Mouse Cntnap2 and Human CNTNAP2 ASD Alleles Cell Autonomously Regulate PV+ Cortical Interneurons

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Abstract

Human mutations in CNTNAP2 are associated with an array of neuropsychiatric and neurological syndromes, including speech and language disorders, epilepsy, and autism spectrum disorder (ASD). We examined Cntnap2’s expression and function in GABAergic cortical interneurons (CINs), where its RNA is present at highest levels in chandelier neurons, PV+ neurons and VIP+ neurons. In vivo functions were studied using both constitutive Cntnap2 null mice and a transplantation assay, the latter to assess cell autonomous phenotypes of medial ganglionic eminence (MGE)-derived CINs. We found that Cntnap2 constitutive null mutants had normal numbers of MGE-derived CINs, but had reduced PV+ CINs. Transplantation assays showed that Cntnap2 cell autonomously regulated the physiology of parvalbumin (PV)+, fast-spiking CINs; no phenotypes were observed in somatostatin+, regular spiking, CINs. We also tested the effects of 4 human CNTNAP2 ASD missense mutations in vivo, and found that they impaired PV+ CIN development. Together, these data reveal that reduced CNTNAP2 function impairs PV+ CINs, a cell type with important roles in regulating cortical circuits.

Key words: CNTNAP2, Cortical interneuron, fast-spiking, MGE, parvalbumin

Introduction

Human CNTNAP2 encodes the CASPR2 protein, a member of the neurexin family of cell adhesion molecules. In recent years, the scope of human disorders associated with CNTNAP2 has grown (for reviews see: Rodenas-Cuadrado et al. 2014; Poot 2015, 2017). Notably, CNTNAP2 dysfunction in humans has been implicated in speech and language disorders, including Gilles de la Tourette syndrome, stuttering, and selective mutism (Verkerk et al. 2003; Petrin et al. 2010; Stein et al. 2011; Zhao et al. 2015). Notably, there is evidence that CNTNAP2 is directly repressed by FoxP2, a gene implicated in speech and language disorders. In addition, CNTNAP2 mutations have been discovered in individuals with cortical dysplasia-focal epilepsy syndrome (Strauss et al. 2006), Pitt-Hopkins like syndrome (Zweier et al. 2015), Schizophrenia (Friedman et al. 2008; Ji et al. 2013), obsessive compulsive disorder (Verkerk et al. 2003), attention deficit hyperactivity disorder (Elia et al. 2010) as well as autism spectrum disorder (ASD) (Alarcon et al. 2008; Arking et al. 2008; Bakkaloglu et al. 2008; Poot et al. 2010).

In mice and zebraﬁsh, Cntnap2 has been implicated in many biological processes, including clustering potassium channels at the juxtaparanodes of myelinated axons, trafﬁcking Glutamatergic AMPA receptors, positively regulating synaptic strength and the number of GABAergic neurons (Horosh et al. 2008; Anderson et al. 2012; Varea et al. 2015; Hoffman et al. 2016).

Cntnap2 is broadly expressed in the developing and mature central nervous system (Gordon et al. 2016). Due to the number of disorders implicated in CNTNAP2 dysfunction, one idea is that CASPR2 may serve as a hub at the cell membrane that
integrates multiple brain regions and coordinates several developmental and maturational processes through its association with both extracellular- and intracellular-binding partners (Rodenas-Cuadrado et al. 2014; Poot 2015, 2017).

Of note, recent reports have implicated CNTNAP2 in inhibitory (GABAergic) neuron development/maturaition. While cortical excitation is primarily mediated by glutamatergic projection neurons and thalamic afferents, cortical inhibition is primarily mediated by locally projecting GABAergic cortical interneurons (CINs) and basal telencephalic GABAergic projection neurons. CINs have diverse morphologies, molecular makeup and electrophysiological properties; they mediate inhibition in distinct ways (Wonders and Anderson 2006; Kepecs and Fishell 2014; Kessaris et al. 2014). For instance, parvalbumin (PV)+ basket CINs are fast spiking, and innervate the cell body of pyramidal neurons, whereas chandelier neurons (many of which are PV+) innervate the initial axon segment (Huang et al. 2007). Mouse mutants lacking Cntnap2 have been reported to have decreased CINs, including the parvalbumin (PV)+, neuropeptide-Y (NPY)+, and calretinin (CR) subgroups (Peñagarikano et al. 2011). Moreover, the number of GABAergic neurons is decreased in zebrafish with Cntnap2 deletion (Hoffman et al. 2016). Interestingly, loss of function of another neurexin family member, Cntnap4, led to decreased GABAergic activity and increased dopaminergic activity, and resulted in PV− CINs that had diminished firing properties (Karayannis et al. 2014). These data suggest that CNTNAP family members may control aspects of CIN development/maturaition. However, little is known about CNTNAP2’s function within CINs, in part because of its broad expression, making it impossible to ascertain its cell autonomous functions in the Cntnap2 constitutive null mouse.

Deficits in CINs have been identified in both humans diagnosed with ASD patients and in genetic animal models of ASD (Peñagarikano et al. 2011; Han et al. 2012; Karayannis et al. 2014; Vogt, Cho et al. 2015; Hoffman et al. 2016; Hashemi et al. 2017). Thus, it is important to rigorously establish whether and how human neurological disease risk genes regulate CIN development and function. Moreover, many types of mutations (e.g., nonsense, missense, and synonymous) have been discovered in humans with neuropsychiatric disorders but their potentially diverse impacts on protein function is not well understood. Of note, missense mutations have been the most difficult to evaluate as they are not obviously deleterious. Notably, in some individuals both alleles of CNTNAP2 are mutated and are either known to, or predicted to, lead to no functional protein (Strauss et al. 2006; Zweier 2012; Watson et al. 2014; Rodenas-Cuadrado et al. 2016). While these and other mutations discovered in CNTNAP2 have been either studied in detail or predicted to be deleterious, there are many missense mutations for which little is known. In addition, there is little understanding as to whether these mutations result in functional changes in CNTNAP2. Moreover, the role that CNTNAP2 dysfunction may play in ASD symptoms is still under investigation.

Herein, CNTNAP2 function was assessed in mouse CIN development and maturation using a constitutive Cntnap2 null mouse. In conjunction, we used a transplantation method to assess cell autonomous roles for mouse Cntnap2 in CINs. While no gross alterations were observed in the expression of several potassium channels (KV1.1, KV3.1, Kv4.2, and Kv4.3) in the null mice, there were changes in the expression of specific CIN markers and the fast-spiking CINs had altered physiology. Notably, Cntnap2 mutant mice had reduced numbers of MGE-derived (PV+) and CGE-derived (REELIN+/SST+) CINs. The transplantation assay showed that Cntnap2 is necessary to autonomously establish the number and electrophysiological properties of PV+ CINs. Finally, we assessed the impact of human ASD CNTNAP2 mutations in CIN development using a recently developed transduction/transplantation assay. We found that these human alleles were either hypomorphic or loss of function in autonomously promoting PV+ CIN development.

**Materials and Methods**

**Animals**

Cntnap2 knockouts (Poliaik et al. 2003), Ai14 Cre-reporters (Madisen et al. 2010), and Nkx2.1-Cre (Xu et al. 2008) mouse strains have been published. Mice were initially on a mixed C57Bl/6J, CD-1 background. All lines were backcrossed to C57BL6/J, CD-1 background. For single cell RNA analysis, we used the following combination of mice: chandelier cells (CH) and PV basket cells in the cortex were labelled using the Nkx2.1-CreER (Taniguchi et al. 2013) (Tamoxifen induced at E17.5) and PV-IRES-Cre (Hippchenmeyer et al. 2005) lines, which were crossed with the Ai14 reporter mouse. Intersectional labeling was achieved by breeding each of the following separately to the Ai65 intersectional reporter (Madisen et al. 2015) to label (1) Sst-Flp; Nosi1-CreER for Long projecting cells (He et al. 2016), (2) Sst-Flp; CR-Cre (He et al. 2016) for Martinotti cells, (3) VIP-Flp (He et al. 2016), CR-Cre for Interneuron selective cells and (4) VIP-Flp; Ckk-cre (He et al. 2016) for Cck-basket cells. For these latter mice, the Cold Spring Harbor Laboratory animal husbandry protocol was followed (IACUC 16-13-09-8).

**Cell Counting and Statistical Analysis**

For cell counts from the somatosensory cortex in constitutive mouse mutants, Image-J was used to calculate the number of CINs and then this number was divided by the area of the cortex to determine cell density. Two sections were counted from each mouse and averaged to generate each n. Cell density and western blots were statistically analyzed using one-way ANOVA, followed by the Tukey’s post test to determine significance. For post-hoc analysis, normality, homogeneity and independence were met. For transplanted cell counts, Image-J was also used, and those cells that were co-labeled for tdTomato and the CIN marker were counted. Since the transplanted cell data are normalized to the number of total cells transplanted, we used a nonparametric test, the Chi-squared analysis, to determine significance. For these counts, cells were assessed in multiple parts of the neocortex, due to the low number, and data were only included if at least 50 cells were counted per sample. Statistics were analyzed using Prism 6.

**DNA Vector Generation**

To generate the Dbl12b-BG-MCS-IRE-S-Cre vector, the T2a site was excised from a previously reported vector (Vogt, Cho et al. 2015) and replaced with an IRES sequence. The IRES sequence was amplified from the pIRE5-EGFP vector (Clontech) with primers (5′ GAGATGTACAACCGGGATCCGCCCCTCT, 3′ GAGAGAA TTCTGTGGCCATATTATCATCG) that introduced BsrGI and
EcoRI sites, and then inserted into these sites in the previous vector. Next, human CNTNAP2 cDNA (ABM) was used as a template to PCR amplify the gene with introduced 5′ XbaI and 3′ BsrGI sites, using the primers: (5′-GAGATCTAGATGCA GGCAGCTCGCCG; 3′-GAGATGTACATCAGGACATCTCCT). The human CNTNAP2 PCR fragment was cloned into these sites within the MCS of the vector. To introduce human ASD allele mutations, extension-overlap PCR was used to introduce each mutation using the following primers with introduced mutations (underlined) in combination with flanking primers (those used to amplify full length CNTNAP2, above): N407>G (5′-mutant GGAACCCGACTGCCTCTGTCCACTAATTTT; 3′-mutant CAGGAGACCACTGGGTTTGCTTCCAGTAAAAGC); N418>D (5′-mutant CTTTCGGGATATGCGCATTGCAGGTATG; 3′-mutant CATTGACCTATCTCCGCAAAGTGACAGGAGACCA). The human CNTNAP2 PCR fragment was cloned into these sites expressing procedure as previously described (Vogt et al. 2012).

**HEK293T Cell Cultures**

HEK293T cells were maintained in DMEM supplemented with 10% fetal bovine serum. For analysis of the human CNTNAP2 expression vectors, the vectors were transfected into HEK293T cells using Lipofectamine2000 (ThermoFisher). After 48 h, the cells were fixed with 4% paraformaldehyde (PFA) and immunolabeled with a rabbit anti-CASPR2 (Millipore) antibody. The appropriate Alexa-conjugated secondary was used to detect CASPR2+ cells and DAPI was used to detect cell nuclei. Lentiviral production using HEK293T cells were performed as previously described (Vogt et al. 2015).

**Immunofluorescence tissue staining**

Either P30 Cntnap2 constitutive KO mice and controls, or P35 MGE-transplanted mice, were transcardially perfused, first with phosphate-buffered saline, followed by 4% PFA. After perfusion, brains were removed and postfixed in PFA for 30 min, then sunk in 30% sucrose overnight before embedding in OCT and freezing. About 25 μm coronal brain sections were made using a cryostat. Immunofluorescence labeling was performed on these cryosections with the following primary antibodies: rabbit anti-parvalbumin (Swant), rat anti-somatostatin (Millipore), rabbit anti-VIP (Immunostar), mouse anti-Reelin (Millipore). The appropriate 488, 594, or 647 Alexa-conjugated secondary antibodies were from Life Technologies. Sections were covered slided with Vectashield containing DAPI (Vector labs).

**MGE Transplantation**

MGE transplantations were done as described (Vogt et al. 2015). Briefly, E13.5 MGE tissue from either Cntnap2−/− or Cntnap2+/− embryos were dissociated, then transduced with lentiviruses for 30 min. Next, the cells were washed to remove excess virus, pelleted and then transplanted into the cortices of WT P1 host mice. The cells developed for 35 days in vivo, and were then assessed via native tdTomato fluorescence.

**Lentiviral Production**

Lentiviral production was performed as previously described (Vogt et al. 2015). Briefly, HEK293T cells were transfected using Lipofectamine2000 (Invitrogen) with 4 plasmids to generate lentivirus particles as previously described, including the lentiviral expression vector, pVSV-g, pRSVr, pMDLg-pRRE, Media containing virus was collected and ultracentrifuged at 100 000 x g for 2.5 h at 4 °C. After the ultracentrifuge step, supernatant was removed and the pellet was resuspended in sterile PBS then stored at −80 °C until use.

**Single Cell RNA Analysis**

**Manual Cell Sorting**

Single cells were collected from P28–35 animals by manual sorting procedure as previously described (Paul et al. 2012). Brains were sectioned at 300 μm thickness, micro-dissected, and dissociated. Single RFP-positive cells were collected using patch pipette capillary and dispensed individually into separate
single tubes prefilled with RNaseOUT (Invitrogen), ERCC spike-in RNAs in 1:400 K dilution, sample specific RT primers. Process was repeated to collect 32–64 cells in one manual cell sorting session. Cells were flash frozen in liquid nitrogen and stored at −80 °C until processed. Patch pipette was single use only and fresh pipettes were used for every single cell collected.

**Linear RNA Amplification, Illumina Library Prep, and Sequencing**

RNA was linearly amplified by T7 RNA polymerase using 2 rounds of in vitro transcription (MessageAmp-II kit Life Technologies) according to the manufacturer’s recommended protocol with some modifications to make aRNA. Second round aRNAs were fragmented chemically using NEBNext Magnesium RNA Fragmentation Module (Cat#E6150S), column purified using RNA MinElute (Qiagen) for final Illumina cDNA library preparation steps using Illumina TruSeq small RNA kit (Cat#RS-200-0012) (Hashimshony et al. 2012). The resulting library was paired-end sequenced for 101 bp in Illumina HiSeq.

**Mapping and Tag Counting**

Bowtie was used for sequence alignment of read2 (polyA primed) to the mouse reference genome (mm9), and read1 sequences were used for UMI (variety-tag) counting. Using a custom python script (https://github.com/maggiecrow/scCoexp), multiple reads to the same gene with the same tag sequences were rejected and only counted as one, such that only mapped sequences with unique tags were retained and tallied for each mRNA for each cell.

**Western Blotting**

E13.5 MGE tissue was dissected and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate in 50 mM Tris) supplemented with protease (Halt protease inhibitor, Pierce) and phosphatase (PhosSTOP, Roche) inhibitors. ~20 μg of total protein was loaded into an SDS-PAGE gel, separated, and transferred to a nitrocellulose membrane. The membrane was probed with the following antibodies: rabbit

**Figure 1.** Cntnap2−/− mice exhibit an increased trend for pERK1/2 in embryonic MGE but no gross changes in MGE-derived CIN numbers or lamination. Western blots from E13.5 MGE tissue lysates showing CASPR2, pERK1/2Thr202/Tyr204, total ERK1/2, pAKTSer473, total AKT, and βIII-tubulin (loading control) (a). (b) quantification of the levels of pAKT or pERK divided by the total amount of AKT or ERK expressed as arbitrary units (AU). (c–e) Immunofluorescence images in coronal sections showing MGE-derived CINs, tdTomato+ and co-labeled with DAPI, in the somatosensory cortex at P30. (f) Quantification of tdTomato+ cell density in the somatosensory cortex at P30. (g) Quantification of the proportion of tdTomato+ cells per lamina at P30. Data are expressed as mean ± SEM. n = 3, all groups. Scale bar in (e) = 100 μm.
anti-CASPR2 (Millipore), rabbit anti-pAKTSer473 (Cell Signaling), rabbit anti-total AKT (Cell Signaling), pERK1/2Threonine 202/Tyrone 204 (Cell Signaling), total ERK1/2 (Cell Signaling), mouse anti-βIII-tubulin (Covance), and the species-appropriate HRP-conjugated secondary antibodies (Bio-rad). HEK293T cells were transfected with human CNTNAP2 expression vectors using Lipofectamine2000 (ThermoFisher), and cell lysates were collected after 48 h in the same manner described above. The rabbit anti-CASPR2 (Millipore), rabbit anti-GFP (ThermoFisher) and appropriate HRP-conjugated antibodies (BioRad) were used to detect proteins.

Results

Cnnap2 Constitutive Mutants have Normal Numbers of CINs in the Nkx2-1 Lineage, but Decreased PV and Reelin Expression

Towards obtaining a deeper understanding of the role of Cnnap2 in CIN development and function, we first studied the constitutive null mutant (Polia et al. 2003). We concentrated on CINs derived from the medial ganglionic eminence (MGE). Thus, we began by assaying CASPR2 protein expression in embryonic day (E) 13.5 MGE tissue from WT, Cnnap2+/+; and Cnnap2−/− using western blotting. We found that CASPR2 was expressed in the MGE at this age, and its expression was not detectable in Cnnap2−/− MGE tissue (Fig. 1a).

Little is known about whether the CASPR2 protein regulates intracellular signaling. Thus, we compared various signaling pathways from WT, Cnnap2+/+; and Cnnap2−/− in E13.5 MGE tissue. We focused on signaling pathways that are implicated in various CINs and their functional properties. Therefore, we assessed numbers of CINs and the different CIN subgroups in adult mouse brains at P30. Our primary goal was to understand if the number of CINs derived from the MGE and Preoptic Area (POA) were altered in Cnnap2−/− mice, since these populations comprise ~70% of all CINs. To this end, we crossed the Nkx2.1-Cre allele (Xu et al. 2008) into mice that had the Cnnap2 null allele. In addition, the Cre-dependent reporter, Ai14 (Madisen et al. 2010), was also utilized to follow tdTomato (red fluorescence) MGE and POA-derived cells. Notably, loss of Cnnap2 did not change the number of tdTomato+ CINs in the P30 somatosensory cortex (Fig. 1c-f), or their laminar distribution at this age (Fig. 1g). Finally, we examined the number of MGE and POA-derived cells in the striatum, and pallium but found no differences (data not shown). Overall, these results suggest that Cnnap2−/− mice generate and maintain normal numbers of MGE and POA-derived CINs by P30.

While Cnnap2 is expressed in MGE tissue (Fig. 1), it is unknown whether it is also expressed in postnatal CINs. To assess this, we performed single cell RNA sequencing from known Cre-driver lines to assess the level of Cnnap2 transcript in cells from P28-P35 as previously described (Paul et al. in press). Nkx2.1-CreER and PV-ires-Cre crossed to Ai14 reporter mice were used to isolate adult chandelier cells (CHC) and PV+ cells, respectively. These cells had high levels of Cnnap2 transcripts in the adult cortex compared with other CIN subtypes (Fig. 2a,b). Next, we used a combination of Sst-Flp and Nos1-CreER lines to isolate SST+ long projecting cells or Sst-Flp with the calretinin (CR)-Cre to label SST+ Martinotti cells. While there was Cnnap2 transcript in these populations (Fig. 2a,b), it was lower than in the CHC and PV lineage groups. Finally, we examined CGE-derived VIP+ CINs by utilizing a combination of the VIP-Flp with CR-Cre to isolate interneurons or the VIP-Flp with CCK-Cre to label CCK+ basket cells. Each of these VIP+ groups also had high levels of Cnnap2 transcript (Fig. 2a,b). Overall, many CINs express Cnnap2 in the postnatal cortex, with higher levels of expression in the CHC/PV+ and VIP− subgroups and lower expression in the SST− subgroups. Due to a lack of Cre-driver lines to specifically isolate the REELIN+/ SST− CINs derived from the CGE, we do not know if Cnnap2 is expressed in these cells postnatally.

Next, we assessed whether distinct groups of CINs were affected in Cnnap2 mutants. To this end, we first examined parvalbumin (PV+) and somatostatin (SST+) CINs; the main CINs derived from the MGE. There was ~24% decrease in PV-expressing CINs in Cnnap2−/− cortices (Fig. 3a–d, P = 0.006), similar to a previous report (Peñagarikano et al. 2011). We found no change in SST+ CINs (Fig. 3e–h).

We then investigated CGE-derived CINs, including those expressing vasoactive intestinal peptide (VIP) or REELIN (but that do not express SST) (Wonders and Anderson 2006; Miyoshi et al. 2010). Notably, there was ~13% decrease in REELIN+/SST− CINs (Fig. 3e–g, i, P = 0.049). However, no differences in VIP+ CINs were evident (Fig. 3j–m). Thus, specific subgroups of both MGE and CGE-derived CINs were reduced in Cnnap2−/− mutants.

Cell Autonomous Role of CNTNAP2 in Mediating Cell Intrinsic Properties of PV+ Fast-Spiking CINs

It is unknown whether Cnnap2 loss of function alters the molecular and/or physiological properties of CINs via cell
autonomous mechanisms, and/or by secondary defects induced by other cells. A Cntnap2 conditional mouse could help solve this problem, however, this mouse does not currently exist to our knowledge. To overcome this roadblock, we employed an MGE cell transplantation approach (Vogt, Wu et al. 2015), in which E13.5 Cntnap2\(^{-/-}\) MGEs (containing immature CINs) are transplanted into the normal environment of a P1 WT cortex, where they develop and mature \(\text{in vivo}\). We fluorescently labeled WT, Cntnap2\(^{+/−}\), and Cntnap2\(^{-/-}\) MGE cells with tdTomato expression, by including the Nkx2.1-Cre and Ai14 alleles, and assessed their electrophysiological properties at 6-8 weeks post transplantation (see schema, Fig. 4).

The transplanted tdTomato\(^{+}\) CINs were separated into 2 subgroups based on their spike responses to depolarizing current pulses: accommodating (non fast-spiking, likely SST\(^{+}\) CINs) or nonaccommodating (fast-spiking, likely PV\(^{+}\) CINs) (see Materials and Methods section). Notably, the nonaccommodating, presumed PV\(^{+}\) CINs, had multiple parameters that were changed in Cntnap2\(^{-/-}\) mutants. Based on their responses to a series of current injections, the nonaccommodating interneurons lacking Cntnap2 displayed wider spikes, smaller maximum rate of rise of membrane voltage during spike rising phase, slower membrane time constants, greater adaptation ratios, and more depolarized resting membrane potentials compared to controls (Fig. 4b-f, WT compared to null: half-width \(P = 0.02\), max slope \(P = 0.002\), tau 

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P = 0.02, \text{ISI } P = 0.01.
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Example traces for nonaccommodating CINs are shown in Figure 4i. These changes suggest a role for Cntnap2 in the properties of voltage-dependent sodium and/or potassium channels, which mediate action potentials and repolarization in nonaccommodating CINs. Interestingly, in Cntnap2 heterozygote CINs several of these properties had values intermediate between those of the WT and Cntnap2 null CINs. While one (action potential half-width) was significantly different between the heterozygotes and nulls (Fig. 4b, \(P = 0.02\)), there were other parameters for which both heterozygotes and knockouts were significantly different compared to WTs (Fig. 4h, f:I slope, heterozygote \(P = <0.0001\), null \(P < 0.0001\)). Thus, loss of one Cntnap2 allele is sufficient to elicit physiological changes in nonaccommodating, presumed PV\(^{+}\), CINs.

In contrast, the accommodating (likely SST\(^{+}\)) Cntnap2 null CINs did not show differences in the same cell intrinsic properties (Fig. 4j–p). Together, our data provide evidence that Cntnap2 preferentially regulates the physiological properties of presumed PV\(^{+}\), fast-spiking CINs.

**Cell Autonomous Role for Human ASD CNTNAP2 Alleles in Regulating the Number of CINs that Express PV**

To probe the functional consequences of missense mutations discovered in CNTNAP2, we cloned the WT human CNTNAP2
gene, as well as 4 reported CNTNAP2 missense alleles discovered in individuals diagnosed with ASD (Bakkaloglu et al. 2008), into a Dlx12b-IRES-Cre lentiviral vector before the IRES sequence and the Cre-recombinase gene (Fig. 5a). Dlx12b is an enhancer that is preferentially expressed in GABAergic neurons (Potter et al. 2009; Arguello et al. 2013). We chose to investigate 4 missense CNTNAP2 alleles discovered in populations with ASD (Bakkaloglu et al. 2008), as little is known about whether these missense alleles alter CNTNAP2 function. Expression of each of the CNTNAP2 alleles was verified by western blotting following transfection of the vector into HEK293T cells (Fig. 5b). We also verified the Cre activity from each vector by its ability to induce expression of GFP from a Cre-dependent GFP-expression plasmid (Fig. 5b). Finally, we expressed the WT and mutant CNTNAP2 alleles in HEK293T cells and found that both the WT and mutant proteins were enriched at cell membranes (Fig. 5c–h).

Next, we used a modification of the MGE transplantation assay (Vogt, Wu et al. 2015) to study the functional properties of human CNTNAP2 mutant alleles on CINs. To this end, MGE cells from Cntnap2+/− or Cntnap2−/− E13.5 mouse embryos were dissociated and transduced with the Dlx12b-IRES-Cre lentivirus (schema, Fig. 5a), before being transplanted into the cortices of P1 WT pups. The MGE cells also harbor the Ai14 allele, to visualize tdTomato after Cre recombination in the transduced cells. Virus encoding either the “empty” vector (Cre only), or a virus encoding one of the human CNTNAP2 alleles and Cre were transduced into mouse E13.5 Cntnap2−/− or Cntnap2+/− MGE cells before transplantation and assessed for PV expression at 35 days post-transplant (DPT). Notably, Cntnap2−/− transplanted MGE cells had ~33% reduction in PV+ CINs compared with Cntnap2+/− transplants (Fig. 6h), demonstrating that reduced PV+ CINs is a cell autonomous phenotype. Of note, WT human CNTNAP2, transduced into Cntnap2−/− MGE cells, completely rescued the number of PV-expressing cells (Fig. 6b,c,h, P = 0.004). However, transduction of human CNTNAP2 into heterozygous Cntnap2 cells did not alter the number of PV-expressing
Thus, human CNTNAP2 complemented the decreased PV+ interneuron phenotype in mouse MGE cells that lacked the gene but did not act in a dominant fashion in the heterozygous cells.

Next, we tested whether the 4 human CNTNAP2 missense mutations, could rescue the reduction in PV+ CINs in the transplanted Cntnap2−/− MGE cells. None of the mutant alleles showed a significant rescue of PV+ CIN numbers and were significantly different than WT CNTNAP2 transduction (Fig. 6d–h, N407>S P < 0.0001; N418>D P = 0.006; G731>S P = 0.0003; T1278>I P = 0.007, compared to WT CNTNAP2 transduction). To explore if the mutant alleles act in a dominant-interfering fashion, we transduced them into heterozygote (Cntnap2+/−) MGE cells. Only G731>S showed a trend towards reduced PV+ CINs in Cntnap2+/− CINs, but this effect did not reach statistical significance (Fig. 6i–o, G731>S P = 0.31). We also examined SST+ CINs in these assays, but did not observe any differences between groups (data not shown). In sum, 4 human CNTNAP2 missense mutations acted like null/hypomorphic alleles based on their inability to rescue the number of PV+ CINs.

We also explored whether transplanted MGE cells lacking Cntnap2 or those transduced with human CNTNAP2 alleles mutated in ASD resulted in elevated ERK1/2 activity but found no changes in adult brains (data not shown), suggesting that this may be a transient phenotype.

**Discussion**

Herein, we focused on cell autonomous roles for CNTNAP2 in CIN development and physiology. As described previously, we found
that Cntnap2−/− mice had reduced numbers of PV+ MGE-derived CINs (Peñagarikano et al. 2011). We extended this analysis in many ways (see below), including by demonstrating that Cntnap2−/− mice also had reduced numbers of REELIN+/SST− (CGE-derived) CINs. We also showed the specificity of this phenotype as the number of other MGE (SST+), and CGE (VIP+)-derived CINs were normal.

We provided evidence that the reduction of PV+ CINs was not due to a reduction in the number MGE-derived CINs by using Nkx2.1-Cre-fate mapping (Fig. 1). This suggests that the reduction in PV+ CINs was not due to a defect in their production, migration, and/or survival, but probably due to a defect in their differentiation and/or activity, that secondarily reduced expression of the PV protein. Indeed, PV expression in CINs does not begin until ~P10–P14, which may depend, at least in part, on neural activity (Vogt Weisenhorn et al. 1998; Patz et al. 2004). While we did not detect a decrease in MGE-derived CINs, it should be noted that others have noted a reduction in GABAergic cells in zebrafish that have Cntnap2 deletion (Hoffman et al. 2016). The discrepancies between our findings could be due to several mechanisms, such as species differences or experimental approaches. Fate mapping in the mouse CNTNAP2 mutant using a Cre that is active in all/most CINs (e.g., GAD2-Cre) could help resolve the issue of whether there is a global reduction of cortical CINs.

Importantly, our MGE transplantation experiments showed that Cntnap2−/− CINs, which had differentiated in a WT cortex, also generated fewer CINs that were PV+ (Fig. 6). This provides evidence that Cntnap2 is autonomously required during CIN maturation to promote PV expression, perhaps because of abnormal activity within these cells.
Cntnap2\(^{-/-}\) MGE-derived CINs had multiple abnormal cell intrinsic physiological properties (Fig. 4). The results provide evidence that Cntnap2 is particularly important in PV\(^+\), nonaccommodating, fast-spiking CINs, rather than in accommodating/likely SST\(^+\) CINs. Consistent with this, Cntnap2 RNA is more highly expressed in PV\(^+\) fast-spiking CINs than in SST\(^+\) CINs (Fig. 2). Interestingly, in the constitutive Cntnap4 loss of function mouse, the action potential width in PV\(^+\) CINs was significantly increased (Karayannis et al. 2014), similar to our observations in transplanted Cntnap2\(^{-/-}\) fast-spiking CINs (Fig. 4). Moreover, while other cell intrinsic properties (i.e., input resistance, tau, and firing rate) in the constitutive Cntnap4 mutant did not reach significance, changes in their values trended in the same direction as the changes we found in the Cntnap2\(^{-/-}\) fast-spiking CINs. These data suggest these 2 neurexin family members have some overlapping functions in fast-spiking CINs.

The changes in half-width, maximum slope, resting membrane potential, and adaptation ratio suggest that the Cntnap2\(^{-/-}\) fast-spiking CINs could have alterations in potassium and/or sodium channels that are associated with the fast-spiking/nonaccommodating electrophysiological phenotype. CASPR2 is known to co-localize with Kv1.1 and Kv1.2 potassium channels (Poliak et al. 1999). However, our analyses of Kv1.1, Kv3.1, Kv4.2, and Kv4.3 expression, and their intracellular localization, did not reveal differences between WT and Cntnap2\(^{-/-}\) CINs (data not shown).

Cntnap2\(^{-/-}\) transplanted CINs exhibited intermediate phenotypes between transplanted WT and Cntnap2\(^{-/-}\) CINs (Fig. 4). Thus, the demonstration that Cntnap2\(^{-/-}\) heterozygote CINs have electrophysiological alterations in PV\(^+\) CINs may be clinically relevant as some individuals have heterozygous mutations in CNTNAP2. Finally, while physiological parameters were altered in the mouse Cntnap2\(^{-/-}\) heterozygous CINs, CIN numbers and molecular CIN markers were only altered in Cntnap2\(^{-/-}\) nulls, suggesting that CIN physiological parameters may be more sensitive to the Cntnap2-heterozygous state. This is interesting in relation to humans that harbor CNTNAP2 mutations, as they can exist in both the heterozygous or homozygous state. We hypothesize that individuals with heterozygous mutations may potentially have alterations in the physiological properties of PV\(^+\) CINs but may lack more severe phenotypes associated with complete loss of functional CNTNAP2. Of note, individuals that have homozygous mutations that result in nonfunctional protein exhibit severe phenotypes including epilepsy, speech and language impairment as well as intellectual disability (Strass et al. 2006; Zweier et al. 2009; Watson et al. 2014; Rodenas-Caudrado et al. 2016).

Human CNTNAP2 disease alleles have been discovered in individuals with ASD and other neurological disorders. It is important to interrogate CNTNAP2 function encoded by these alleles using relevant assays, particularly in vivo. Herein, we focused on a subset of CNTNAP2 missense alleles reported in ASD individuals (Bakkaloglu et al. 2008).

Previously, we validated an efficient in vivo approach to determine the impact of ASD alleles; we first applied this to PTEN (Vogt, Cho et al. 2015). Here, we utilized this approach to evaluate the function of CNTNAP2 ASD mutations in MGE-derived CINs that did not express mouse Cntnap2. Notably, none of the mutant human CNTNAP2 alleles could rescue the reduction of PV\(^+\) CINs. In addition, none of them induced phenotypes when expressed in Cntnap2\(^{-/-}\) cells, providing evidence that they did not have a dominant effect. Together, these data provide evidence that these mutant alleles are either hypofunctional or loss of function, with respect to promoting the development of PV\(^+\) CINs. Furthermore, our results support a functional consequence of these human missense mutations on human CIN development. Likewise, we previously found PTEN ASD missense alleles to be hypo/loss of function but not dominant-interfering (Vogt, Cho et al. 2015).

Many ASD mouse models have alterations in PV\(^+\) CINs (Selby et al. 2007; Gogolla et al. 2009; Martins et al. 2011; Takanoue 2015), suggesting that dysfunction or alterations in this cell type is a common lesion found in ASD. While we currently do not understand why all 4 CNTNAP2 missense alleles resulted in decreased CINs expressing PV, PV expression is a late event in the development of these CINs and any number of developmental insults may contribute to this phenotype. Thus, our defining a role for CNTNAP2 in PV\(^+\) CIN development and maturation elucidates a mechanism that may be part of a common pathway that leads to cortex dysfunction in some forms of ASD, and potentially in other disorders caused by CNTNAP2. We propose that our findings have implications for understanding how CNTNAP2 disease alleles result in specific phenotypes in ASD, perhaps through reducing cortical inhibition and thereby disrupting the E/I balance (Rubenstein and Merzenich 2003; Sudhof et al. 2009). Future studies are needed to understand if mutations in CNTNAP2 associated with the wider spectrum of CNTNAP2 disorders (Rodenas-Caudrado et al. 2014; Poot 2015, 2017) have similar phenotypes.

We also detected trends towards increased pERK1/2 activity in Cntnap2\(^{-/-}\) MGE tissue (Fig. 1a) that should be examined in future studies. Disruptions in RAS/MAPK signaling cause multiple disorders (known as RASopathies) that have a high comorbidity with ASD (Adiento et al. 2014). Thus, it is possible that human CNTNAP2 may regulate MAPK signaling. At this point, we do not know whether the increased pERK1/2 activity contributes to the decreased number of PV\(^+\) and REELIN/‘SST’ CINs, and/or to the alterations in intrinsic electrophysiological properties. However, it is intriguing that ERK1/2 can target potassium channels (Schrader et al. 2006, 2009), which could explain the altered electrophysiological properties found in the fast-spiking CINs.

In sum, our results provide new insights into the cell autonomous functions of CNTNAP2 in the development and physiology of CINs. They highlight CNTNAP2’s function in PV\(^+\) CINs, a cell type whose dysfunction may have a central role in ASD as well as other neurological disorders.

Authors’ Contributions

D.V., K.K.A.C., S.M.S., and A.P. performed experiments and analyzed data. A.P. and J.H. contributed the single cell adult CIN RNA-seq data. All authors contributed to writing the manuscript.

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