Intragenic *CNTNAP2* Deletions: A Bridge Too Far?

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Keywords

CNTNAP2 · Intragenic deletions · Neurodevelopmental disorders

Abstract

Intragenic deletions of the contactin-associated protein-like 2 gene (*CNTNAP2*) have been found in patients with Gilles de la Tourette syndrome, intellectual disability (ID), obsessive compulsive disorder, cortical dysplasia-focal epilepsy syndrome, autism, schizophrenia, Pitt-Hopkins syndrome, stuttering, and attention deficit hyperactivity disorder. A variety of molecular mechanisms, such as loss of transcription factor binding sites and perturbation of penetrance and expressivity, have been proposed to account for the phenotypic variability resulting from *CNTNAP2* mutations. Deletions of both *CNTNAP2* alleles produced truncated proteins lacking the transmembrane or some of the extracellular domains, or no protein at all. This observation can be extended to heterozygous intragenic deletions by assuming that such deletion-containing alleles lead to expression of a Caspr2 protein lacking one or several extracellular domains. Such altered forms of Caspr2 proteins will lack the ability to bridge the intercellular space between neurons by binding to partners, such as CNTN1, CNTN2, DLG1, and DLG4. This presumed effect of intragenic deletions of *CNTNAP2*, and possibly other genes involved in connecting neuronal cells, represents a molecular

basis for the postulated neuronal hypoconnectivity in autism and probably other neurodevelopmental disorders, including epilepsy, ID, language impairments and schizophrenia. Thus, *CNTNAP2* may represent a paradigmatic case of a gene functioning as a node in a genetic and cellular network governing brain development and acquisition of higher cognitive functions.

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The contactin-associated protein-like 2 gene (*CNTNAP2*) is located in the chromosomal region 7q35, spans 2.3-Mb genomic DNA, with a full-length open reading frame of 3,996 nucleotides, and encodes a transcript of 24 exons. The *CNTNAP2*-encoded Caspr2 protein of 1,331 residues consists of a signal peptide, an F5/8 discoidin/neuropilin homology domain, 4 laminin G-binding domains, 2 EGF-like domains, a fibrinogen-like region, a transmembrane domain, and a short intracellular domain terminating with a PDZ interaction domain. Caspr2 contains 12 N-glycosylation sites and 36 cysteine residues allowing 18 disulfide bonds [Rubio-Marrero et al., 2016]. Thus, the extracellular domain of Caspr2 has a compact conformation with a likely 3-domain clover leaf-like feature and 2 somewhat extended domains (Fig. 1) [Rubio-Marrero et al., 2016]. In addition, 2 alternative transcripts (*CNTNAP2*-005 and

Fig. 1. Diagram depicting the putative in solution structure of the extracellular domains of Caspr2 [for details, see Rubio-Marrero et al., 2016]. FBG, fibrinogen-like region; F5/8, discoidin/neuropilin homology domain; LG, laminin G-binding domains.

CNTNAP2-006) encode proteins of 390 and 119 amino acid residues, respectively (Ensembl release 86; October 2016). Being one of the largest genes in the human genome, *CNTNAP2* is a likely target for structural rearrangements, including inversions and translocations, copy number variations (CNVs), mutations in exons and transcription factor binding sites, and for epigenetic modifications. In addition, the genomic DNA of *CNTNAP2* overlaps with one validated and several inferred micro-RNAs (GENCODE v24 release; March 28, 2016).

In one family with cortical dysplasia and focal epilepsy (CDFE), mild gross motor delay, leading to regression of learning abilities, language and social behaviors and signs of attention deficit hyperactivity disorder (ADHD) as well as autism (ASD), a cosegregating *CNTNAP2* mutation (3709delG) has been described [Straus et al., 2006]. In several isolated patients, CNVs affecting both *CNTNAP2* alleles, leading to complete loss of *CNTNAP2* function, have been found [Zweier, 2012; Watson et al., 2014; Rodenas-Cuadrado et al., 2016]. In addition, heterozygous genomic rearrangements and CNVs have implicated *CNTNAP2* in a variety of neurodevelopmental disorders such as Gilles de la Tourette syndrome (GTS), intellectual disability (ID), obsessive compulsive disorder, CDFE, ASD, schizophrenia, Pitt-Hopkins syndrome,

stuttering, and ADHD [Malhotra and Sebat, 2012; Rodenas-Cuadrado et al., 2014; Poot, 2015].

In this review, the phenotypic spectrum of patients with intracellular deletions of *CNTNAP2* and *Cntnap2*-null animals is discussed together with results from biochemical and cellular studies of *CNTNAP2* function. The latter indicate interaction of the Caspr2 protein with the proteins encoded by *CNTN1*, *CNTN2*, *DLG1* and *DLG4*. Taking these interactions into account, a model will be put forward in which the Caspr2 protein may serve as a bridge to connect different cell types. Disruptions of such bridges are considered in order to account for the various clinical phenotypes associated with intracellular deletions of *CNTNAP2* and the roles of the intact protein in model animals.

Organ and Tissue Expression Patterns in Humans and Rodents

The *CNTNAP2*-encoded Caspr2 protein is expressed in both the myelinated axons of the spinal cord and in the central nervous system. In the spinal cord, the Caspr2 protein localizes to the juxtaparanodes on the axons, which depends on axon-glia interactions and the generation of barriers along the axon [Poliak et al., 1999, 2001]. At the juxtaparanodes, Caspr2 associates with TAG-1 (*CNTN2*) and is involved with clustering of potassium channels [Poliak et al., 1999, 2003; Traka et al., 2002, 2003]. Since myelination is not apparent in humans until 28 weeks of gestation, these data suggest that *CNTNAP2* may be involved in the development of myelinated neurons [Panaitof et al., 2010]. Thus far, a deletion of exon 4 of *CNTNAP2* has been described in a single patient with the peripheral neuropathy Charcot-Marie-Tooth disease [Høyer et al., 2015].

In the human fetal brain, *CNTNAP2* is predominantly expressed in the anterior frontal and temporal perisylvian regions, the striatum, and the thalamus [Abrahams et al., 2007; Alarcón et al., 2008]. From the 18th to the 20th week of gestation, fetal brains show a striking enrichment of *CNTNAP2* signals in the frontal gray matter, roughly between the orbital gyrus and superior frontal cortical anlagen, in the Broca area, and other perisylvian brain regions [Abrahams et al., 2007; Alarcón et al., 2008]. Strong signals were also seen in the basal ganglia of the thalamus, amygdala, caudate, cortical plate, dorsal thalamus, and in the putamen [Alarcón et al., 2008].

Marked differences in cerebral cortical expression between humans, nonhuman primates, and rodents were

observed [Abrahams et al., 2007; Alarcón et al., 2008; Peñagarikano et al., 2011; Schneider et al., 2012]. In contrast to the enrichment in the human frontal cortex, mice showed only limited expression in the cortical plate during the corresponding developmental period, embryonic day 17 (E17), with highest levels in the posterior region. The highest levels of *Cntnap2* expression were in the olfactory bulb, ventricular zones, striatum, thalamus, and the hippocampus [Abrahams et al., 2007; Peñagarikano et al., 2011]. In addition, *Caspr2* is expressed in brain regions involved in sensory signal processing and in all primary sensory organs. Olfaction-based behavioral tests demonstrated abnormal responses to sensory stimuli and lack of preference for novel odors in *Caspr2*-null mice [Gordon et al., 2016].

CNTNAP2 expression in the developing rat brain was remarkably similar to that observed in mice, except that there was much less expression in the rat olfactory bulb [Abrahams et al., 2007]. Also in the rat, the signal was broadly distributed throughout the brain and uniformly low or absent in the cortical plate [Abrahams et al., 2007]. It should be noted that the anterior temporal and prefrontal regions, in which *CNTNAP2* expression is high in humans and low or absent in rodents, are much more developed in human and nonhuman primates. *CNTNAP2* expression in perisylvian language-related structures and circuits is consistent with the involvement of *CNTNAP2* in language development and higher cognitive functions.

To delineate the possible mechanisms underlying the distinct patterns of *CNTNAP2* expression, Abrahams et al. [2007] analyzed *CNTNAP2* loci in the human, mouse, and rat genomes for possible structural differences. The gene structure was conserved across the 3 species with each ortholog having 24 coding exons distributed across roughly 2 Mb of genomic DNA. Similarly, the protein motif structure (100% identity among human, mouse, and rat), amino acid composition (94% human vs. mouse, 92% human vs. rat), and coding DNA (87% human vs. mouse, 87% human vs. rat) were highly conserved among the 3 species. These findings suggest that, in addition to the differential regulation of overall transcript abundance, disparate distribution of transcripts is likely to be an important driver of cerebral cortical evolution. Reviewing studies in songbirds, Rodenas-Cuadrado et al. [2014] indicated that *Cntnap2* shows highly differential expression patterns within the specialized cortico-striato-thalamic circuit that makes up the vocal pathway in songbirds. Thus, *CNTNAP2* appears to be highly conserved with respect to its biological function(s).

Cellular Functions of *Caspr2*

Apart from its involvement with myelinated axons, *Caspr2* has an organizing function in developing neurons, which is essential for the assembly of neural circuits in the central nervous system [Anderson et al., 2012]. RNAi-mediated knockdown of *Caspr2* protein levels in cultures of cortical neurons from newborn mice of the CD1 strain produced a cell-autonomous decrease in dendritic arborization and spine development in pyramidal neurons. This leads to a global decline in numbers of excitatory and inhibitory synapses and a decrease in synaptic transmission [Anderson et al., 2012]. Notably, a decrease in total dendritic length and branching as well as a decreased spine size in mature *CNTNAP2* knockdown neurons was observed [Anderson et al., 2012]. Thus, *Caspr2* performs an early organizational function in developing neurons, essential for neural circuit assembly and operating at a point in brain development that coincides with the time of ASD pathogenesis [Anderson et al., 2012]. In cultures of cortical neurons, obtained from newborn *Cntnap2*-null mice of the CD1 strain, mature neurons with fully developed synapses but reduced spine density and alterations in spine morphology have been observed, similar to those in a previous study with RNAi-mediated knockdown of *Caspr2* protein levels [Anderson et al., 2012; Varea et al., 2015]. Also in cultures of cortical neurons from *Cntnap2*-null C57BL/6J mice, new dendritic spines were formed at normal rates but failed to stabilize [Gdalyahu et al., 2015]. Thus, cellular functions of *CNTNAP2* in C57BL/6J and CD1 mice are congruent, which is in stark contrast to the marked differences regarding behavioral and cognitive features with relevance to ASD found among inbred mouse strains [Kas et al., 2014]. These observations indicate that *CNTNAP2* is most likely required for the maturation and maintenance of spiny synapses, but not for earlier stages of postnatal development of pyramidal neurons. Such findings may suggest a potential mechanism for the delayed appearance of symptoms in some clinical cases with intragenic *CNTNAP2* CNVs and gene truncating mutations.

Mutations Affecting Both *CNTNAP2* Alleles

In an Old Order Amish family, a homozygous truncating mutation of *CNTNAP2* (3709delG) in exon 22 was found in 3 patients with a 7.1-Mb region of autozygosity (homozygosity) in the chromosomal region 7q36 [Strauss et al., 2006]. All patients were homozygous for the muta-

Fig. 2. Postulated proteins in patients with homozygous *CNTNP2* mutations. The yellow bar represents the PDZ interaction domain. CDFE, cortical dysplasia/focal epilepsy; E, EGF-like domain; FBG, fibrinogen-like region; F5/8, discoidin/neuropilin homology domain; LG, laminin G-binding domains; SP, signal peptide; TM, transmembrane domain.

tion, and their parents were heterozygous. The frameshift mutation results in a premature stop codon and is predicted to encode a nonfunctional protein, lacking the transmembrane and cytoplasmic domains of CNTNAP2. The clinical phenotypes included CDFE, relative macrocephaly, diminished deep-tendon reflexes, and intractable focal seizures from early childhood on. Later on, all children developed language regression, hyperactivity, impulsive and aggressive behavior, as well as mental retardation. Temporal-lobe specimens showed evidence for abnormalities of neuronal migration and structure, widespread astrogliosis, and reduced expression of Caspr2 [Strauss et al., 2006; Jackman et al., 2009].

After this family with an autosomal recessive *CNTNAP2* syndrome, 3 more cases with homozygous *CNTNAP2* deletions have been reported (Fig. 2) [Zweier et al., 2009; Watson et al., 2014; Rodenas-Cuadrado et al., 2016]. A homozygous exon 2–3 deletion and a homozygous exon 3 deletion, which introduce premature stop codons, are likely to elicit nonsense-mediated mRNA decay [Watson et al., 2014; Rodenas-Cuadrado et al., 2016]. In the third case, a homozygous exon 2–9 deletion most likely produces a truncated protein product, which is due to an in-frame loss of amino acids 33–500 [Zweier et al., 2009]. All these patients present with severe ID, early-onset drug-resistant epilepsy with concomitant regression of language, communicative impairments, and autistic features, albeit with some degree of variability. On the basis of the *CNTNAP2* deletions in these patients, the minimally mutated form of Caspr2 would be a protein retaining its membrane-anchoring domain but lacking

the F5/8 discoidin/neuropilin homology domain and 2 of the N-terminal laminin G-binding domains [Zweier et al., 2009; Watson et al., 2014; Rodenas-Cuadrado et al., 2016]. The truncated protein from the Old Order Amish family (CASPR2-1253*) would be nonfunctional, since it is no longer anchored in the cell membrane but secreted (Fig. 2) [Strauss et al., 2006; Falivelli et al., 2012]. Conceivably, loss of a membrane-anchored form of Caspr2 or at least the F5/8 discoidin/neuropilin homology domain and the 2 N-terminal laminin G-binding domains would be sufficient to elicit a syndrome consisting of ID, early-onset drug-resistant epilepsy with concomitant regression of language, communicative impairments, and autistic features.

Heterozygous Disruptions and Intragenic Deletions of *CNTNAP2*

By classical karyotyping with 3 patients with features of GTS shared a chromosome 2p21p23 insertion into chromosome 7q35q36, which interrupts intron 8 of *CNTNAP2* (Fig. 3) [Verkerk et al., 2003]. GTS is a complex neuropsychiatric disorder characterized by involuntary motor and vocal tics, which is often accompanied by disorders such as obsessive compulsive disorder and ADHD. Subsequently, in a patient with ASD, an inversion 7q21q35, which disrupted both *AUTS2* and *CNTNAP2* (Fig. 3), and in a boy with language delay and ASD, a complex insertion-translocation of intron 1 through 3 of *CNTNAP2* into chromosomal region 1q31.1

Fig. 3. Intragenic *CNTNAP2* deletions. Orange bars: heterozygous de novo deletions. Purple bar: homozygous deletion inherited from both heterozygous carrier parents. Cross-hatched bars: inherited heterozygous deletions. The arrows indicate the breakpoints of *CNTNAP2* disruptions in translocation and inversion patients. The double black arrow connects a maternally inherited deletion of exon 1 and a paternally inherited deletion of exons 4–20 in a patient reported by Smogavec et al. [2016]. ADHD, attention deficit hyperactivity disorder; GTS, Gilles de la Tourette syndrome; ID, intellectual disability; Schizo, schizophrenia.

and an additional deletion in region 1q41 was found (Fig. 3) [Bakkaloglu et al., 2008; Poot et al., 2010]. Finally, in a healthy individual, a translocation disrupting intron 13 of *CNTNAP2* had been detected (Fig. 3) [Belloso et al., 2007]. Taken together, these genetic data suggest that proximal *CNTNAP2* disruptions, up to intron 8, may cause a neurodevelopmental disorder, while distal disruptions may be inconsequential.

Intragenic de novo heterozygous deletions of *CNTNAP2* were described in patients with ASD [Alarcón et al., 2008], Pitt Hopkins syndrome [Zweier et al., 2009], ADHD [Elia et al., 2010], stuttering [Petrin et al., 2010], and ID (Fig. 3) [Mikhail et al., 2011]. In a patient with neonatal seizures, an in-frame deletion of both alleles of exons 2–4 (153 amino acid residues) of a stretch of DNA spanning intron 2 and 3 of *CNTNAP2*, due to the inheri-

tance of heterozygous losses from both healthy parents has been described (Fig. 3) [Mefford et al., 2010]. A patient with ID and epilepsy and a heterozygous loss of similar size has been reported [Smogavec et al., 2016]. Another patient with ID and epilepsy harbored a maternally inherited 56-kb deletion of exon 1, arr[hg19] 7q35(145795795_145824743)×1, and a paternally inherited 1.23-Mb deletion of exons 4–20, arr[hg19] 7q35q36.1(146730472_147928239)×1 [Smogavec et al., 2016]. A deletion of exon 1, as found in 2 patients from this study, is predicted to result in the loss of the transcription start codon. The loss of exons 4–20 may result in frameshifting and thus truncation of the protein. Hence, this patient would express a truncated protein encoded by exons 1 through 4 only, since the deletion of exon 1 of the other allele would not produce any protein

at all [Smogavec et al., 2016]. In 2 patients with schizophrenia from 2 independent families with multiple psychiatric disorders, transmitted heterozygous losses of part of *CNTNAP2* have been found, and 2 ASD patients had small proximal losses in *CNTNAP2*, which they inherited from their healthy mothers (Fig. 3) [Friedman et al., 2008; Gregor et al., 2011]. These findings link disruptions of *CNTNAP2* to several distinct neurodevelopmental disorders.

Models to Explain the Clinical Variability of Intragenic *CNTNAP2* Deletions

In view of the data on intragenic deletions, the impression from the results of karyotyping that *CNTNAP2* may harbor “disorder-specific domains,” i.e., disruptions up to intron 8 of *CNTNAP2* will lead to GTS or ASD, needs reconsideration [Rodenas-Cuadrado et al., 2014; Poot, 2015; Pippucci et al., 2015]. As is evident from Figure 3, intragenic deletions in patients with epilepsy, ASD, schizophrenia, ID, and language delay are scattered throughout the entire gene, such that no disorder-specific domains can be pinpointed. The sheer variability in clinical phenotypes also complicates attempts to identify a single molecular mechanism that may explain all cases. Conceivably, de novo deletions may introduce premature stop codons, which would in turn activate nonsense-mediated mRNA decay of the deletion containing alleles [Lou et al., 2014]. In such cases, no mRNA with the deleted allele would persist, and the same clinical phenotypes would result regardless of the position and size of the deletion involved. This is clearly not the case (Fig. 3).

CNTNAP2 is poorly transcribed in cultured fibroblasts and lymphoblastoid cell lines, such that no mRNAs have been examined for most cases of intragenic deletions. In a single case with an inherited intragenic deletion of exons 14 and 15, inducible pluripotent stem cells (iPSC) were generated and induced to neuronal differentiation [Lee et al., 2015]. The deleted allele was preferentially transcribed in the cells derived from the patient, but not in those of the healthy father. By transcriptome sequencing, allele-biased expression has been described in both T lymphocytes and in neurons derived from iPSC [Heap et al., 2010; Lin et al. 2012]. This apparently general phenomenon may be due to genetic or epigenetic factors, i.e., imprinting, which have as yet not been explored. Nevertheless, selective expression of *CNTNAP2* alleles containing intragenic deletions needs due consideration.

The preferential expression of the *CNTNAP2* allele with the intragenic deletion of exons 14 and 15 provoked in iPSC significantly reduced neural migration, as demonstrated by a neurosphere migration assay [Lee et al., 2015]. On the other hand, mutations of several residues in laminin G-binding domains and fibrinogen-like domains of Caspr2, which have been found in patients with ASD, caused a local misfolding of the protein [Falivelli et al., 2012]. Thus, the protein did not mature in the endoplasmic reticulum and Golgi apparatus and was consequently not transported to the cell membrane [Falivelli et al., 2012]. More studies are needed to fully understand the functional consequences of point mutations and intragenic deletions of *CNTNAP2*.

Gene Haploinsufficiency and Nonallelic Noncomplementation

If a gene is transcribed from both alleles, loss of one of those would be inconsequential if the gene product, i.e., the protein, functions as an isolated entity. Yet, in many cases, e.g., cell surface receptors, signal transduction pathways, transcription, ion channels, and chromatin, proteins form complexes with other proteins [Veitia and Birchler, 2010]. If the relative amount of proteins making up these complexes is out of balance, due to not enough or too much of one of the proteins, the total amount of protein complexes will be insufficient for normal cell function. In this way, haploinsufficiency for a single gene will produce a dominant phenotypic effect as has been observed in patients with, among other conditions, epilepsy, ASD, and schizophrenia [Poot et al., 2011].

Following classical biochemical reasoning, the gene dosage balance hypothesis postulates that stoichiometric imbalances in macromolecular complexes and cellular networks may be responsible for abnormal phenotypes [Birchler and Veitia, 2012]. Veitia [2010] extended this hypothesis to constellations of mutations at 2 different loci, i.e., genes. In a constellation of hemizygosity at both loci, due to deletions, or of heterozygosity at both loci, due to recessive mutations, in which single locus hemizygosity or heterozygosity is not sufficient to elicit a phenotype, nonallelic noncomplementation (NANC) arises. Thus, halving the gene dosage or a heterozygotic loss of function at both gene loci is responsible for the abnormal phenotype. In such cases of combined haploinsufficiency, the level of the remaining functional protein complex is very small and the resulting phenotype likewise severe. Thus, 2 heterozygotic mutations in or deletions of reces-

sive genes, which each individually do not produce a phenotype, would become dominant negative through the NANC mechanism.

While the NANC model has not yet been rigorously tested in an experimental setting, it is supported by evidence from observational studies. In healthy individuals and in patients with ID, ASD, and other neurodevelopmental disorders, individuals with 2 or more unique or recurrent CNVs have been reported [Girirajan et al., 2010; Poot et al., 2010]. The finding that 2 or even more recurrent CNVs occur in healthy individuals indicates that not just any pair of recurrent CNVs will produce a phenotype. Rather pairs of CNVs which affect genes that functionally interact will be deleterious. Such pairs of CNVs may elicit a phenotype, due to NANC. The resulting phenotype(s) may be variable depending on which pairs of CNVs occur [Girirajan and Eichler, 2010; Girirajan et al., 2012]. Since roughly 10% of all patients with ID carry more than a single CNV, a genome-wide screen should be performed to search for additional CNVs after a first one has been detected [Poot et al., 2011; Girirajan et al., 2012]. NANC is not restricted to CNVs, but also applies to single nucleotide variants (SNVs) causing loss-of-function mutations [Leblond et al., 2012; Mercati et al., 2016].

Negative Transdominance in Relation to Intragenic Deletions

Thus far, only mechanisms of action of heterozygous deletions were discussed, but also expressed alleles with intragenic deletions may exert a dominant phenotypic effect. Let us assume that a dimer AB of proteins A and B has to be formed for proper functioning. Consider that AB formation is compromised by the expression of mutant alleles a and b, which is a case of negative transdominance. If 50% of AB formation is still compatible with a normal phenotype, individuals with a heterozygous intragenic deletion of a single gene will be healthy. Individuals, in whom alleles with intragenic deletions for both genes are heterozygously expressed will have only one-fourth of the normal AB-dimer level. Thus, these polypeptides with partial deletions will disrupt the activity of the wild-type full-length polypeptides. This will inevitably produce a phenotypic impact. To identify individuals in whom this may apply for the case of *CNTNAP2*, as “gene A,” we first have to pinpoint the “partners” which bind to the *CNTNAP2*-encoded Caspr2 protein, i.e., the “genes B.”

Identification of *CNTNAP2* Partners by Association and by Biochemical Studies

If 2 genes have to interact to assure normal functioning, their “normal” alleles should occur in the healthy population more frequently than the risk alleles that associate with a given disorder. Conversely, in association studies, the individual risk alleles occur more frequently in the patient population. In cases of gene interaction, combinations of risk alleles will occur more frequently among patients than would be expected under random segregation. Thus far, in only one association study, a search for genes interacting with *CNTNAP2* has been performed [Poot, 2014]. In a cohort of ASD patients with additional comorbidities, a combination of risk alleles of SNPs in *CNTN6* (rs9878022) and in *CNTNAP2* (rs7804520) was found more frequently than would be expected under random segregation. This association is consistent with a polygenic disease model, but does not necessarily indicate a molecular mechanism. Indeed, an association of a locus may also involve mechanisms not directly related to the protein-coding part of a nearby gene, but for instance an eQTL [Nicolae et al., 2010]. Nevertheless, in a survey of 2 large cohorts of patients with ASD, a single patient was found to carry mutations in both *CNTNAP2* and *CNTN6*, which he inherited from his healthy mother [Mercati et al., 2016]. Furthermore, the association of *CNTNAP2* with *CNTN6* was not confirmed in terms of a physical interaction of Caspr2 with CNTN6 [Rubio-Marrero et al., 2016]. These results indicate that genetic marker-based associations need independent replication and “material,” i.e., experimental confirmation.

In myelinated axons, Caspr2 binds to TAG-1 (CNTN2) and thus aids in the formation of axon-glia contacts at the juxtaparanodes, which enables clustering of Kv1.1 channels, via the cytoskeletal adapter protein 4.1B and accumulation of scaffolding proteins DLG4 and DLG1 [Horresh et al., 2008, 2010]. In hippocampal lysates from adult Balb/c57/J mice at postnatal days P56–P70, Caspr2 localizes abundantly in the lipid raft fraction, in the synaptosome, and the synaptic membrane but is depleted from the postsynaptic density [Chen et al., 2015]. In the latter fraction, glutamate receptor proteins, such as GRIN2B and GRIA2, localize. The expression level of Caspr2 is constant from birth until adulthood. By immunoprecipitation experiments of subcellular fractions, Caspr2 was found together with TAG-1 (CNTN2), ADAM22, LGI1, subunits of the Kv1.1 channel (*KCNA1*) and the scaffolding protein DLG4 [Chen et al., 2015]. In-

Fig. 4. Proteins binding to Caspr2. The black bar on the left-hand side represents the extracellular domain to which CNTN1, CNTN2, DLG1, and DLG4 bind. The black bar on the right-hand side represents the intracellular domain to which ADAM22, LGI1, and KCNA1 bind. For abbreviations, see Figure 2.

Interestingly, in *CNTNAP2*-null mice a short isoform 2, consisting of the C-terminal region of Caspr2, was expressed and found to bind to ADAM22 and LGI1, and to a lesser extent to the Kv1.1 channel (*KCNA1*) [Chen et al., 2015]. Furthermore, Caspr2 forms a complex with the G-protein-coupled receptor 37 (GPR37) with its intracellular PDZ domain [Tanabe et al., 2015]. This binding is abrogated by the R558Q mutation of the GPR37 protein, which has been found in patients with ASD [Tanabe et al., 2015]. The biochemically confirmed binding partners of the intracellular and the extracellular part of Caspr2 are depicted in Figure 4.

The interaction studies of Caspr2 with its ligands CNTN1 and TAG-1 (CNTN2) have been controversial. Early in vivo immunoprecipitation studies and in vitro cell-based assays showed that the interaction of Caspr2 with TAG-1 (CNTN2) appears to be required for the clustering of potassium channels at the juxtaparanodes [Poliak et al., 1999, 2003; Traka et al., 2002, 2003]. According to these studies, this “trans” interaction requires the simultaneous presence of both Caspr2 and TAG-1 on the same membrane. Recent biophysical and biochemical experiments, using purified recombinant proteins, have shown that Caspr2 interacts with the micromolar affinity with CNTN1 [Rubio-Marrero et al., 2016]. In this study, the 3 central domains of CNTN1 bind to the 6 N-terminal domains of Caspr2 (the F5/8 discoidin/neuropilin homology domain, 2 laminin G-binding domains, 1 EGF-like domain, a fibrinogen-like region, and a third laminin G-binding domain; amino acid residues 47–945; exons 2–17) [Rubio-Marrero et al., 2016]. This study did not confirm the interaction of CASPR2 with TAG-1. Another study demonstrated subsequently that purified recombinant Caspr2 binds directly to CNTN2 with low nanomolar affinity but without the need for a *cis* interaction with TAG-1. This study also did not confirm an interaction with CNTN1 [Lu et al., 2016]. Further studies are

needed to resolve the apparent discrepancies between the earlier immunoprecipitation studies and in vitro cell-based assays and those using recombinant Caspr2. Nevertheless, the *CNTNAP2* encoded protein Caspr2 may serve as a “bridge” between specific intracellular and extracellular proteins and may bind to different “partners” at different anatomical locations. Conceivably, perturbation of different binding patterns may elicit distinct clinical phenotypes.

The Phenotypic Scope of *CNTNAP2* Deletion Disorders

While intragenic deletions are consistent with involvement of *CNTNAP2* in a variety of neurodevelopmental disorders (Fig. 3), association studies may help to narrow down and to better circumscribe the phenotypic scope of *CNTNAP2* function. For instance, *CNTNAP2* polymorphisms may affect neural development as related to vocal communication and language processing [Koeda et al., 2015]. The genotype of SNP rs7794745 (A/A or A/T) in intron 2 significantly affects voice-specific brain function in the right middle frontal gyrus and in the bilateral superior temporal gyrus in healthy individuals [Koeda et al., 2015]. The SNP rs2710102 in intron 13 of *CNTNAP2* associates with impaired language development, specific language impairment, delayed oral and written language skills, dyslexia, age of first words as well as speech and sound disorder (Fig. 5) [Alarcón et al., 2008; Newbury et al., 2011; Peter et al., 2011; Whitehouse et al., 2011; Poot, 2014; Zhao et al., 2015]. Initially the same SNP showed an association with ASD, but not in subsequent studies of patient cohorts with “pure” ASD, ASD with normal to high IQ, formerly known as Asperger syndrome, or ASD with accompanying comorbidities [Anney et al., 2012; Sampath et al., 2013; Toma et al.,

Fig. 5. Associating SNPs (green), DNA methylation site (orange) [Schneider et al., 2012], and transcription binding sites (blue) are shown. The downward arrow indicates inhibition, and the upward arrows indicate stimulation of transcription by binding of the transcription factor.

2013; Poot, 2014, 2015; Werling et al., 2016]. This phenomenon occurs frequently and emphasizes the need for replication studies with at least one independent patient cohort [Curran et al., 2011]. In agreement with the lack of association of *CNTNAP2* with ASD, no elevated burden of SNVs or ASD-specific SNVs were found by resequencing all exons of *CNTNAP2* in a large cohort of sporadic ASD patients from families without any neurodevelopmental disorders [Murdoch et al., 2015]. A patient with language impairment and ASD and a complex chromosomal rearrangement disrupting *CNTNAP2* carries the risk allele for rs2710102, as do both his healthy parents [Poot et al., 2010]. Hence, it is tempting to attribute the presence of the risk allele for rs2710102 to the language-related problems of this patient and not to his ASD per se. These association studies also raise the question whether the risk allele for rs2710102 indicates language-related problems as an endophenotype of ASD.

After genotyping a large sample of families with a single child with ASD according to ADI-R, ADOS-G, and DSM-IV criteria, 7 variants in the 5' core promoter region 600 bp upstream from the transcription start site of *CNTNAP2* were identified [Chiocchetti et al., 2015]. Nominal association with ASD was found for rs34712024[G] and with language development for rs71781329GCG[7]. The 7 variants were located in tran-

scription factor binding sites, and by luciferase assays, the respective minor alleles showed effects on *CNTNAP2* promoter activation. The authors hypothesized that reduced *CNTNAP2* transcription during neuronal development increases liability for ASD [Chiocchetti et al., 2015].

In the Chinese Han population, *CNTNAP2* associates with schizophrenia and major depression [Ji et al., 2013]. In 2 patients with schizophrenia and seizures from 2 independent families with multiple psychiatric disorders, heterozygous deletions of part of *CNTNAP2* were transmitted from healthy parents (Fig. 3) [Friedman et al., 2008]. Third, in a single case, an inherited intragenic deletion of exons 14 and 15 was preferentially transcribed in the iPSC cells derived from the patient, but not in those of the healthy father [Lee et al., 2015]. These findings stress the importance of transmitted alleles that may predispose to schizophrenia and other neurodevelopmental disorders. For the families reported by Friedman et al. [2008] and by Lee et al. [2015], several explanations can be envisaged for the fact that the transmitting parent is healthy, but the proband affected. First, the heterozygous parent may have other protective alleles not transmitted to the proband. Second, affected probands may carry additional deleterious alleles, which were either inherited from the other healthy parent or arose de novo. Intragenic *CNTNAP2* deletions may have incomplete pene-

trance and variable expressivity, due to the expression of variable levels of the deleted *CNTNAP2* allele.

CNTNAP2 and the neural cell-adhesion molecules contactin 4, 5, and 6 show comparable functions during brain development [Mercati et al., 2013]. Disruptions in these contactin genes, such as those in *CNTNAP2*, confer an increased risk for ASD [Alarcón et al., 2008; Zweier et al., 2009; Poot et al., 2010; van Daalen et al., 2011; Nava et al., 2014]. Commensurate with its negative association with ASD, mice with a homozygous loss of *Cntn4* did not show any significant ASD-related behavioral disturbances [Poot, 2014; Molenhuis et al., 2016]. In contrast, patients with CNVs of or SNVs in the coding sequence of *CNTN5* and *CNTN6* show auditory phenotypes, such as hyperacusis [Mercati et al., 2016]. In 14 out of 3,724 patients with neurodevelopmental disorders, such as developmental delay, ASD, seizures, and ADHD, CNVs of *CNTN6* were found [Hu et al., 2015]. This widens the phenotypic spectrum of *CNTN6* CNVs considerably.

A girl with moderate ID, autistic features, epileptiform activity, language delay and psychomotor problems carried an inherited duplication within intron 1 of *CNTNAP2*, encompassing the *FOXP2* binding sites (Fig. 5) [Polimanti et al., 2016]. In 3 out of 5 patients with a deletion of intron 1 of *CNTNAP2*, covering the *FOXP2* binding sites, language delay was found, while 4 were autistic (Fig. 2). These findings underscore the importance of a balance between transcription factor binding sites and transcription factor gene dosage [Vernes et al., 2008]. However, the phenotypic consequences of deletions or duplications of either are not straightforward.

Phenotypes of *Cntnap2*-Null Mice

Cntnap2-null C57BL/6J mice showed behavioral deficits such as hyperactivity and epileptic seizures, akin to the phenotypes of patients with CDFE syndrome, as well as in the 3 core ASD behavioral domains, similar to those reported in humans with full *CNTNAP2* gene deletions [Peñagarikano et al., 2011]. Neuropathological and physiological analyses of these mice before the onset of seizures revealed abnormal neuronal migration, lowered numbers of interneurons, and abnormal neuronal network activity. However, not all of the phenotypes found in the human patients, such as mislocalization of potassium channels, were observed in these *Cntnap2*-null mice. This may be due to the different anatomical localization of *Caspr2* in human and rodent brains (see above). *Cntnap2*-null mice make fewer social approaches and engage in less vocaliza-

tion and nesting. In contrast, perseveration, grooming, and digging (used to indicate repetitive behaviors) are enhanced, as are overall levels of activity. Treatment with risperidone, an atypical antipsychotic drug licensed for the treatment of autism, increases nesting and decreases grooming, perseveration, and hyperactivity. However, risperidone has no effect on social approach or vocalization [Lord, 2011; Peñagarikano et al., 2011; Peñagarikano and Geschwind, 2012]. In contrast, exogenous and evoked oxytocin restores social behavior in the *Cntnap2*-null mice [Peñagarikano et al., 2015].

In *Cntnap2*-null mice, excitatory transmission was close to normal, but inhibition onto CA1 pyramidal cells was altered [Jurgensen and Castillo, 2015]. Specifically, putative perisomatic-, but not dendritic-, evoked inhibitory postsynaptic potentials were significantly reduced. Whereas both inhibitory short-term plasticity and miniature inhibitory postsynaptic potential frequency and amplitude were normal in *Cntnap*-null mice, an unexpected increase in the frequency of spontaneous, action potential-driven was found. Such altered neuronal inhibition may account for some of the behavioral phenotypes of *Cntnap2*-null mice later in life. The authors conclude that *Cntnap2* deletion selectively impairs perisomatic hippocampal inhibition, while sparing excitation provides support for synaptic dysfunction as a common mechanism underlying ASD and other neurodevelopmental phenotypes [Jurgensen and Castillo, 2015].

Studies of learning behavior and auditory processing in *Cntnap2*-null mice have generated novel insights relevant to ASD and other neurodevelopmental disorders [Kloth et al., 2015; Truong et al., 2015; Rendall et al., 2016]. These transgenic mice showed reduced silent gap detection but superior pitch-related discrimination as compared to wild-type controls. By stereological analysis, a reduced number and density of neurons, as well as a shift toward smaller neurons in the medial geniculate nucleus was found in *Cntnap2*-null mice [Truong et al., 2015]. These findings indicate that *CNTNAP2* is involved in the ontogeny and function of neural systems participating in auditory processing. Conversely, disruption of these neural systems during development may contribute to the atypical language phenotype seen in some patients with ASD [Boddaert et al., 2003; Ceponiene et al., 2003; Poot et al., 2010; Truong et al., 2015; Mercati et al., 2016].

Cntnap2-null mice showed normal sensory responsiveness, gross motor function, and noncerebellar learning and memory functioning, but conditional responses and cerebellum-based learning were significantly reduced [Kloth et al., 2015]. In addition, they showed sig-

nificant deficits in working and reference memory during acquisition of a task, while *Cntnap2*-null mice performed comparably to wild-type mice during the retention period [Rendall et al., 2016]. Taken together these studies suggest that *CNTNAP2* may be involved in the development of neural systems that are important for learning and crossmodal integration. Conversely, disruption of these functions may be associated with delayed learning as observed in ASD patients [Poot et al., 2010; Rendall et al., 2016].

Concluding Remarks

In the human fetal brain, *CNTNAP2* is expressed in the anterior frontal and temporal perisylvian regions, in the Broca area, the striatum, amygdala, caudate nucleus, putamen, and thalamus [Abrahams et al., 2007; Alarcón et al., 2008]. *CNTNAP2* expression in perisylvian language-related structures and circuits is consistent with the involvement in language development and higher cognitive functions. Studies of cellular functions indicate that *CNTNAP2* is probably required for the maturation and maintenance of spiny synapses, but not for earlier stages of postnatal development of pyramidal neurons. This is consistent with the delayed appearance of symptoms in some clinical cases with intragenic deletions or *CNTNAP2* truncating mutations. Furthermore, SNP association data and intragenic deletions covering transcription factor binding sites link *CNTNAP2* with disor-

ders of language acquisition and development. Also the scope of *CNTNAP2* in humans and in mice is consistent with pivotal functions of *CNTNAP2* in auditory processing and task-learning skills. By perturbation of complex mechanisms of penetrance, expressivity and effects on interactions with other alleles and loci *CNTNAP2* may be related to ASD, epilepsy, schizophrenia, and ID. *CNTNAP2* alleles with expressed intragenic deletions may interfere with the ability to bridge the intercellular space between neurons by binding to specific partners, such as *CNTN1*, *CNTN2*, *DLG1*, and *DLG4*. This postulated effect of intracellular deletions of *CNTNAP2*, and possibly other genes encoding proteins involved in connecting neuronal cells, represents a molecular basis for the presumed neuronal hypoconnectivity in ASD and possibly other neurodevelopmental disorders.

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