Methods for more information).

PAR3 and CNTNAP2 interact via PDZ domains. In heterologous cells, this interaction drives CNTNAP2 into enlarged vesicles that are associated with clathrin-dependent endocytosis. Endogenous CNTNAP2 is also clustered by exogenous PAR3 in primary neurons.

Keywords

Interneurons; Cos7 cells; Protein-protein interactions; Trafficking

Introduction

CNTNAP2 is a prominent disease susceptibility gene associated with Autism Spectrum Disorder (ASD), Intellectual Disability (ID), Schizophrenia (SCZ), epilepsy, and is causative for Cortical Dysplasia Focal Epilepsy (CDFE) syndrome (Rodenas-Cuadrado *et al.*, 2014). *Cntnap2* knockout rat, mice, and zebrafish have recapitulated several disease endophenotypes including impaired social interaction, communication, and seizures (Penagarikano *et al.*, 2011; Hoffman *et al.*, 2016; Thomas *et al.*, 2017). *CNTNAP2* encodes a type I transmembrane cell adhesion molecule whose most prominent role is in its localization to the juxtaparanode, where it clusters potassium channels (Poliak *et al.*, 2003); however, it is also involved in neuronal migration and promotes dendritic branching (Rodenas-Cuadrado *et al.*, 2014). While CNTNAP2 is expressed in both excitatory and inhibitory neurons, several reports have demonstrated that CNTNAP2 and its close family members are more prominently expressed in interneurons (Karayannis *et al.*, 2014; Mo *et al.*, 2015; Gao *et al.*, 2018). Indeed, interneuron dysfunction and altered inhibition may be crucial factors for behavioral endophenotypes in *Cntnap2* null mice (Jurgensen & Castillo, 2015; Bridi *et al.*, 2017; Vogt *et al.*, 2017). Despite this, the cellular and molecular consequences of CNTNAP2 dysfunction in interneurons remain poorly understood.

Polarity is an organizing process that is essential for cellular development, allowing cells to form asymmetrical specializations critical for cellular differentiation (Goldstein & Macara, 2007). The partitioning defective (PAR) proteins participate in an evolutionarily-conserved

complex consisting of three members: Partitioning-defective protein 3 (PAR3, a.k.a. PARD3), Partitioning-defective 6 (PAR6), and atypical protein kinase C (aPKC). This complex initiates many of its essential processes through interactions of its members with downstream guanine nucleotide exchange factors (Johansson *et al.*, 2000; Lin *et al.*, 2000). Through this interplay, PAR3 assists in regulation of multiple polarity events including apical-basal establishment in epithelial cells, axon specification in neurons, asymmetric mitotic spindle positioning during cell division, and astrocyte migration (Macara, 2004; Mertens *et al.*, 2006). Conversely, PAR3 dysfunction leads to loss of polar organization, abnormal GTPase signaling, and carcinogenesis/metastasis in some cell types (McCaffrey *et al.*, 2012).

In addition to its canonical role with PAR6 and aPKC, PAR3 also has PAR complex-independent functions. For example, PAR3 can spatially localize the Rac-GEF TIAM1 to the membrane, a process important for events requiring cytoskeletal rearrangement, such as spinogenesis (Zhang & Macara, 2006; Duman *et al.*, 2013), migration (Narayanan *et al.*, 2013), and tight junction formation (Mizuno *et al.*, 2003; Chen & Macara, 2005; Narayanan *et al.*, 2013). PAR3 also is involved in mammalian cell survival and phosphoinositide signaling through membranous interactions with exocyst docking proteins and PTEN respectively (Feng *et al.*, 2008; Ahmed & Macara, 2017). Previous studies have also implicated cell adhesion molecules, such as JAMs, Ephrin receptors, and GPCRs, as critical upstream effectors for recruitment of PAR3 (Lin *et al.*, 1999; Itoh *et al.*, 2001; Duman *et al.*, 2013). Thus, PAR3, in both canonical and non-canonical roles, serves as a central recruitment scaffold for a multitude of interactors, many of which likely remain unknown.

We discovered that PAR3 is a novel CNTNAP2 interactor by using a yeast two-hybrid screen using the CNTNAP2 C-terminus. We validated this interaction by co-immunoprecipitating CNTNAP2 and PAR3 from mouse cortex. Through domain mapping in HEK293T cells, we demonstrated this association to be dependent on PDZ domain binding, specifically between CNTNAP2's PDZ binding domain and PAR3's third PDZ domain. Immunocytochemical analysis in COS7 cells revealed both molecules in association within abnormally large clathrin-dependent endocytic vesicles, while live imaging showed that this association reduced CNTNAP2 mobility. Finally, overexpressed PAR3 formed large vesicular structures in primary cultured interneurons, in which endogenous CNTNAP2 was localized. Taken together, we characterized PAR3 as a novel CNTNAP2 interaction partner, which may contribute to a deeper fundamental understanding of CNTNAP2's molecular functions and associated biological pathways that contribute to neurodevelopmental disorders.

Materials and Methods

Ethical Standards

All experiments were approved by the Institutional Animal Care and Use Committee of Northwestern University, protocol number IS0005992.

Antibodies and Plasmids

A detailed antibody and primer list is presented in the Supplementary Information. pEGFP-N2 plasmid was purchased from Clontech (Mountain View, CA, USA). pEBFP2-N1 was purchased from Addgene (Cambridge, MA, USA; #54595). Dlx-GFP is a GFP expression plasmid driven by a Dlx5/6 enhancer (gift from Dr. Daniel Vogt; Michigan State University). FLAG-CNTNAP2 was generated by subcloning human *CNTNAP2* cDNA (gift from Dr. Elior Peles, Weizmann Institute of Science, Israel) into the pEGFP-N2 vector (restriction sites BamHI and NotI, removing EGFP) using the Infusion cloning system from Clontech. The FLAG sequence was then inserted downstream of the signaling peptide (amino acids 1–27) using the same technique. mCherry-CNTNAP2 was constructed similarly, with mCherry being inserted after the signal peptide. CNTNAP2 truncations were created by deleting the C-terminus, 4.1-binding site, or PDZ-binding site.

Myc-PAR3 was created by subcloning human *Myc-PAR3* from the *pK-Myc-Par3b* plasmid (Addgene #19388) into the pEGFP-N2 vector as described above using BamHI and NotI restriction sites. PAR3 truncation mutants were created by deleting the first (amino acids 271–359), second (amino acids 461–546), third (amino acids 590–677), or all PDZ domains (PDZ_{all}; amino acids 271–677). GFP-PAR3 and GFP-PAR3 PDZ_{all} were generated by inserting EGFP into Myc-PAR3 between the BglII and BamHI restriction sites.

Neuronal and Cell Line Culture and Transfection

High density (300,000 cells/cm²) cortical neuron cultures were prepared from Sprague-Dawley rat E18 embryos and plated on poly-D-lysine coated coverslips (18mm diameter, #1.5 glass). Cortical neurons were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations. Experiments were performed 3 days post-transfection. Any visual signs of poor neuronal health meant the exclusion of the cell from quantification.

COS7 or HEK293T cells (ATCC, Manassas, VA, USA; lines authenticated before shipment) were transfected using Lipofectamine 2000 following the manufacturer's instructions. Experiments were performed 48h post-transfection.

Immunocytochemistry

Endogenous PAR3 was detected as described (Zhang & Macara, 2006). Briefly, neurons were fixed in 4% formaldehyde-sucrose-PBS for 15 min at room temperature (RT), followed by permeabilization with 0.2% Triton-X-100 for 5 minutes at RT, blocking in 10% bovine serum albumin (BSA) for 1 hour at RT, and incubation of primary antibody in 3% BSA overnight at 4°C. Secondary antibodies were applied in 3% BSA 1 hour at room temperature. For detection of all other targets, ICC was performed as described (Gao *et al.*, 2018). COS7 cells were permeabilized as above and blocked in 5% normal goat serum (NGS) for 1 hour. Primary and secondary antibodies were added in the conditions described for neurons, with 5% NGS as a blocking agent. Coverslips were mounted using ProLong Gold and imaged after curing.

Immunoprecipitation

Mouse cortex or transfected HEK293T cells were homogenized in immunoprecipitation buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton X-100), with protease inhibitor cocktail (Roche, Basel, Switzerland) and solubilized for 1 hour at 4°C. Solubilized material was centrifuged at 20,000 g for 10 minutes at 4°C and the supernatant was precleared with protein A/G sepharose beads (Thermo Fisher Scientific) for 30 minutes. Proteins were then immunoprecipitated with 3 µg of antibody overnight at 4°C, followed by a 1 hour incubation with protein A/G sepharose beads the following day. Beads were then washed 3 times with IP buffer before adding 2x Laemmli buffer (Biorad, Hercules, CA, USA). Samples were analyzed by SDS-PAGE and Western blotting.

Fractionation

Subcellular fractionation was performed as previously described (Nakagawa *et al.*, 2005). Briefly, cortices from 6-week old mice were homogenized in cold sucrose buffer (20 mM HEPES pH 7.4, 320 mM sucrose, 5 mM EDTA) with protease inhibitor cocktail (Roche). Homogenates were centrifuged at 3,000 g for 20 minutes at 4°C to pellet nuclei. Supernatant (S1) was then centrifuged at 38,000 g for 30 min at 4°C to obtain a crude membrane pellet (P2). P2 was resuspended in potassium iodide buffer (20 mM HEPES pH 7.4, 1 M KI, 5 mM EDTA) to remove membrane-associated proteins (S3). Membranes were again collected by centrifugation (38,000 g for 20 min at 4°C). Membranes were washed (20 mM HEPES pH 7.4, 5 mM EDTA) and pelleted once more (S4) before solubilizing in CHAPS buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% CHAPS) supplemented with protease inhibitors for 2 hours at 4°C. Solubilized membranes were clarified by centrifugation at 100,000 g for 30 min at 4°C (S5). The final CHAPS-insoluble pellet was resuspended in SDS buffer (50mM TRIS pH 7.4, 150 mM NaCl, 1% SDS) supplemented with protease inhibitors, solubilized at 37°C for 20 mins and clarified by centrifugation (S6).

Yeast Two-Hybrid Screening

Y2H screening was performed using the Matchmaker Gold System (Clontech). Briefly, the C-terminal region of CNTNAP2 (amino acids 1284–1331) was cloned into the bait vector (pGBKT7) and transformed into yeast. Bait-positive yeast were then mated with yeast containing cDNA (pGADT7) from a mouse brain cDNA library. Mated yeast were then plated onto double amino acid (-Leu/- Trp) dropout, Aureobasidin A positive (A), and X- α -Gal positive (X) plates (DDO/X/A), a low stringency medium that selects for mated yeast containing both bait and prey vectors. DDO/X/A colonies that were blue, indicative of a possible interaction between bait-prey, were streaked onto quadruple amino acid (-Leu/- Trp/-His/-Ade) dropout, Aureobasidin-A positive (A), and X- α -Gal positive (X) plates (QDO/X/A), which require a genuine bait-prey interaction for growth. The blue colonies from QDO/X/A plates were isolated and plasmids sequenced.

Confocal Microscopy

Confocal images of neurons were acquired using a Nikon C2 confocal microscope using a 60X oil immersion objective with numerical aperture (NA) = 1.4 with 0.4 µm z-stacks. All images were acquired in the linear range of fluorescence intensity. The acquisition

parameters were kept the same for all conditions and images were analyzed using Fiji software (for more detail on analysis, see below).

Colocalization analysis

Colocalization highlighter images and Manders' colocalization coefficients were determined in ImageJ after thresholding (Figure 3). Total immunofluorescence intensity of region of interest (ROI) was measured automatically. For the size of puncta quantification, a threshold was applied to the maximum projection images to include all detectable puncta. The puncta restricted to objects with areas greater than $0.08 \mu\text{m}^2$, were manually detected, and the puncta area was measured.

For object-based binary classification of colocalization (Figure 4), ROIs were drawn using ImageJ on single planes encompassing whole cell cytoplasm and excluding nuclei using BFP cell-fill as a guide. Images were processed and analyzed using Python 3.6 with the scikit-image package (van der Walt *et al.*, 2014). Briefly, images were pre-processed using a 3×3 median filter and 20-pixel disk white tophat transformation to reduce noise and background, respectively. Drawn ROIs were imported and thresholding was performed on the ROI histogram for each channel using the Triangle algorithm (Zack *et al.*, 1977) and images were converted to binaries. Small objects ($<12 \text{ pixels}^2$) were excluded from analysis. Puncta were segmented into objects using a Watershed algorithm. Colocalization analysis was performed as follows: For two-channel colocalization of PAR3 and CNTNAP2, an object in the first channel was considered colocalized with the second channel if $>50\%$ of its included pixels were also above threshold in channel 2. For triple colocalization of CNTNAP2⁺/PAR3⁺ puncta with trafficking markers, the intersection of the above-threshold CNTNAP2/PAR3 signal was generated and segmented as described. These objects were then used as channel 1 for colocalization with markers (channel 2). See Supplemental Figure 2 for illustration.

Live Imaging and Analysis

COS7 cells for FRAP experiments were grown on glass bottom culture dishes from MatTek (Ashland, MA, USA) and transfected with mCherry-CNTNAP2 and GFP-PAR3 or GFP-PAR3 PDZ_{all}. Cells were imaged with a confocal microscope (Nikon) in a CO2 incubator stage. Images were taken every 1s for 300s after bleaching. 100% laser power pulses of 1ms for 1 min were used to bleach GFP-PAR3. Fluorescence intensity of bleached GFP-PAR3 was measured and normalized to the fluorescence before bleaching within the same area. Recovery data points were then fitted to a one-phase association exponential in GraphPad Prism (Version 7).

Blinding and Statistical Analysis

Data from cell line and primary neuron studies were obtained and analyzed under blinded conditions (coverslip identity hidden, cells selected randomly, filenames randomized). Cells of visibly poor health were excluded from quantification. Sample sizes for cell line studies were between 20–28 cells from multiple experiments. For primary neuronal studies, over 30–40 neurons were imaged and the best representative neuron was used for display.

All statistical tests were performed with GraphPad Prism (Version 7) or in Python using the SciPy library. 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. Post-hoc tests were always used in multiple comparison analysis. P values < 0.05 were considered significant.

Code Availability

Python code for trafficking marker analysis will be made available upon request.

Data Accessibility

Original yeast two-hybrid results can be found in supplementary information of Gao et al. (2018). Supplementary data can be found in the online version of this publication.

Results

A yeast two-hybrid screen reveals PAR3 as a novel CNTNAP2 interaction partner

We performed a yeast two-hybrid screen of a mouse brain cDNA library with the entire CNTNAP2 intracellular domain (ICD) as bait (amino acids 1284–1331) to find intracellular interactors of CNTNAP2 (Figure 1a). Out of three million candidates screened, we identified 41 unique clones (Gao *et al.*, 2018), with PAR3 being one of the most intriguing, given its role as a spatial organizer (Zhang & Macara, 2006; Duman *et al.*, 2013; Ahmed & Macara, 2017). We confirmed this positive result by demonstrating yeast growth on high stringency plates only when expressing both bait/prey plasmids, indicating a direct physical interaction (data not shown). We then validated that CNTNAP2 and PAR3 are in the same protein complex *in vivo* by co-immunoprecipitation experiments from mouse cortex homogenates (Figure 1b). To determine the protein domains involved in their interaction, we co-expressed various CNTNAP2 truncation mutants (Figure 1a; red lines) with PAR3 in HEK293T cells and performed co-immunoprecipitations. We found that the C-terminal PDZ-binding motif of CNTNAP2 is necessary for interaction with PAR3, as its deletion prevented PAR3 pull-down (Figure 1c). Conversely, the PDZ domains of PAR3 are also necessary for CNTNAP2 interaction. Deletion of any of the three domains (Figure 1a, red lines) reduces co-immunoprecipitation efficiency, with PDZ3 having a complete loss (Figure 1d). Moreover, PDZ3 lies within the realm of the original PAR3 prey clone (Figure 1a). From these results, we conclude that the C-terminal PDZ-binding domain of CNTNAP2 interacts primarily with the third PDZ domain on PAR3, a site previously observed to also bind PAR6 (Renschler *et al.*, 2018). These experiments demonstrate that PAR3 is a novel CNTNAP2 interactor that utilizes PDZ binding for physical association. For ease of interpretation, we opted to use a PAR3 mutant lacking all PDZ sites (PAR3 PDZ_{all}) for subsequent experiments.

Subcellular compartmentalization of PAR3 and CNTNAP2

We proceeded to characterize the spatial properties of both proteins by performing an in-depth subcellular fractionation procedure using salt extraction and detergent solubilization (Figure 2). To confirm fractionated compartments, we probed the fractions with GluA1, PSD95, and β -tubulin antibodies. GluA1 (synaptic membrane protein) was highly enriched in the crude membrane (S5) and synaptic (S6) fractions while PSD95 (synaptic cytosolic

protein) was only enriched in the S6 fraction; β -tubulin (non-synaptic cytosolic protein) was absent from either S5 or S6 fractions (Figure 2b, bottom). We found both PAR3 and CNTNAP2 in the S6 (synaptic) fraction, consistent with previous publications (Lin *et al.*, 2000; Oiso *et al.*, 2009; Chen *et al.*, 2015; Gao *et al.*, 2018). We also validated previous studies and showed strong colocalization of PAR3 with PSD95 (Supplementary Figure 1). Moreover, PAR3 was also highly enriched in the crude membrane-associated (S3) fraction but is almost nonexistent in S5, while CNTNAP2 was most abundant in the S5 fraction, as expected. Together, these data suggest that PAR3 is a cytosolic protein, which can transiently associate with membrane proteins such as CNTNAP2.

PAR3 redistributes CNTNAP2 in COS7 cells

We next used COS7 cells to ascertain localization patterns for both molecules. When CNTNAP2 was expressed alone, we noted distribution patterns at the cell periphery, perinuclear region, and within small cytoplasmic vesicles, some of which were small and punctate (Figure 3a; mCherry-CNTNAP2 alone). PAR3 alone, on the other hand, displayed a primarily diffuse cytoplasmic localization pattern or of that bordering endoplasmic reticulum but lacking puncta (Figure 3a; Myc-PAR3 alone) (Lin *et al.*, 2000). Upon co-expression, respective expression patterns changed drastically, where both molecules were found mostly within large punctate cytoplasmic structures (arrows, Figure 3a; mCherry-CNTNAP2 + Myc-PAR3). Moreover, CNTNAP2 puncta size increased significantly upon PAR3 co-expression (mCherry-CNTNAP2 + Myc-PAR3: $1.16 \pm 0.04 \mu\text{m}^2$ vs. mCherry-CNTNAP2 alone: $0.46 \pm 0.014 \mu\text{m}^2$; Figure 3d). These patterns were abolished by replacement of CNTNAP2 or PAR3 with CNTNAP2 PDZ or PAR3 PDZ_{all} mutants, respectively (Figure 3a; mCherry-CNTNAP2 PDZ + Myc-PAR3 and mCherry-CNTNAP2 + Myc-PAR3 PDZ_{all}). Quantitative Manders' colocalization analysis confirmed these observations (Figure 3b–c; mCherry-CNTNAP2 + Myc-PAR3 M2: 0.921 ± 0.02 vs. mCherry-CNTNAP2 + Myc-PAR3 PDZ_{all} M2: 0.176 ± 0.03). Our data therefore suggest a potential role of PAR3 in regulating CNTNAP2 subcellular localization.

CNTNAP2 and PAR3 co-associate with clathrin-mediated endocytic vesicles

Because the structures containing both CNTNAP2 and PAR3 resembled trafficking compartments, we next determined the identity of CNTNAP2⁺/PAR3⁺ structures by co-staining exogenously-expressed proteins in COS7 cells with endogenous trafficking markers. Cells were transfected with mCherry-CNTNAP2, Myc-PAR3, and a Blue Fluorescent Protein (BFP) cell fill. Markers for endocytosis (Caveolin-1 [CAV1], Clathrin heavy chain [CHC]), very early endosomes (APPL1), early endosomes (EEA1, RAB5), and the trans-Golgi network (TGN) secretory pathway (GOPC, Syntaxin-6 [STX6]) were examined. Association of CNTNAP2⁺/PAR3⁺ puncta with these markers was measured using profile plots and a novel object-based algorithm (Supplementary Figure 2a, also see Materials and Methods).

We found that CNTNAP2⁺/PAR3⁺ vesicles were increasingly associated with CHC, EEA1, and RAB5, suggesting a localization in the clathrin-dependent endocytosis pathway (Figure 4a). Profile plots through putatively colocalized puncta show synchronous peaks of all three fluorescent channels (Figure 4b). In contrast, the CAV1, APPL1, GOPC and STX6 show a

lower degree of fractional colocalization (Supplementary Figure 2b–e). Analysis of the aggregate fractional data by Kruskal-Wallis test with Dunn's post-hoc test show that the putative interactors (CHC, EEA1, RAB5) are not statistically distinct from each other, but each is significantly higher than CAV1, APPL1, and STX6 (Table 1). Furthermore, EEA1 localization is significantly increased compared to GOPC, and RAB5 is very nearly significant ($p=0.055$; Table 1). Collectively, our analysis shows CNTNAP2⁺/PAR3⁺ vesicles may be involved in clathrin-mediated endocytosis and subsequent endosomal trafficking in COS7 cells.

PAR3 alters CNTNAP2 dynamics in intracellular compartments

Since PAR3 appeared to localize CNTNAP2 to intracellular vesicles, we wanted to determine if CNTNAP2 is actively recruited by PAR3 and if this association affects mobility. We used live cell imaging and fluorescence recovery after photobleaching (FRAP) to study these characteristics of CNTNAP2⁺/PAR3⁺ and CNTNAP2⁺/PAR3 PDZ_{all}⁺ vesicles in COS7 cells. We observed that GFP-PAR3 formed distinctively large punctate clusters when co-expressed with mCherry-CNTNAP2 while GFP-PAR3 PDZ_{all} retained a diffuse cytoplasmic staining (Figure 5a), consistent with our previous observations (See Figure 3).

Time-lapse imaging of overexpressed mCherry-CNTNAP2 and GFP-PAR3 in COS7 cells revealed a mixed population of large and immobile CNTNAP2⁺/PAR3⁺ vesicles intermixed with smaller, more mobile ones (Supplemental Movie 1). Conversely, the immobile CNTNAP2⁺/PAR3⁺ structures were abolished when GFP-PAR3 was replaced with the GFP-PAR3 PDZ_{all} mutant (Supplemental Movie 2). Using a one-minute bleaching cycle for maximum effect, we found that mCherry-CNTNAP2 recovery in the bleached region was decreased with co-expression of GFP-PAR3 compared to the GFP-PAR3 PDZ_{all} mutant. Five minutes following bleaching, mCherry-CNTNAP2 fluorescence recovered to twice the extent when co-expressed with GFP-PAR3 PDZ_{all} compared to WT (Figure 5a–b). Additionally, the rate of recovery was nearly 20% faster with the PAR3 PDZ_{all} mutant (WT: $\tau = 426.2s$, PDZ_{all}: $\tau = 361.3s$). These data suggest that PAR3 restricts CNTNAP2 mobility in a manner dependent on the PDZ domains of PAR3.

PAR3 affects the localization of CNTNAP2 in primary interneuronal dendrites

Having shown that PAR3 can influence CNTNAP2's localization in COS7 cells, we set out to understand this relationship in cultured rat neurons. Because our previous study and others show CNTNAP2 to be more highly expressed in interneurons (Karayannis *et al.*, 2014; Gao & Penzes, 2015; Mo *et al.*, 2015), and that interneurons are critical for shaping cortical circuitry (Markram *et al.*, 2004), we focused our analyses on these cells. We overexpressed PAR3 in inhibitory neurons, which were labeled with Dlx-GFP, and studied its expression patterns as well as that of endogenous CNTNAP2. We confirmed the specificity of our Dlx-GFP expression by co-staining with an antibody specific to GABA, a molecule found exclusively within interneurons (Supplementary Figure 3a). In line with our observations in cell lines, PAR3 was largely diffuse in the cytoplasm but also formed large vesicular-like clusters (Figure 6a–b), consistent with endogenous staining patterns (Supplementary Figure 3b) (Zhang & Macara, 2006). On the other hand, overexpression of the PAR3 PDZ_{all} mutant produced a diffuse cytosolic pattern, suggesting the PDZ

interaction is critical for clustering (Figure 6a–b). Finally, as in COS7 cells, we occasionally found large aggregates of endogenous CNTNAP2 that clustered strongly with overexpressed PAR3, but not PAR3 PDZ_{all} (Figure 6a–b). To ensure that our observations are robust, we confirmed the specificity of the CNTNAP2 antibody by staining *Cntnap2*^{-/-} mouse neurons (Supplementary Figure 4). While some apparent signal remains in the KO neurons, it has been recently observed that at least one CNTNAP2 isoform remains detectable in this model (Chen *et al.*, 2015). Altogether, our data indicate that PAR3 can direct localization of CNTNAP2 to punctate structures in interneurons.

Discussion

PAR3 is part of a conserved signaling complex required for inducing polarity events critical for cellular function and survival, such as migration, apical-basal polarity establishment, and axon specification (Mertens *et al.*, 2006). PAR3, with its multi-domain scaffold partners, serves as a central recruiter of downstream signaling molecules in these processes. Our work expands on our knowledge of this function by characterizing the existence of a previously unknown interaction with CNTNAP2 identified by unbiased yeast two-hybrid screening.

CNTNAP2 has multi-faceted roles in neuronal migration, development, and maintenance; PAR3 may serve as a spatial regulator for these functions (Zhang & Macara, 2006; Ahmed & Macara, 2017). In support of this model, we show that PAR3 clusters CNTNAP2 into large vesicles via PDZ binding in heterologous cells. These CNTNAP2⁺/PAR3⁺ vesicles appear to be components of the clathrin-dependent endosomal trafficking pathway, within which PAR3 stabilizes and spatially restricts CNTNAP2. Interestingly, we observed two distinct populations of vesicles with unique mobilities: a large immobile pool and a small mobile population of PAR3/CNTNAP2-containing vesicles. Future studies will address the molecular distinctions and significance of these vesicles.

While CNTNAP2 and PAR3 are expressed in both excitatory and inhibitory neurons (Karayannis *et al.*, 2014; Mo *et al.*, 2015; Gao *et al.*, 2018; Kim *et al.*, 2019; Lazaro *et al.*, 2019), we chose to focus on the latter since CNTNAP2 is more highly expressed in interneurons (Karayannis *et al.*, 2014; Mo *et al.*, 2015; Gao *et al.*, 2018) and because inhibitory circuits are a critical but underexplored aspect of disease pathogenesis in neuropsychiatric disorders (Markram *et al.*, 2004; Marin, 2012). However, PAR3 and CNTNAP2 are also expressed in excitatory neurons (Allen Brain Atlas), particularly within the postsynaptic density (Figure 2b, Supplementary Figure 1). Thus, it is possible that PAR3's effect on CNTNAP2 is influenced by cellular environment. For example, PAR3 within pyramidal spines may affect local CNTNAP2 levels and function in that area (Zhang & Macara, 2006; Duman *et al.*, 2013), leading to alterations in synaptic density, dynamics, or signaling (Gdalyahu *et al.*, 2015; Varea *et al.*, 2015). Likewise, since interneurons are mostly aspiny and we observed CNTNAP2 and PAR3 colocalize in inhibitory dendrites (Figure 6), the complex here may be more influential on dendritic architecture. Equally as speculative, PAR3 may be involved in CNTNAP2 trafficking in both cell types given our COS7 observations (Figure 3). Future cell-specific characterization and manipulation is therefore warranted.

Previous results from our lab (Gao *et al.*, 2018) show that CNTNAP2 also interacts with calcium/calmodulin-dependent serine protein kinase (CASK) at the plasma membrane. Since both CASK and PAR3 appear to regulate CNTNAP2 localization via PDZ domains and because CNTNAP2 has only one PDZ binding region, there may be competitive binding that serve to localize CNTNAP2 to specific regions in a context-dependent manner. Based on their positioning within interneuron dendrites, the PAR3/CNTNAP2 structures identified here appeared to be intracellular rather than plasma membrane-localized, in agreement with our observations in COS7 cells. Thus, CNTNAP2 may be spatially regulated or compartmentalized depending on its target interactor. Such preference may be influenced by neuronal activity, as neurexin phosphorylation can induce conformational change and alter partner binding affinities (LaConte *et al.*, 2016).

Mechanistically, we found the third PDZ domain of PAR3 to be required for CNTNAP2 binding. However, it also appears individual removal of either the first two PAR3 PDZ domains reduces the interaction strength. While multiple interactions may be the result of artificial steric disruption, it is possible that such architecture exists to hold multiple CNTNAP2 molecules together within a tight spatial confine (Renschler *et al.*, 2018). This could explain the abnormally large size of CNTNAP2⁺/PAR3⁺ vesicles upon overexpression and suggest the possibility of a network assembly (Hayashi *et al.*, 2009). This theory is further bolstered by our evidence of occasional endogenous CNTNAP2 clustering by overexpressed PAR3 (Figure 6). This configuration may also be necessary to offset the naturally weak CNTNAP2-PAR3 interaction, as seen by our fractionation results (Figure 2). It is possible that the multiple PDZ domains of PAR3 can coordinate the binding of multiple partners, which may further tune CNTNAP2 function and localization. It is also unlikely that our tagged proteins are forming aggregates, as the variants lacking PDZ domains fail to form these structures. PAR3 has been shown to self-assemble through its N-terminal CR1 domain (Mizuno *et al.*, 2003); however, this is unlikely to play a role in the appearance of large structures since the PAR3 PDZ_{all} mutant retains the relevant N-terminal domain.

Yet, how exactly does PAR3 mechanistically regulate CNTNAP2? The answer is difficult to determine since CNTNAP2 has multiple regions critical to trafficking. It has been established that threonine 1292 on CNTNAP2's intracellular domain regulates endocytosis through protein kinase C (Bel *et al.*, 2009) and that proper surface targeting of close family member neurexin-1 requires its PDZ-binding motif (Fairless *et al.*, 2008). Furthermore, specific sites on CNTNAP2's extracellular region are also critical for proper secretory and axonal trafficking (Falivelli *et al.*, 2012; Pinatel *et al.*, 2017). Our immunocytochemistry experiments link PAR3-CNTNAP2 complexes with clathrin-coated endosomes, suggesting that PAR3 may, via PDZ binding, be another component critical for CNTNAP2 internalization from the surface membrane. Furthermore, PAR3 has been shown to interact with the kinesin family member KIF3A and link other molecular partners to this motor (Nishimura *et al.*, 2004). It is possible that CNTNAP2 can also be trafficked through a PAR3-KIF3A interaction. These questions will be addressed in future studies.

Both CNTNAP2 and PAR3 play important roles in several steps of cortex development, including cell proliferation, migration, myelination, cell adhesion and synapse development (Bultje *et al.*, 2009; Famulski *et al.*, 2010; Penagarikano *et al.*, 2011; Tep *et al.*, 2012; Varea

et al., 2015; Scott *et al.*, 2017; Liu *et al.*, 2018). While defects in all of these processes have been implicated in neurodevelopmental disorders, further work is needed to determine the specific processes where their interaction plays a key role. Future work will focus on the molecular pathways dependent on this newly identified CNTNAP2-PAR3 interaction, and to elucidate the specific processes in which this partnership operates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants NS100785 and MH097216 from the NIH-NIMH to P.P and F30MH096457 to R.G.

Abbreviations

aPKC	Atypical protein kinase C
APPL1	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1
ASD	Autism Spectrum Disorder
BFP	Blue fluorescent protein
BSA	Bovine serum albumin
CASK	Calcium/calmodulin-dependent serine protein kinase
CAV1	Caveolin-1
CDFE	Cortical Dysplasia Focal Epilepsy
CHC	Clathrin heavy chain
CNTNAP2	Contactin-associated protein-like 2
EEA1	Early endosome antigen 1
FRAP	Fluorescent recovery after photobleaching
GABA	γ -aminobutyric acid
GluA1	Glutamate receptor 1
GOPC	Golgi associated PDZ And coiled-coil motif containing
GPCR	G protein-coupled receptor
ICD	Intracellular domain
ID	Intellectual Disability

JAM	Junctional adhesion molecule
KIF3A	Kinesin-like protein KIF3A
NGS	Normal goat serum
PAR3	Partitioning-defective protein 3
PAR6	Partitioning-defective protein 6
PSD95	Postsynaptic density protein 95
PTEN	Phosphatase and tensin homolog
RAB5	Ras-related protein Rab-5A
ROI	Region of interest
RT	Room temperature
SCZ	Schizophrenia
STX6	Syntaxin-6
TIAM1	T-lymphoma invasion and metastasis-inducing protein 1
Y2H	Yeast two-hybrid

References

- Ahmed SM & Macara IG (2017) The Par3 polarity protein is an exocyst receptor essential for mammary cell survival. *Nat. Commun*, 8, 14867. [PubMed: 28358000]
- Bel C, Oguievetskaia K, Pitaval C, Goutebroze L, & Faivre-Sarrailh C (2009) Axonal targeting of Caspr2 in hippocampal neurons via selective somatodendritic endocytosis. *J Cell Sci*, 122, 3403–3413. [PubMed: 19706678]
- Bridi MS, Park SM, & Huang S (2017) Developmental Disruption of GABAAR-Mediated Inhibition in Cntnap2 KO Mice. *eNeuro*, 4, ENEURO.0162–17.2017.
- Bultje RS, Castaneda-Castellanos DR, Jan LY, Jan YN, Kriegstein AR, & Shi SH (2009) Mammalian Par3 regulates progenitor cell asymmetric division via notch signaling in the developing neocortex. *Neuron*, 63, 189–202. [PubMed: 19640478]
- Chen N, Koopmans F, Gordon A, Paliukhovich I, Klaassen RV, van der Schors RC, Peles E, Verhage M, Smit AB, & Li KW (2015) Interaction proteomics of canonical Caspr2 (CNTNAP2) reveals the presence of two Caspr2 isoforms with overlapping interactomes. *Biochim. Biophys. Acta BBA - Proteins Proteomics, Neuroproteomics: Applications in Neuroscience and Neurology*, 1854, 827–833.
- Chen X & Macara IG (2005) Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. *Nat. Cell Biol*, 7, 262–269. [PubMed: 15723052]
- Duman JG, Tzeng CP, Tu YK, Munjal T, Schwechter B, Ho TS, & Tolias KF (2013) The adhesion-GPCR BAI1 regulates synaptogenesis by controlling the recruitment of the Par3/Tiam1 polarity complex to synaptic sites. *J Neurosci*, 33, 6964–6978. [PubMed: 23595754]
- Fairless R, Masius H, Rohlmann A, Heupel K, Ahmad M, Reissner C, Dresbach T, & Missler M (2008) Polarized targeting of neuroligins to synapses is regulated by their C-terminal sequences. *J Neurosci*, 28, 12969–12981. [PubMed: 19036990]

- Falivelli G, De Jaco A, Favaloro FL, Kim H, Wilson J, Dubi N, Ellisman MH, Abrahams BS, Taylor P, & Comoletti D (2012) Inherited genetic variants in autism-related CNTNAP2 show perturbed trafficking and ATF6 activation. *Hum Mol Genet*, 21, 4761–4773. [PubMed: 22872700]
- Famulski JK, Trivedi N, Howell D, Yang Y, Tong Y, Gilbertson R, & Solecki DJ (2010) Siah regulation of Pard3A controls neuronal cell adhesion during germinal zone exit. *Science*, 330, 1834–1838. [PubMed: 21109632]
- Feng W, Wu H, Chan L-N, & Zhang M (2008) Par-3-mediated junctional localization of the lipid phosphatase PTEN is required for cell polarity establishment. *J. Biol. Chem*, 283, 23440–23449. [PubMed: 18550519]
- Gao R & Penzes P (2015) Common mechanisms of excitatory and inhibitory imbalance in schizophrenia and autism spectrum disorders. *Curr Mol Med*, 15, 146–167. [PubMed: 25732149]
- Gao R, Piguel NH, Melendez-Zaidi AE, Martin-de-Saavedra MD, Yoon S, Forrest MP, Myczek K, Zhang G, Russell TA, Csernansky JG, Surmeier DJ, & Penzes P (2018) CNTNAP2 stabilizes interneuron dendritic arbors through CASK. *Mol Psychiatry*,.
- Gdalyahu A, Lazaro M, Penagarikano O, Golshani P, Trachtenberg JT, & Geschwind DH (2015) The Autism Related Protein Contactin-Associated Protein-Like 2 (CNTNAP2) Stabilizes New Spines: An In Vivo Mouse Study. *PLoS One*, 10, e0125633. [PubMed: 25951243]
- Goldstein B & Macara IG (2007) The PAR proteins: fundamental players in animal cell polarization. *Dev Cell*, 13, 609–622. [PubMed: 17981131]
- Hayashi MK, Tang C, Verpelli C, Narayanan R, Stearns MH, Xu RM, Li H, Sala C, & Hayashi Y (2009) The postsynaptic density proteins Homer and Shank form a polymeric network structure. *Cell*, 137, 159–171. [PubMed: 19345194]
- Hoffman EJ, Turner KJ, Fernandez JM, Cifuentes D, Ghosh M, Ijaz S, Jain RA, Kubo F, Bill BR, Baier H, Granato M, Barresi MJ, Wilson SW, Rihel J, State MW, & Giraldez AJ (2016) Estrogens Suppress a Behavioral Phenotype in Zebrafish Mutants of the Autism Risk Gene, CNTNAP2. *Neuron*, 89, 725–733. [PubMed: 26833134]
- Itoh M, Sasaki H, Furuse M, Ozaki H, Kita T, & Tsukita S (2001) Junctional adhesion molecule (JAM) binds to PAR-3: a possible mechanism for the recruitment of PAR-3 to tight junctions. *J. Cell Biol*, 154, 491–497. [PubMed: 11489913]
- Johansson A, Driessens M, & Aspenstrom P (2000) The mammalian homologue of the *Caenorhabditis elegans* polarity protein PAR-6 is a binding partner for the Rho GTPases Cdc42 and Rac1. *J Cell Sci*, 113 (Pt 18), 3267–3275. [PubMed: 10954424]
- Jurgensen S & Castillo PE (2015) Selective Dysregulation of Hippocampal Inhibition in the Mouse Lacking Autism Candidate Gene CNTNAP2. *J Neurosci*, 35, 14681–14687. [PubMed: 26511255]
- Karayannis T, Au E, Patel JC, Kruglikov I, Markx S, Delorme R, Heron D, Salomon D, Glessner J, Restituito S, Gordon A, Rodriguez-Murillo L, Roy NC, Gogos JA, Rudy B, Rice ME, Karayiorgou M, Hakonarson H, Keren B, Huguet G, Bourgeron T, Hoeffler C, Tsien RW, Peles E, & Fishell G (2014) Cntnap4 differentially contributes to GABAergic and dopaminergic synaptic transmission. *Nature*, 511, 236–240. [PubMed: 24870235]
- Kim J-W, Park K, Kang RJ, Gonzales ELT, Kim DG, Oh HA, Seung H, Ko MJ, Kwon KJ, Kim KC, Lee SH, Chung C, & Shin CY (2019) Pharmacological modulation of AMPA receptor rescues social impairments in animal models of autism. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol*, 44, 314–323.
- LaConte LEW, Chavan V, Liang C, Willis J, Schönhense E-M, Schoch S, & Mukherjee K (2016) CASK stabilizes neurexin and links it to liprin- α in a neuronal activity-dependent manner. *Cell. Mol. Life Sci. CMLS*, 73, 3599–3621. [PubMed: 27015872]
- Lazaro MT, Taxidis J, Shuman T, Bachmutsky I, Ikrar T, Santos R, Marcello GM, Mylavarapu A, Chandra S, Foreman A, Goli R, Tran D, Sharma N, Azhdam M, Dong H, Choe KY, Peñagarikano O, Masmanidis SC, Rácz B, Xu X, Geschwind DH, & Golshani P (2019) Reduced Prefrontal Synaptic Connectivity and Disturbed Oscillatory Population Dynamics in the CNTNAP2 Model of Autism. *Cell Rep*, 27, 2567–2578.e6. [PubMed: 31141683]
- Lin D, Edwards AS, Fawcett JP, Mbamalu G, Scott JD, & Pawson T (2000) A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat Cell Biol*, 2, 540–547. [PubMed: 10934475]

- Lin D, Gish GD, Songyang Z, & Pawson T (1999) The carboxyl terminus of B class ephrins constitutes a PDZ domain binding motif. *J. Biol. Chem*, 274, 3726–3733. [PubMed: 9920925]
- Liu WA, Chen S, Li Z, Lee CH, Mirzaa G, Dobyns WB, Ross ME, Zhang J, & Shi SH (2018) PARD3 dysfunction in conjunction with dynamic HIPPO signaling drives cortical enlargement with massive heterotopia. *Genes Dev*, 32, 763–780. [PubMed: 29899142]
- Macara IG (2004) Parsing the polarity code. *Nat Rev Mol Cell Biol*, 5, 220–231. [PubMed: 14991002]
- Marin O (2012) Interneuron dysfunction in psychiatric disorders. *Nat Rev Neurosci*, 13, 107–120. [PubMed: 22251963]
- Markram H, Toledo-Rodriguez M, Wang Y, Gupta A, Silberberg G, & Wu C (2004) Interneurons of the neocortical inhibitory system. *Nat. Rev. Neurosci*, 5, 793–807. [PubMed: 15378039]
- McCaffrey LM, Montalbano J, Mihai C, & Macara IG (2012) Loss of the Par3 polarity protein promotes breast tumorigenesis and metastasis. *Cancer Cell*, 22, 601–614. [PubMed: 23153534]
- Mertens AE, Pegtel DM, & Collard JG (2006) Tiam1 takes PARt in cell polarity. *Trends Cell Biol*, 16, 308–316. [PubMed: 16650994]
- Mizuno K, Suzuki A, Hirose T, Kitamura K, Kutsuzawa K, Futaki M, Amano Y, & Ohno S (2003) Self-association of PAR-3-mediated by the conserved N-terminal domain contributes to the development of epithelial tight junctions. *J. Biol. Chem*, 278, 31240–31250. [PubMed: 12756256]
- Mo A, Mukamel EA, Davis FP, Luo C, Henry GL, Picard S, Urich MA, Nery JR, Sejnowski TJ, Lister R, Eddy SR, Ecker JR, & Nathans J (2015) Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. *Neuron*, 86, 1369–1384. [PubMed: 26087164]
- Nakagawa T, Cheng Y, Ramm E, Sheng M, & Walz T (2005) Structure and different conformational states of native AMPA receptor complexes. *Nature*, 433, 545–549. [PubMed: 15690046]
- Narayanan AS, Reyes SB, Um K, McCarty JH, & Tolias KF (2013) The Rac-GAP Bcr is a novel regulator of the Par complex that controls cell polarity. *Mol Biol Cell*, 24, 3857–3868. [PubMed: 24152735]
- Nishimura T, Kato K, Yamaguchi T, Fukata Y, Ohno S, & Kaibuchi K (2004) Role of the PAR-3-KIF3 complex in the establishment of neuronal polarity. *Nat. Cell Biol*, 6, 328–334. [PubMed: 15048131]
- Oiso S, Takeda Y, Futagawa T, Miura T, Kuchiiwa S, Nishida K, Ikeda R, Kariyazono H, Watanabe K, & Yamada K (2009) Contactin-associated protein (Caspr) 2 interacts with carboxypeptidase E in the CNS. *J. Neurochem*, 109, 158–167. [PubMed: 19166515]
- Penagarikano O, Abrahams BS, Herman EI, Winden KD, Gdalyahu A, Dong H, Sonnenblick LI, Gruver R, Almajano J, Bragin A, Golshani P, Trachtenberg JT, Peles E, & Geschwind DH (2011) Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. *Cell*, 147, 235–246. [PubMed: 21962519]
- Pinatel D, Hivert B, Saint-Martin M, Noraz N, Savvaki M, Karagogeos D, & Faivre-Sarrailh C (2017) The Kv1-associated molecules TAG-1 and Caspr2 are selectively targeted to the axon initial segment in hippocampal neurons. *J Cell Sci*, 130, 2209–2220. [PubMed: 28533267]
- Poliak S, Salomon D, Elhanany H, Sabanay H, Kiernan B, Pevny L, Stewart CL, Xu X, Chiu SY, Shrager P, Furley AJ, & Peles E (2003) Juxtaparanodal clustering of Shaker-like K⁺ channels in myelinated axons depends on Caspr2 and TAG-1. *J Cell Biol*, 162, 1149–1160. [PubMed: 12963709]
- Renschler FA, Brueckner SR, Salomon PL, Mukherjee A, Kullmann L, Schutz-Stoffregen MC, Henzler C, Pawson T, Krahn MP, & Wiesner S (2018) Structural basis for the interaction between the cell polarity proteins Par3 and Par6. *Sci Signal*, 11.
- Rodenas-Cuadrado P, Ho J, & Vernes SC (2014) Shining a light on CNTNAP2: complex functions to complex disorders. *Eur J Hum Genet*, 22, 171–178. [PubMed: 23714751]
- Scott R, Sanchez-Aguilera A, van Elst K, Lim L, Dehorter N, Bae SE, Bartolini G, Peles E, Kas MJH, Bruining H, & Marin O (2017) Loss of Cntnap2 Causes Axonal Excitability Deficits, Developmental Delay in Cortical Myelination, and Abnormal Stereotyped Motor Behavior. *Cereb Cortex*, 29, 586–597.
- Tep C, Kim ML, Opincariu LI, Limpert AS, Chan JR, Appel B, Carter BD, & Yoon SO (2012) Brain-derived neurotrophic factor (BDNF) induces polarized signaling of small GTPase (Rac1) protein at

the onset of Schwann cell myelination through partitioning-defective 3 (Par3) protein. *J Biol Chem*, 287, 1600–1608. [PubMed: 22128191]

Thomas AM, Schwartz MD, Saxe MD, & Kilduff TS (2017) Cntnap2 Knockout Rats and Mice Exhibit Epileptiform Activity and Abnormal Sleep-Wake Physiology. *Sleep*, 40.

van der Walt S, Schönberger JL, Nunez-Iglesias J, Boulogne F, Warner JD, Yager N, Gouillart E, Yu T, & scikit-image contributors (2014) scikit-image: image processing in Python. *PeerJ*, 2, e453. [PubMed: 25024921]

Varea O, Martin-de-Saavedra MD, Kopeikina KJ, Schürmann B, Fleming HJ, Fawcett-Patel JM, Bach A, Jang S, Peles E, Kim E, & Penzes P (2015) Synaptic abnormalities and cytoplasmic glutamate receptor aggregates in contactin associated protein-like 2/Caspr2 knockout neurons. *Proc. Natl. Acad. Sci. U. S. A.*, 112, 6176–6181. [PubMed: 25918374]

Vogt D, Cho KKA, Shelton SM, Paul A, Huang ZJ, Sohal VS, & Rubenstein JLR (2017) Mouse Cntnap2 and Human CNTNAP2 ASD Alleles Cell Autonomously Regulate PV+ Cortical Interneurons. *Cereb Cortex*, 1–12. [PubMed: 28365777]

Zack GW, Rogers WE, & Latt SA (1977) Automatic measurement of sister chromatid exchange frequency. *J Histochem Cytochem*, 25, 741–753. [PubMed: 70454]

Zhang H & Macara IG (2006) The polarity protein PAR-3 and TIAM1 cooperate in dendritic spine morphogenesis. *Nat Cell Biol*, 8, 227–237. [PubMed: 16474385]

Figure 1:

PAR3 binds with CNTNAP2 through PDZ interaction. (a) Cartoon showing the domain topology of CNTNAP2 and PAR3, with black lines representing the bait/prey regions derived from the yeast-two hybrid screen and red lines indicating the truncated mutants used for experimentation. (b) Representative western blot showing PAR3 co-immunoprecipitation with CNTNAP2 from mouse cortex homogenates (representative image from 2 independent experiments). (c) Western blot showing co-immunoprecipitation of PAR3 co-expressed with various CNTNAP2 truncations to demonstrate this interaction is specific to CNTNAP2's PDZ-binding domain (representative image from 3 independent experiments) (d) Western blot showing coimmunoprecipitation of CNTNAP2 with various PAR3 truncations, demonstrating the third PDZ domain is critical the interaction (representative image from 3 independent experiments).

Figure 2:

CNTNAP2 and PAR3 are localized to membranes in mouse brain. (a) Schematic showing the subcellular fractionation protocol. (b) Immunoblots of subcellular fractionations from adult mouse forebrain probed with CNTNAP2 and PAR3. Purple and red boxes highlight fractions of interest. GluA1, PSD95, and β -tubulin served as compartment-specific markers.

Figure 3:

Co-expressed CNTNAP2 and PAR3 associate with large vesicles in COS7 cells. (a) Representative confocal images of COS7 cells expressing mCherry-CNTNAP2/Myc-PAR3 alone, together, or with various truncation mutants shown to affect protein-protein interactions. Arrows point to the abnormally large mCherry-CNTNAP2 + Myc-PAR3 aggregates induced by co-expression of the two (scale bar = 25 μm) (representative image from 2 independent experiments). (b) Selective representative images showing co-localization (white) between mCherry-CNTNAP2 + GFP-PAR3 (WT) or mCherry-CNTNAP2 + GFP-PAR3 Δ PDZ_{all} (scale bar = 10 μm). (c) Quantification of Mander's colocalization (WT: 9 cells, Δ PDZ_{all}: 10 cells from 1 independent experiment) by unpaired two-tailed t-test. (d) Measurement of the size of mCherry-CNTNAP2 vesicles alone or when GFP-PAR3 is co-expressed (n = 9 cells for both conditions from 1 independent experiment) by unpaired two-tailed t-test. Values are means $\pm$ SEM. *** P < 0.001.

Figure 4: CNTNAP2 and PAR3 co-localize in clathrin-dependent endocytic vesicles. (a) Representative images of COS7 cells transfected with mCherry-CNTNAP2 (red channel) and Myc-PAR3 (green channel) and stained with the indicated markers (far red channel). Colocalized puncta are shown with yellow arrowheads and white boxes (Scale bars = 15 μ m). Insets at bottom are magnified images of the white boxes with line scans shown in yellow. Bottom shows a magnified view of the merged images. (b) Profile plots from line-scans in (a) showing overlap of CNTNAP2, PAR3, and markers. Profiles are normalized to maximum fluorescence. (c) Box plots with individual data points showing the fraction of thresholded CNTNAP2⁺/PAR3⁺ puncta with inclusion of marker (see methods and Supplementary

Figure 2). Color-matched text below indicates relevant intracellular compartments. Each marker was compared to every other marker using Kruskal-Wallis test with Dunn's post hoc test (See table 1).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5:

PAR3 stabilizes CNTNAP2 within intracellular vesicles. (a) FRAP and time-lapse imaging (5 minutes) of live COS7 cells co-expressing mCherry-CNTNAP2 + GFP-PAR3 (WT) or mCherry-CNTNAP2 + GFP-PAR3 PDZ_{all} (scale bar = 5 μm). Green channel represents expression patterns of GFP-PAR3 or GFP-PAR3 PDZ_{all}. Pseudo-color channel represents mCherry-CNTNAP2 before (−1'), during (0), or after (5') FRAP bleaching in either condition. (b) Quantification of post-FRAP recovery in either condition by two-way ANOVA (WT: n = 8 cells, PDZ_{all}: n = 10 cells from 1 independent experiment). Values are means ± SEM. *** P < 0.001. WT τ =426.2s, PDZ_{all} τ =361.3s

Figure 6:

Overexpressed PAR3 clusters endogenous CNTNAP2 in primary inhibitory neurons. (a) Representative images (from at least 3 independent experiments) of cultured primary inhibitory neurons transfected with blue fluorescent protein (BFP), alone or together with Myc-PAR3 or Myc-PAR3 Δ PDZ_{all} (scale bar = 5 μ m). (b) Maximum projections of select BFP and Myc-PAR3 expressing neurons immunostained for Myc and endogenous

CNTNAP2. BFP was used as a 3D mask to exclude signal from outside of the cell. Arrowheads show colocalizing regions in dendrites. Scale bars 40 μm .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1:

Results of Dunn's multiple comparison post hoc test of figure 4 showing summarized results. Outliers visible in Figure 4c were not excluded from analysis.

Comparison Groups	Mean rank diff.	Adj. P Value	Summary
CAV1 vs. CHC	-61.42	0.0001	***
CAV1 vs. RAB5	-64.26	<0.0001	****
CAV1 vs. EEA1	-85.64	<0.0001	****
CAV1 vs. GOPC	-23.22	>0.9999	ns
CAV1 vs. APPL1	5.855	>0.9999	ns
CAV1 vs. STX6	-16.79	>0.9999	ns
CHC vs. RAB5	-2.839	>0.9999	ns
CHC vs. EEA1	-24.23	>0.9999	ns
CHC vs. GOPC	38.2	0.1428	ns
CHC vs. APPL1	67.27	<0.0001	****
CHC vs. STX6	44.63	0.0329	*
RAB5 vs. EEA1	-21.39	>0.9999	ns
RAB5 vs. GOPC	41.04	0.0554	ns
RAB5 vs. APPL1	70.11	<0.0001	****
RAB5 vs. STX6	47.46	0.0106	*
EEA1 vs. GOPC	62.43	0.0003	***
EEA1 vs. APPL1	91.5	<0.0001	****
EEA1 vs. STX6	68.85	<0.0001	****
GOPC vs. APPL1	29.07	0.869	ns
GOPC vs. STX6	6.425	>0.9999	ns
APPL1 vs. STX6	-22.65	>0.9999	ns