

Connecting the *CNTNAP2* Networks with Neurodevelopmental Disorders

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Key Words

Autism · *CNTNAP2* · CNV · DNA methylation · Epilepsy · Intellectual delay · Multigenic networks · Neurodevelopmental disorders · Schizophrenia · SNV · Specific language impairment

Abstract

Based on genomic rearrangements and copy number variations, the contactin-associated protein-like 2 gene (*CNTNAP2*) has been implicated in neurodevelopmental disorders such as Gilles de la Tourette syndrome, intellectual disability, obsessive compulsive disorder, cortical dysplasia-focal epilepsy syndrome, autism, schizophrenia, Pitt-Hopkins syndrome, and attention deficit hyperactivity disorder. To explain the phenotypic pleiotropy of *CNTNAP2* alterations, several hypotheses have been put forward. Those include gene disruption, loss of a gene copy by a heterozygous deletion, altered regulation of gene expression due to loss of transcription factor binding and DNA methylation sites, and mutations in the amino acid sequence of the encoded protein which may provoke altered interactions of the *CNTNAP2*-encoded protein, Caspr2, with other proteins. Also exome sequencing, which covers <0.2% of the *CNTNAP2* genomic DNA, has revealed numerous single nucleotide variants in healthy individuals and in patients with neurodevelopmental

tal disorders. In some of these disorders, disruption of *CNTNAP2* may be interpreted as a susceptibility factor rather than a directly causative mutation. In addition to being associated with impaired development of language, *CNTNAP2* may turn out to be a central node in the molecular networks controlling neurodevelopment. This review discusses the impact of *CNTNAP2* mutations on its functioning at multiple levels of the combinatorial genetic networks that govern brain development. In addition, recommendations for genomic testing in the context of clinical genetic management of patients with neurodevelopmental disorders and their families are put forward.

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The *CNTNAP2* gene, being located in chromosomal region 7q35, spans 2.3 Mb of genomic DNA with a full-length open reading frame of 3,996 nucleotides distributed among 24 exons and encoding a protein of 1,331 amino acids. In addition, 2 alternative transcripts (*CNTNAP2*-201 and *CNTNAP2*-002) encode proteins of 390 and 185 amino acid residues, respectively (Ensembl release 75, February 2014). Furthermore, *CNTNAP2* encodes 2 non-coding transcripts (*CNTNAP2*-003, 6,065 bp, and *CNTNAP2*-004, 1,749 bp). Thus, 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), altered transcription factor binding, mutations in exons, and epigenetic modifications. In addition, the genomic DNA of *CNTNAP2* overlaps with 1 validated and several inferred microRNAs (miRNAs).

Surprisingly, no recessive or dominant Mendelian syndrome linked to *CNTNAP2* has yet been listed in Online Mendelian Inheritance in Man (OMIM). Thus far, in 1 family with cortical dysplasia and focal epilepsy (CDFE), mild gross motor delay, leading to regression of learning ability, language and social behaviors, and signs of attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD), a co-segregating *CNTNAP2* mutation (3709delG) has been described [Strauss et al., 2006]. In addition, heterozygous *CNTNAP2* disruptions have been found in individuals with intellectual disability (ID), seizures, signs of ASD, e.g. repetitive behaviors, and language problems, including dysarthric language, delayed or absent speech or language acquisition [Rodenäs-Cuadrado et al., 2014]. In recent reviews of human and mouse studies, *CNTNAP2* has also been related to neurodevelopmental disorders such as autism [Poot et al., 2011; Peñagarikano and Geschwind, 2012; Poot, 2013; Kas et al., 2014]. Although *CNTNAP2* is clearly associated with impaired development of language, this gene may also be a node in one or several molecular networks governing neurodevelopment [Cristino et al., 2014]. Here, *CNTNAP2* is discussed as part of combinatorial molecular networks governing gene expression and protein-protein interactions, and how this paradigm affects clinical management of patients with *CNTNAP2* mutations.

***CNTNAP2* Expression and Biological Functions**

CNTNAP2 encodes 5 transcripts, 3 of which are translated into proteins of 1,331 (transcript *CNTNAP2*-001), 390 (transcript *CNTNAP2*-201, 1,944 bp, 9 exons) and 185 amino acid residues (transcript *CNTNAP2*-002, 556 bp, 4 exons), respectively (Ensembl release 75, February 2014) (fig. 1). In addition, *CNTNAP2* encodes 2 transcripts of 4 non-coding exons (*CNTNAP2*-003, 6,065 bp, and *CNTNAP2*-004, 1,749 bp; Ensembl release 75, February 2014). *CNTNAP2*-201 is 1.6-fold upregulated in human cortex, compared to the chimpanzee [Schneider et al., 2014]. Transcripts *CNTNAP2*-001, -002 and -003 did not show skewed allelic expression, which argues against *CNTNAP2* imprinting in the adult human brain [Schneider et al., 2014].

The full-length transcript *CNTNAP2*-001 encodes a protein composed of 1,331 amino acid residues, Caspr2. This is a single-pass transmembrane protein composed of 8 extracellular, 1 transmembrane and 2 C-terminal intracellular domains (InterPro, <http://www.ebi.ac.uk/interpro/protein/Q9UHC6>; April 2014). The extracellular domains are 4 laminin G domains, 2 EGF-like domains, 1 discoidin homology domain, and 1 fibrinogen-like domain. These extracellular domains mediate cell-cell interactions and binding with receptors, ligands and with the extracellular matrix. Intracellularly, Caspr2 has 2 domains: 1 protein 4.1 binding domain and 1 PDZ interaction domain, which may be involved in protein-protein interactions (fig. 1).

Expression, Evolutionary Conservation and Functions of CNTNAP2

The expression pattern of *CNTNAP2* can provide clues regarding its biological function in relation to neurodevelopmental disorders of patients with mutated *CNTNAP2*. In situ hybridization studies showed frontal and anterior localization, in Broca's area and other perisylvian brain regions, while in the subcortical structures, *CNTNAP2* was expressed at high levels in the dorsal thalamus, the caudate, the putamen and the amygdala [Abrahams et al., 2007]. There were marked differences in cerebral cortical expression between humans and rodents, however. In contrast to the enrichment observed in human frontal cortex, mice showed only limited expression in the cortical plate, with highest levels of expression posteriorly, during the corresponding developmental period (embryonic day 17). Highest levels of *CNTNAP2* expression were in the olfactory bulb, ventricular zones, striatum, thalamus, and hippocampus [Abrahams et al., 2007; Peñagarikano et al., 2011]. Expression in the developing rat brain was remarkably similar to that observed in mice. 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 non-human primates. These results indicate restricted *CNTNAP2* expression in perisylvian language-related structures and circuits, which is consistent with the involvement of *CNTNAP2* in language development as suggested by the genetic results described below.

To find possible mechanisms underlying the distinct patterns of *CNTNAP2* expression, Abrahams et al. [2007] compared *CNTNAP2* loci in the human, mouse and rat

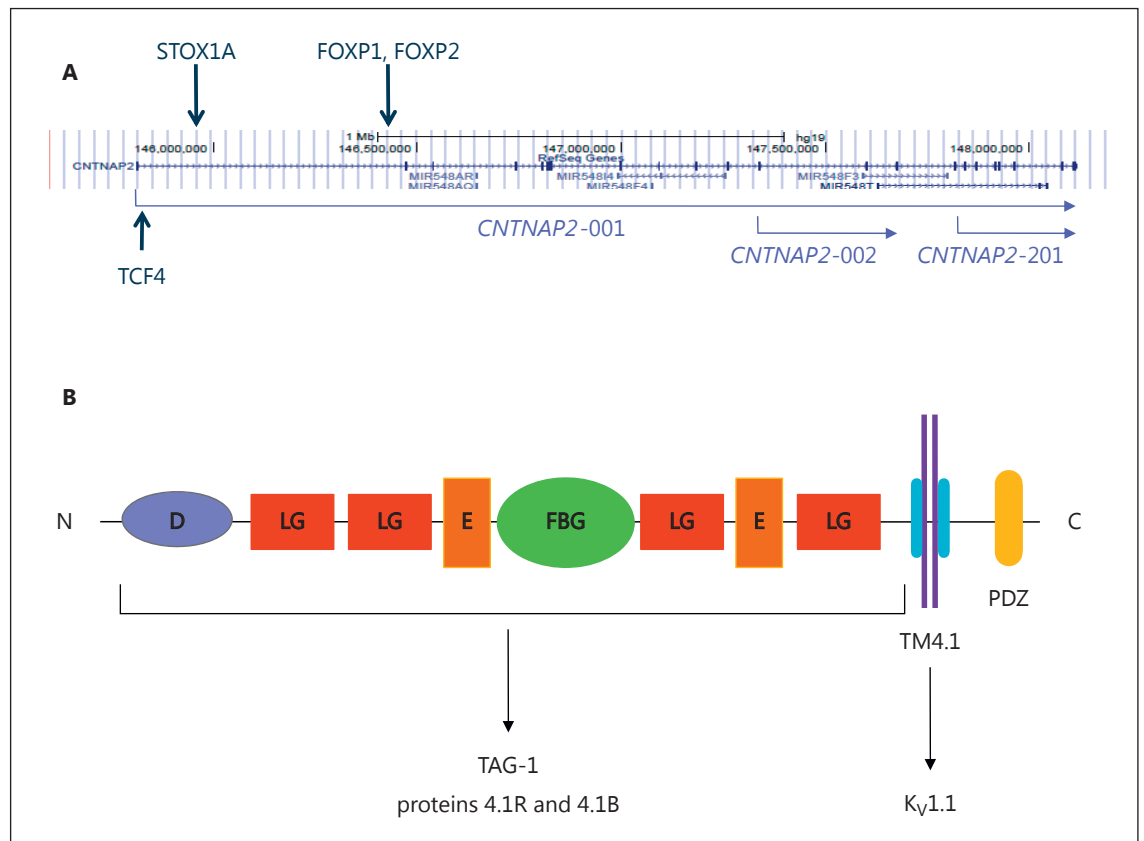


Fig. 1. Transcriptional regulation network of *CNTNAP2* (**A**) and the Caspr2 protein with its interaction partners (**B**). In **A**, dark blue arrows indicate transcription factor binding sites; downward arrow indicates transcription-inhibiting and upward arrows transcription-enhancing activity. Light blue arrows indicate the 3 *CNTNAP2* transcripts. In **B**, D = discoidin homology domain;

LG = laminin G binding domain; E = EGF-like domain; FBG = fibrinogen-like region; TM4.1 = transmembrane and protein 4.1 binding domain; PDZ = PDZ interaction domain; TAG-1 = *CNTN2*-encoded TAG-1 protein; Kv1.1 = hippocampal potassium channel Kv. Purple vertical bars indicate the position of the cell membrane.

genomes for possible structural differences. Gene structure was conserved across the 3 species with each ortholog having 24 coding exons distributed across roughly 2 Mb of genomic DNA. Similarly, 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 between these 3 species. These findings suggest that, in addition to 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 some of its biological function(s).

CNTNAP2 is also highly expressed in the spinal cord, where the Caspr2 protein is involved in the clustering of potassium channels to juxtaparanodes on axons [Poliak et al., 1999, 2003; Traka et al., 2003]. Since in humans myelination is not apparent until 28 weeks of gestation, these data suggest that *CNTNAP2* may have an additional, as yet not appreciated, function in the development of myelinated neurons [Panaitof et al., 2010].

Caspr2 also has an organizing function in developing neurons, which is essential for the assembly of neural circuits [Anderson et al., 2012]. RNAi-mediated knock-down of Caspr2 protein levels produced a cell-autonomous decrease in dendritic arborization and spine development in pyramidal neurons. This led to a global decline

in excitatory and inhibitory synapse numbers and a decrease in synaptic transmission [Anderson et al., 2012]. This function of Caspr2 in neural development is comparable to the involvement of the neural cell-adhesion molecules contactin 4, contactin 5 and contactin 6 in brain development [Mercati et al., 2013]. Disruptions in these contactin genes confer an increased risk for ASD [van Daalen et al., 2011; Nava et al., 2014]. Likewise, loss of Caspr2 function may contribute to the ASD found in several patients with CNVs in *CNTNAP2* [Zweier et al., 2007; Alarcon et al., 2008; Poot et al., 2010].

Protein-Protein Interactions Involving Caspr2

Apart from evolutionary conservation, the specific localization and interactions of Caspr2 with other proteins may provide clues regarding its biological function(s). In myelinated axons, Kv1 (Shaker) voltage-gated potassium (Kv1.1) channels are clustered in the juxtaparanodal regions flanking the node of Ranvier. Precise localization of axonal ion channels is crucial for proper electrical and chemical functions of axons. This localization can be disrupted by deletion of proteins, such as Caspr2 and transient axonal glycoprotein-1 (TAG-1), a glycosylphosphatidylinositol-anchored cell adhesion molecule, encoded by the contactin 2 (*CNTN2*) gene [Gu and Gu, 2011]. Caspr2 was shown to bind the axonal proteins 4.1R and 4.1B in peripheral myelinated axons, where it is likely to anchor these proteins to the actin-based cytoskeleton [Denisenko-Nehrbass et al., 2003]. Transgenic mice lacking the 4.1-binding sequence of Caspr2 failed to promote Kv1 channel clustering at the juxtaparanodal axonal membrane, while the carboxy-terminal PDZ-binding motif of Caspr2 is dispensable for Kv1 channel clustering [Horresh et al., 2008, 2010]. These observations appear to support the hypothesis of Verkerk et al. [2003] that disruption or decreased expression of *CNTNAP2* may lead to a disturbed distribution of the K(+) channels in the nervous system, thereby influencing conduction and/or repolarization of action potentials, eventually causing unwanted actions or movements seen in patients with Gilles de la Tourette syndrome (GTS).

The *CNTN2*-encoded TAG-1 protein is enriched in the juxtaparanodal regions of the central nervous system and in myelinated fibers [Traka et al., 2002]. Being expressed in both neurons and glia cells, TAG-1 associates in *cis* with Caspr2 and in *trans* with itself. Thus, a tripartite intercellular protein complex, comprised of these TAG-1 and Caspr2, appears critical for axo-glial contacts at juxtaparanodes. This kind of complex formation is

analogous to that described previously at paranodes, suggesting that similar molecules are crucial for different types of axo-glial interactions [Traka et al., 2003]. While the immunoglobulin domains of TAG-1 are necessary and sufficient for the direct, *cis* interaction of this protein with Caspr2 and potassium channels, *CNTNAP2* has not yet been associated with any demyelinating disorder, such as multiple sclerosis [Tzimourakas et al., 2007]. On the other hand, the recent indication of a genetic association of *CNTNAP2* and *CNTN6* in ASD has not yet been corroborated by studies of possible physical interactions between the Caspr2 and the contactin-6-encoded protein [Poot, 2014].

Disruptions Implicate *CNTNAP2* in Several Neurodevelopmental Disorders

CNTNAP2 became for the first time linked to a neurodevelopmental disorder after classical karyotyping of a family with GTS had shown that the affected father and both his affected son and daughter shared an insertion into chromosomal region 7q35q36, which interrupted intron 8 of *CNTNAP2* (fig. 2, red arrow) [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, a translocation disrupting intron 13 of *CNTNAP2* has been found in a healthy individual (fig. 2, red arrow) [Belloso et al., 2007]. Third, Bakkaloglu et al. [2008] detected an inversion 7q21q35 in a patient with autism which disrupted both *AUST2* and *CNTNAP2* (fig. 2, red arrow). Fourth, in a boy with language delay and autism, a complex insertion-translocation of intron 1–3 of *CNTNAP2* into chromosomal region 1q31.1 and an additional deletion in region 1q41 was found (fig. 2; red bar) [Poot et al., 2010]. Taken together, these data suggest that proximal disruptions, up to intron 8, may cause a neurodevelopmental disorder, while distal disruptions may remain inconsequential.

By CNV mapping, several investigators found de novo heterozygous losses of the proximal part of *CNTNAP2* in patients with autism [Alarcon et al., 2008], Pitt-Hopkins syndrome [Zweier et al., 2007], ADHD [Elia et al., 2010], stuttering [Petrin et al., 2010], and ID [Mikhail et al., 2011] (fig. 2, red bars). In a patient with epilepsy, a loss of both alleles of a stretch of DNA spanning introns 2 and 3 of *CNTNAP2*, due to inheritance of heterozygous losses from both healthy parents, has been described (fig. 2, blue

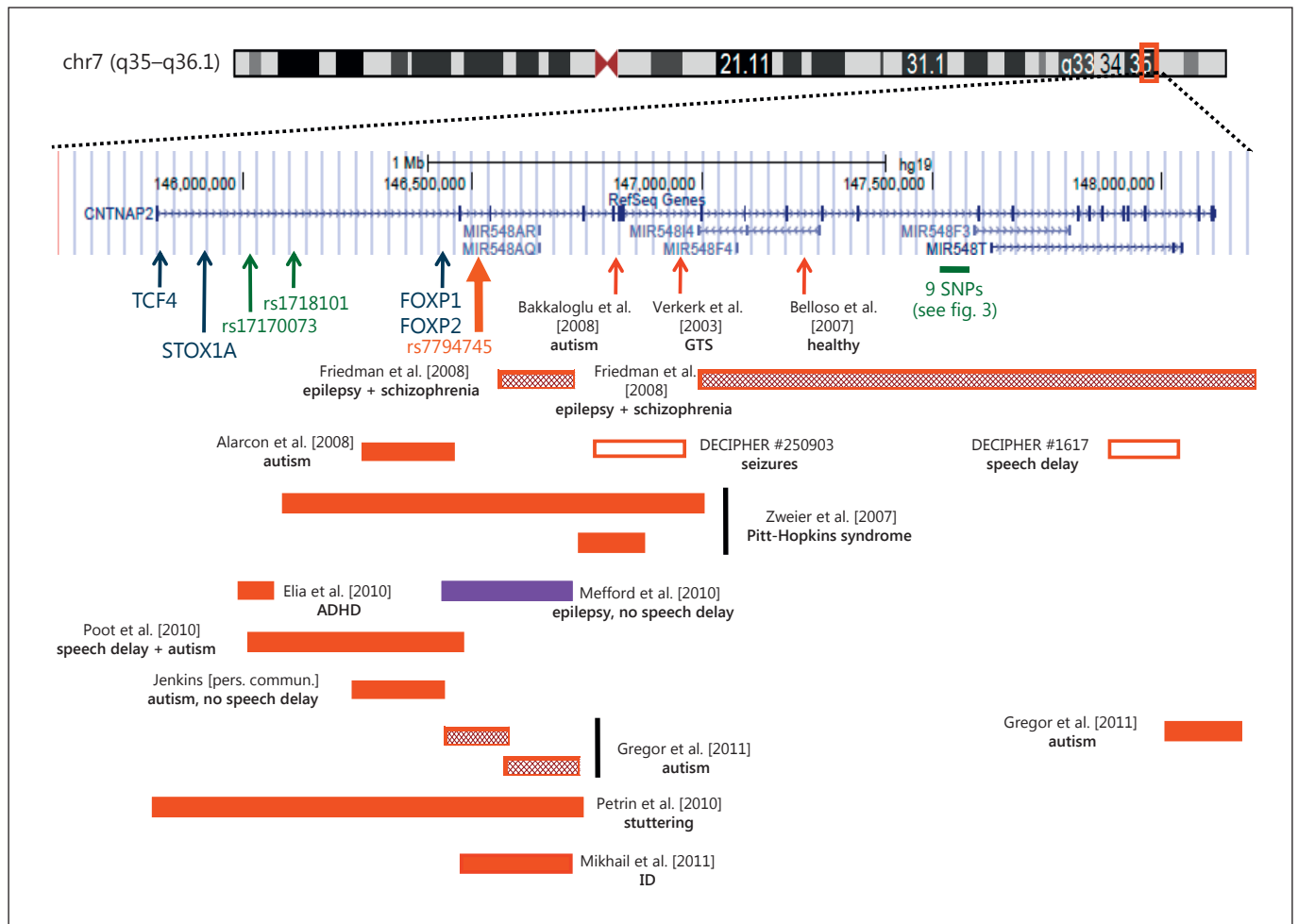


Fig. 2. The *CNTNAP2* gene with structural rearrangements, transcription factor binding sites, DNA methylation site, and associating SNPs. Dark blue arrows indicate transcription factor binding sites, red arrows translocation and inversion breakpoints, filled red bars de novo losses with at least 1 breakpoint located inside

CNTNAP2, cross-hatched red bars losses inherited from a healthy parent, open red bars losses with unknown inheritance, green arrows and a green bar associating SNPs, and the orange arrow the DNA methylation site [Schneider et al., 2014].

bar) [Mefford et al., 2010]. In addition, in 2 patients with schizophrenia from 2 independent families with multiple psychiatric disorders, transmitted heterozygous losses of part of *CNTNAP2* have been found (fig. 2, cross-hatched red bars) [Friedman et al., 2008]. In a cohort of 1,461 ASD patients, 2 large losses, which, among other genes, also included *CNTNAP2*, were found [Rosenfeld et al., 2010]. Also Gregor et al. [2011] reported an ASD patient with a de novo loss of 677 kb, covering exons 21–24 of *CNTNAP2* and the *CUL1* and *EZH2* genes. These findings link disruptions of *CNTNAP2* to several distinct neurodevelopmental disorders. It should be noted, however, that DECIPHER lists disruptions and heterozygous losses

of *CNTNAP2*, which have been inherited from healthy parents (fig. 2, red-framed bars) [Bragin et al., 2014]. While the Database of Genomic Variants [MacDonald et al., 2013] lists a number of phenotypically neutral CNVs within *CNTNAP2*, the burden of CNVs in *CNTNAP2* is not elevated in individuals with ID and autism [Cooper et al., 2011; Sanders et al., 2011]. Also, 2 ASD patients had small proximal losses in *CNTNAP2*, which they inherited from their healthy mothers [Gregor et al., 2011]. Taken together, this indicates that heterozygous disruptions of *CNTNAP2* may by themselves not be sufficient to elicit cellular or organismal phenotypes.

Molecular Effects of *CNTNAP2* Disruptions

Gene disruption is often assumed to engender altered gene expression and thus pathogenic effects by causing haploinsufficiency [Poot et al., 2011]. This mechanism has been presumed after a minimal region of phenotypic and genotypic overlap had been identified by using the results from breakpoint analyses and CNV mapping, e.g. in patients with losses in region 1q44 [Boland et al., 2007; van Bon et al., 2008; Caliebe et al., 2010]. By taking a converse approach, an individual gene or part of a gene can be linked to specific phenotypes in a series of patients with different, but overlapping losses and diverging combinations of phenotypes [Poot and Kas, 2013]. This approach has allowed to successively pinpoint *HNRNPU* and *HNRPU-AS1*, also known as *C1orf199* or *NCRN00201*, as a candidate gene for seizures within region 1q44 [Ballif et al., 2012; Thierry et al., 2012].

Gene Haploinsufficiency

Applying these approaches to *CNTNAP2*, several minimal regions could be considered (fig. 2). One minimal region consists of roughly the final third of intron 1 and exon 2 in patients with autism [Zweier et al., 2007; Alarcon et al., 2008; Poot et al., 2010; Jenkins, pers. commun.]. A likely explanation for the observed autism would be haploinsufficiency for this part of *CNTNAP2* [Poot et al., 2011]. This is not necessarily the only mechanism for the observed autism in these patients, since a patient described by Petrin et al. [2010], exhibiting stuttering, was not autistic. A second region, ranging from exon 2 to the 3' terminus of the gene, may contain 2 sites that predispose to risk for seizures [Zweier et al., 2007; Friedman et al., 2008; Mefford et al., 2010]. Again, the patient described by Petrin et al. [2010] and the one with ID described by Mikhail et al. [2011] do not fit into the picture. If the patient described as DECIPHER #250903 did inherit the loss from a probably healthy parent, this reported patient would further weaken the case for this region. Finally, speech delay can be linked to the terminal third of intron 1 and to a locus at the end of *CNTNAP2* (DECIPHER patient #1617). Again, haploinsufficiency for either of these regions does not fully explain the observed speech delay. Taken together, 3 regions within *CNTNAP2* can, to some extent, be linked to phenotypes such as autism, seizures and speech delay, but haploinsufficiency is not sufficient as an explanation, and alternative hypotheses have to be considered.

Gene Truncation and Loss of Alternative Transcripts

Conceivably, gene disruption due to translocations, inversions or CNVs may lead to the expression of truncated proteins that may exert dominant-negative actions. Such a truncating mutation has been found in the C-terminal part of *CNTNAP2* in a family with children displaying CDFE, mild gross motor delay and frequent seizures, leading to regression of learning abilities and social behavior, such as signs of ADHD and autism [Strauss et al., 2006]. Such mutated alleles lead to the expression of a mutant protein containing only extracellular domains, which become secreted [Falivelli et al., 2012]. These secreted mutated Caspr2 proteins may interact with other ligands or receptors than the cognate *CNTNAP2* partners (see below), which in turn may produce deleterious effects. Since all thus far described disruptions of *CNTNAP2* (fig. 2) are proximal to the mutations investigated by Falivelli et al. [2012], such formation of a secreted protein appears not to be a likely molecular mechanism in most cases.

Intragenic losses may also affect the expression of alternative splicing variants and consequently alter cellular phenotypes [Bertelsen et al., 2014; Wang et al., 2014]. Thus, a de novo heterozygous 677-kb loss in a patient with ASD (figs. 1, 2) may potentially lead to truncation of the alternative transcript *CNTNAP2*-201 [Gregor et al., 2011]. Although this transcript is 1.6-fold upregulated in human cortex compared to the chimpanzee, its biological function is unknown [Schneider et al., 2014].

Loss of Transcription Factor Binding Sites

A third effect of gene disruption may be altered gene expression due to imbalanced regulation by transcription factor binding. Two transcription-inhibiting factors, *STOX1A* and *FOXP2*, and 1 transcription-enhancing factor, *TCF4*, bind to intron 1 of *CNTNAP2* (fig. 1, blue arrows). With the demonstration of *FOXP2* binding sites in intron 1 of *CNTNAP2* [Vernes et al., 2008], a paradigm shift was brought about. *CNTNAP2* became considered as part of a combinatorial gene-expression network. This network is thought to be responsible for precise control of *CNTNAP2* expression both during development and in the adult brain. Disruptions of these networks have been implicated in several disorders of language development [Newbury and Monaco, 2010; Newbury et al., 2010].

STOX1A binds to intron 1 of *CNTNAP2* (fig. 1, blue arrow) and downregulates *CNTNAP2* expression. In the hippocampus of Alzheimer's disease patients, where *STOX1A* expression is upregulated, *CNTNAP2* expres-

sion is downregulated [van Abel et al., 2012]. This is the first finding that implicates *CNTNAP2* in a neurodegenerative disorder.

The intron 1 of *CNTNAP2* also contains binding sites for the transcription-enhancing transcription factor TCF4 (fig. 1, blue arrow) [Forrest et al., 2012]. Heterozygous deletions of *TCF4* cause Pitt-Hopkins syndrome, a disorder of postnatal microcephaly, intellectual and speech delay, epilepsy and hyperventilation [Amiel et al., 2007; Brockschmidt et al., 2007; Zweier et al., 2007]. Also frameshift, nonsense, splice site, and missense mutations have been found in patients with Pitt-Hopkins syndrome [Peippo and Ignatius, 2012]. Mutations in the basic helix-loop-helix (bHLH) domain of TCF4 alter the subnuclear localization of the mutant protein and can attenuate homo- and heterodimer formation, whereas mutations proximal to the bHLH domain do not alter the location of TCF4 or impair heterodimer formation [Forrest et al., 2012]. In addition, TCF4 can transactivate the *NRXN1 β* and *CNTNAP2* promoters. Taken together, missense mutations in distinct domains of TCF4 can have different effects on TCF4 protein function, such that TCF4 may modulate the expression of *NRXN1* and *CNTNAP2* in a regulatory network involved in Pitt-Hopkins syndrome [Forrest et al., 2012]. In the human brain, TCF4 is more highly expressed in the neocortex and hippocampus than in the striatum, thalamus and cerebellum [Whalen et al., 2012]. Thus, lower levels of TCF4 protein or loss of TCF4 binding sites in intron 1 of *CNTNAP2* in these brain regions may mediate neurodevelopmental phenotypes such as intellectual delay, speech development and epilepsy. A patient with stuttering has indeed a heterozygous deletion of the TCF4 binding sites in intron 1 of *CNTNAP2*, which may account for his phenotype [Petrin et al., 2010].

De novo deletions of part or the entire intron 1 of *CNTNAP2* have been found in 2 patients with autism, one with autism and speech delay, one with stuttering, and one with Pitt-Hopkins syndrome [Zweier et al., 2007; Alarcon et al., 2008; Petrin et al., 2010; Poot et al., 2010; Jenkins, pers. commun.]. Since the patients show distinct phenotypes, such deletions by themselves do not indicate a specific disorder. Whether imbalance between TCF4, *STOX1A* and *FOXP2* binding sites, as proposed by Poot et al. [2010], accounts for any of the clinical phenotypes of the patients remains to be seen. Interestingly, this patient carries additional genome rearrangements, which may be considered clinically relevant if one assumes the hypothesis of a polygenic network [Poot et al., 2010].

Imbalanced Regulation of CNTNAP2 Expression Involving miRNAs

A fourth hypothetical mechanism by which gene disruption may affect *CNTNAP2* expression is deletion or duplication of miRNAs. Neurally expressed miRNAs affect neuronal cell differentiation by downregulating non-sense-mediated RNA decay of specific genes that are involved in neurodevelopment [Lou et al., 2014]. A case in point is the demonstration that the schizophrenia candidate MIR137 also binds to the 3' UTR of the retinoic acid-related orphan receptor alpha (*RORA*) gene, which acts as a transcriptional regulator [Devanna and Vernes, 2014]. *RORA* is considered an ASD candidate gene, while MIR137 is linked to schizophrenia. In addition to binding to *RORA*, MIR137 also binds to a large number of other autism candidate genes, thus providing a molecular link between the 2 neurodevelopmental disorders.

Systematic analysis of miRNAs in CNVs in relation to their cognate target genes allowed identifying 10 miRNAs as hub molecules in regulatory networks with transcription factors and their downstream target genes [Vaishnavi et al., 2013]. One of these miRNAs, hsa-miR-548f, which located within *CNTNAP2*, has not yet been linked to any neurodevelopmental disorders, however, among its potential targets are *PTEN*, *SLC1A1*, *GRIK2*, *GABRG1*, *SLC1A3*, *NGF*, and *GABRA4* [Vaishnavi et al., 2013]. By miRNA expression profiling of cell line-derived total RNA, possible transcripts and networks of molecules involved in ASD were evaluated [Ghahramani Seno et al., 2011]. In this way, several dysregulated genes and miRNAs were identified in ASD as compared with controls, which included *HEY1*, *SOX9*, miR-486, and miR-181b. These are all involved in nervous system development and function [Ghahramani Seno et al., 2011]. Therefore, changes in their expression level may affect proper neurodevelopment and consequently entail neurodevelopmental disorders [Sarachana et al., 2010].

Thus far, no *CNTNAP2* expression-regulating miRNA has been identified. However, intron 3 of *CNTNAP2* contains the miRNAs MIR548AQ and MIR548AR, of which 1 copy is deleted in several patients (figs. 1, 2) [Zweier et al., 2007; Friedman et al., 2008; Petrin et al., 2010; Mikhail et al., 2011]. In a patient with epilepsy described by Meford et al. [2010], none of the genomic copies of MIR548AQ and MIR548AR were retained. MIR548F4, MIR548F3, MIR548I4, and MIR548T were all deleted in a second patient with schizophrenia and epilepsy described by Friedman et al. [2008]. miRNAs of the MIR548F-family have not yet been associated with neuronal disorders, yet among their potential targets are

PTEN, *SLC1A1*, *GRIK2*, *GABRG1*, *SLC1A3*, *NGF*, and *GABRA4* [Vaishnavi et al., 2013]. Clearly more research is needed to clarify the possible involvement of miRNAs located within the genomic DNA of *CNTNAP2* in neurodevelopmental disorders.

Altered Regulation of CNTNAP2 Expression by DNA Methylation

A fifth hypothetical mechanism of altered *CNTNAP2* expression may originate from the evolutionarily conserved clustering of genes in chromosomal region 7q31q36 (from 107 to 149 Mb), which are strongly associated with language and communication (e.g. *IMMP2L*, *DOCK4*, *FOXP2*, *CNTNAP2*). Some of these genes are differentially expressed in the cortices of humans and non-human primates (chimpanzee, baboon, and/or marmoset) [Schneider et al., 2012]. In Europeans, but not in the Han Chinese, Japanese, or Yoruba populations, there is strong evidence of selection of *FOXP2* targets, such as *CNTNAP2* and *RBFOX1*, which have been associated with neurodevelopmental disorders involving language dysfunction [Ayub et al., 2013].

Comparing the DNA methylation patterns of the human and chimpanzee *CNTNAP2* orthologs in the cortex, Schneider et al. [2014] identified several differentially methylated regions throughout the gene. In a region 300 bp upstream of human SNP rs7794745 (orange arrow in fig. 2), which has been associated with autism and parent-of-origin effects, 28% DNA methylation in human and 59% in chimpanzee cortex was found. In addition, by quantitative real-time RT-PCR, the authors showed that the protein-coding splice variant *CNTNAP2*-201 is 1.6-fold upregulated in human cortex compared to the chimpanzee. The transcripts *CNTNAP2*-001, -002 and -003 did not show skewed allelic expression, however. This argues against *CNTNAP2* imprinting, at least in the adult human brain. In humans (7 male vs. 3 female samples), none of the transcripts was differentially expressed between sexes. These results contradict possible preferential expression of 1 parental allele of the tested isoforms and genomic imprinting. Taken together, these results suggest widespread changes in the methylation of *CNTNAP2* in the cortex since the human-chimpanzee split, which supports a role for *CNTNAP2* fine-regulation in human-specific language and communication skills. Although this study is fraught with inherent technical limitations, like the number and type of brain samples available, the results suggest that genetic and epigenetic variation in *CNTNAP2* contributes to both interspecies and interindividual differences in brain

development and function. Future study of the DNA methylation status in patients with neurodevelopmental disorders is clearly warranted.

Single Nucleotide Variations, SNP Associations and Their Ramifications

The early discovery of a cosegregating *CNTNAP2* mutation (3709delG) in a family with CDFE, mild gross motor delay, regression of learning ability, language and social behaviors, and signs of ADHD and ASD [Strauss et al., 2006] prompted searches for single nucleotide variations (SNVs) in *CNTNAP2* in numerous cohorts of patients with neurodevelopmental disorders such as ASD, schizophrenia, depression, and impaired language development. In addition, *CNTNAP2* became the subject of linkage and association studies (fig. 3).

CNTNAP2 SNVs in Neurodevelopmental Disorders

The Human Gene Mutation Database (HGMD, <http://www.hgmd.org/>) lists 13 missense/nonsense mutations in *CNTNAP2*. Of those 13 mutations, 8 were discovered in a study of 635 ASD patients and 942 healthy controls [Bakkaloglu et al., 2008]. Among the ASD patients, 27 nonsynonymous SNVs, 13 of which had an allele frequency of <1/4,000, were detected. Of these 13 SNVs, 8 were predicted to be deleterious or were found in regions conserved across all species examined. In 4 cases, these potentially deleterious SNVs were identified in pedigrees with more than 1 affected individual, and 3 of these showed cosegregation with ASD. In the control cohort of 942 healthy individuals, 35 nonsynonymous variants were identified; 11 of these were rare, and 6 were predicted to be deleterious or were conserved across all species examined. The rates of all unique and predicted deleterious/conserved variants were, respectively, 1.75- and 2-fold higher in cases as compared to controls. This did not reflect a statistically significant increase in mutation burden for the ASD cohort.

The other missense/nonsense mutations in *CNTNAP2* were found in ASD patients [O'Roak et al., 2011; Koshimizu et al., 2013] and in patients with Pitt-Hopkins syndrome, childhood apraxia of speech, and ID [Zweier et al., 2009; Gregor et al., 2011; Worthey et al., 2013]. In addition, splice site mutations were reported in patients with ID and Pitt-Hopkins syndrome [Zweier et al., 2009; Gregor et al., 2011]. The SNVs detected in several studies covering single families and small cohorts of patients with neurodevelopmental disorders have been reviewed re-

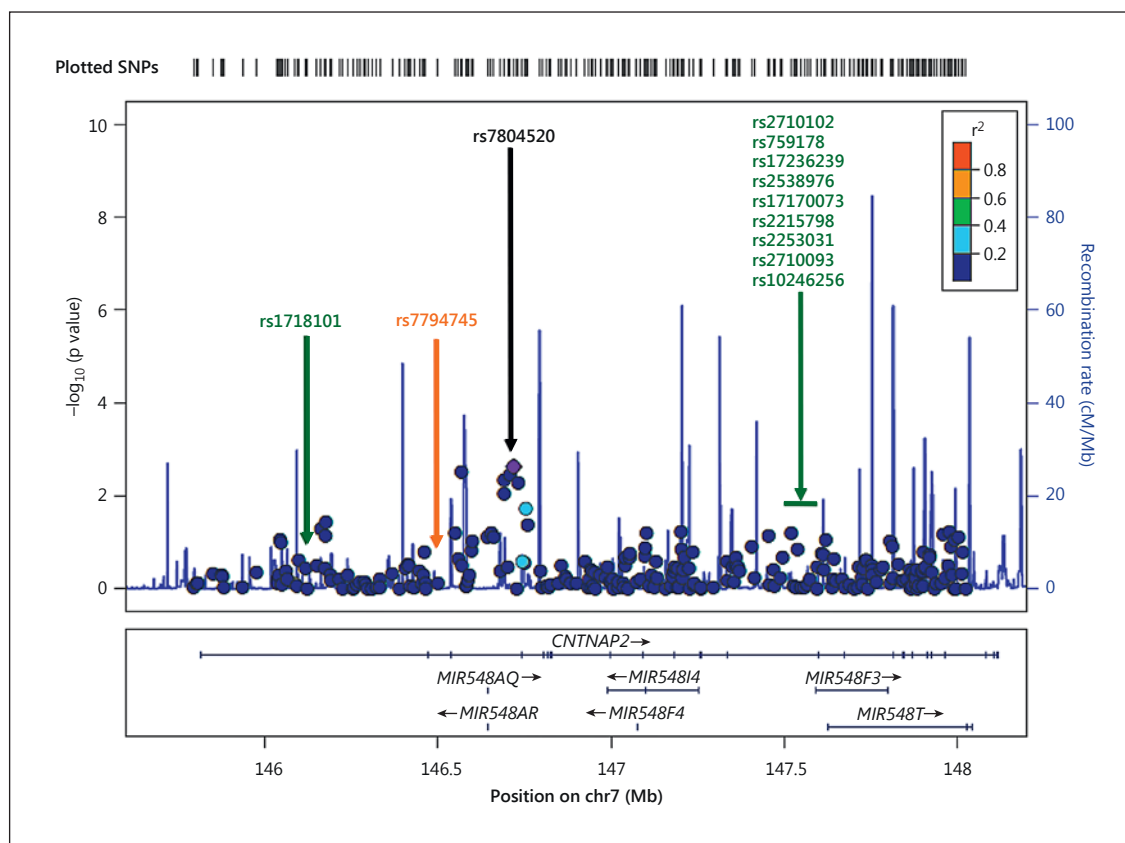


Fig. 3. LocusZoom plot of the association study of contactin genes with ASD [Poot, 2014]. The black arrow indicates the SNP that putatively associates with *CNTN6*, the orange arrow indicates the DNA methylation site [Schneider et al., 2014], and the green arrow and green bar indicate significantly associating SNPs (for further explanations, see table 1 and the text).

cently [Peñagarikano and Geschwind, 2012; Rodenas-Cuadrado et al., 2014]. Thus far, no patients with a complete loss of *CNTNAP2* have been described. Although homozygous knock-out mice exist (see below), it is conceivable that humans without any functional *CNTNAP2* may not be viable or exhibit a hitherto undetected phenotype.

The NHLBI GO Exome Sequencing Project (ESP) aims to discover novel genes and mechanisms contributing to heart, lung and blood disorders by applying next-generation sequencing of the protein-coding regions of the human genome across diverse, well-phenotyped populations in order to extend and enhance diagnosis, management and treatment of heart, lung and blood disorders. The current data set, representing data from more than 200,000 individuals, is available through the Exome Variant Server of the Department of Genome Sciences of the University of Washington, Seattle, USA ([http://evs.](http://evs.gs.washington.edu/EVS/)

[gs.washington.edu/EVS/](http://evs.gs.washington.edu/EVS/)). As of July 1, 2014, the EVS lists 282 SNVs within the *CNTNAP2* gene, of which 274 are also listed in dbSNP. Their genomic evolutionary rate profile scores range from -12.1 to 6.07, i.e. from poorly to highly conserved. Their Grantham matrix scores range from 5 to 184, i.e. very little up to maximal impact on protein structure and function [Grantham, 1974; Cooper et al., 2005].

Whole-exome sequencing studies of over 1,000 ASD families revealed a de novo rate of potentially relevant SNVs in the order of $1-2 \times 10^{-8}$ per nucleotide and per generation [O’Roak et al., 2011, 2012; Iossifov et al., 2012; Neale et al., 2012; Sanders et al., 2012]. While 4 genes, *SCN2A*, *NTNG1*, *KATNAL2*, and *CHD8*, carried more than a single SNV, *CNTNAP2* appeared not to be hit by de novo exonic SNVs in these autism patient cohorts. Since the exons comprise only 0.17% of the genomic DNA of *CNTNAP2*, one stands to miss phenotypically

Table 1. *CNTNAP2* association studies arranged by study phenotype

Primary phenotype	Specific phenotypes	Cohort	Sample size	Study type	SNP	Genome coordinates ^a	<i>CNTNAP2</i>	Model and statistics	Reference
Language	language development	healthy children	606 boys 543 girls	population-based association	rs2710102	7:147877298	exon 13–15	single-point and 4 marker haplotype association	Whitehouse et al., 2011
					rs759178	7:147878020			
					rs17236239	7:147885213			
					rs2538976	7:147888727			
Autism	SLI; oral and written language skills	3-generation SLI families	181 families (grand total of 780 individuals)	family-based association	rs7794745	7:146792514	intron 13	case-control and quantitative association	Newbury et al., 2011
					rs10246256	7:147857715			
					rs2710102	7:147877298			
					rs17236239	7:147885213			
	dyslexia	family trios with a child with dyslexia	188 family trios	quantitative transmission disequilibrium testing (QTDT)	rs2710102	7:147877298	intron 13	QTDT: $p = 0.0174$	Peter et al., 2011
					rs1718101	7:146425696	intron 1		
	age of first phrase	Autism Genome Project (AGP)	2,705 families	family-based association	rs2710102	7:147877298	intron 13	additive p value = $7.783e^{-09}$ OR = 2.13 95% CI: 1.63–2.80	Anney et al., 2012
					rs2710102	7:147877298	intron 13		
	age at first words	Autism Genetic Research Exchange (AGRE)	476 patient-parent trios	family-based association	rs2710102	7:147877298	intron 13	for all $p = 0.028$ for males only $p = 0.005$ for females only $p = 0.87$	Alarcon et al., 2008
					rs2710102	7:147877298	intron 13		
autism	autism	Spanish local	322 patients 524 controls 186 multiplex, 323 simplex families	population-based case-control transmission disequilibrium tests	rs2710102	7:147877298	intron 13	no association	Toma et al., 2013
					rs2710093 rs2253031	7:147935043 7:147934809	intron 14		

^a Genome coordinates are according to dbSNP 141 (21 May 2014). For further explanations, see text.

important variants (e.g. in transcription binding and methylation sites) if studies are limited to exome sequencing only. Nevertheless, loss of gene function due to gene-disrupting mutations such as nonsense, coding indels, and splice acceptor/donor site mutations have been found at significantly elevated frequencies in the genomes of patients with severe ID, epilepsy and ASD [Petrovski et al., 2013].

Association of *CNTNAP2* with Neurodevelopmental Disorders

With the evidence supporting pathogenicity of detected SNVs still being limited, linkage and genome-wide association studies have clearly linked *CNTNAP2* to neurodevelopmental disorders, such as specific language impairment (SLI), dyslexia, autism, schizophrenia, and depression (fig. 3; table 1) [Alarcon et al., 2008; Arking et al., 2008; Newbury et al., 2011; Peter et al., 2011; Villanueva et al., 2011; Whitehouse et al., 2011; Anney et al., 2012; Ji et al., 2013; Sampath et al., 2013; Toma et al., 2013; Poot, 2014; Rodenas-Cuadrado et al., 2014].

Although none of the SNPs in the association studies discussed above reached genome-wide significance (5×10^{-8}), some showed significant association within and across studies. The SNP rs2710102 in intron 13 showed significant association with ASD in the Autism Genetic Research Exchange (AGRE) cohort [Alarcon et al., 2008], but not in the Autism Genome Project (AGP) cohort [Anney et al., 2012] and in 3 smaller studies [Sampath et al., 2013; Toma et al., 2013; Poot, 2014]. This may be due to population effects, such as different allele compositions, or to limited power afforded by too small numbers of participants. The first possible explanation is consistent with the associating SNP rs1718101 in intron 1 found in the AGP cohort of ASD patients [Anney et al., 2012], with the SNPs rs2710093 and rs2253031 in intron 14 [Sampath et al., 2013], and the differences among different European populations reported with SNP rs4141463 in *MACROD2* [Curran et al., 2011; Anney et al., 2012; Prandini et al., 2012].

The SNP rs2710102 in intron 13 consistently associated with SLI [Newbury et al., 2011], non-word repetition [Peter et al., 2011], and as part of specific 4-SNP haplotypes with language development in healthy individuals [Whitehouse et al., 2011]. These findings clearly link the region around intron 13 of *CNTNAP2* to development of language (fig. 3, green arrow). In addition, SNP rs2710102 associates with ASD and with major depression [Alarcon et al., 2008; Ji et al., 2013]. This may either reflect shared phenotypes or endophenotypes in these disorders [Rode-

nas-Cuadrado et al., 2014]. Alternatively, it may indicate that there are subtypes of ASD and major depression, e.g. with and without involvement of impaired language development [Poot, 2013]. Since both hypotheses may have widespread ramifications for patient management and treatment, they need further investigation.

In addition to associating with ASD in the AGRE cohort and with SLI, rs7794745 in intron 2 of *CNTNAP2* showed a parent-of-origin effect and was also associated with a region of differential DNA methylation in a study of human and chimpanzee cortices [Arking et al., 2008; Newbury et al., 2011; Schneider et al., 2014]. These findings indicate that association of a gene with a disorder may also involve mechanisms that are not directly related to the protein-encoding part of the gene [Nicolae et al., 2010]. Therefore, searches for possible epimutational mechanisms should complement exome sequencing efforts in both the research and in the clinical genetic setting.

Finally, a candidate gene association study focusing on the contactin gene family was prompted by the preponderance of CNVs affecting contactin genes in cohorts of ASD patients [van Daalen et al., 2011; Nava et al., 2014]. Upon testing 1,648 SNPs, spanning 12.1 Mb of genomic DNA, significant association was found for only 1 SNP, being located within the *CNTN5* gene (rs6590473 [G], $p = 4.09 \times 10^{-7}$) [Poot, 2014]. None of the 250 *CNTNAP2* SNPs studied showed significant association with ASD. However, a combination of risk alleles of SNPs in *CNTN6* (rs9878022 [C]) and in intron 3 of *CNTNAP2* (rs7804520 [A]) occurred more frequently in the ASD cohort than would be expected under random segregation, albeit this association was only nominally significant (fig. 3, purple diamond). This finding supports the notion that *CNTNAP2* may be a node in a combinatorial genetic network. Given its implications, this finding needs testing in independent larger cohorts using statistically rigorous methods [Won et al., 2014].

Transgenic Mouse Models

Creation of a transgenic animal model in which only a single gene has been mutated, or fully ablated, constitutes a valid approach to studying the phenotypic roles and the effects of possible treatments of the disorders with mutations in that gene, in this case *CNTNAP2*. To do so, the model has to fulfill 3 validation criteria [Chadman et al., 2009; Nestler and Hyman, 2010]. First, such an animal model should be based on a known gene mutation; the

so-called construct validity. Second, the animal should clearly exhibit demonstrable and ideally quantifiable phenotypes of human patients; the face validity. Third, the animal model should respond to treatments that have been shown to be effective in humans; the predictive validity.

The original transgenic mice lacking *Cntnap2* (Caspr2 null mice) were in the ICR outbred strain [Poliak et al., 2003]. After being backcrossed onto the C57BL/6J background, these 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 *CNTNAP2* mutations [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. Not all of the phenotypes found in the human patients, such as mislocalization of potassium channels, were observed in the Caspr2 null mice, however. This may be because the mouse was a complete knockout of Caspr2, while CDFE patients carry a truncated Caspr2 protein product. While Caspr2 null mice exhibit some, but not all features of autism, mice with mutations in *Nlgn3* exhibit an almost complete recapitulation of autism [Poot, 2013; Rothwell et al., 2014]. It should be pointed out, however, that the genetic background of the mouse strain studied may influence what kind of phenotypes can be observed [Kas et al., 2014].

Ramifications for the Clinical Management of Patients with *CNTNAP2* Alterations

A considerable body of genetic, evolutionary, functional, and neuroanatomical evidence firmly established the involvement of *CNTNAP2* in the development of language [Rodenas-Cuadrado et al., 2014]. In addition, structural disruptions of *CNTNAP2* have been found in a broad range of patients with neurodevelopmental disorders (fig. 2). The phenotypic variability of *CNTNAP2* disruptions can either be explained by assuming a common *CNTNAP2*-dependent (endo)phenotype in all patients, or by assuming a polygenic model in which *CNTNAP2* is necessary, but not sufficient for the full phenotypic spectrum of the patient. Such a polygenic model has been proposed to explain the phenotypic variability of patients with ID [Girirajan and Eichler, 2010; Girirajan et al., 2012] and autism [Poot et al., 2011; Leblond et al., 2012;

Poot, 2013]. ‘Natural’ examples of a polygenic model are ASD patients with genome rearrangements involving *CNTNAP2* and at least 1 other gene [Bakkaloglu et al., 2008; Poot et al., 2010]. On the one hand, such genomic rearrangements complicate a straightforward interpretation of the case, but on the other hand, they may provide clues towards other contributing genes if one assumes a polygenic model. The recently reported co-occurrence of pairs of independent alleles with a higher frequency than expected assuming random segregation is also consistent with a polygenic model, which in turn may point towards regulatory networks (e.g. transcription factors, imprinting) or physical interactions between proteins [Poot, 2014]. Although no firm data are currently available for *CNTNAP2*, a polygenic etiology has certainly to be taken into consideration for patients with many neurodevelopmental disorders [Girirajan and Eichler, 2010; Girirajan et al., 2012].

Recommendations for the Management of Patients and Their Families

Given the considerable yield of ‘actionable’ CNVs in cohorts of patients with ID, ASD and schizophrenia, segmental aneuploidy screening with genome-wide arrays is clearly the method-of-choice for genome analysis of patients with neurodevelopmental disorders, including SLI [Hochstenbach et al., 2011]. Not surprisingly, the HGMD lists 25 CNVs and other disruptions of *CNTNAP2* that would be detected with a high-density oligonucleotide or SNP array, while 15 mutations would be found by classical or next-generation sequencing. These data reinforce the current recommendation regarding genome investigation of patients with developmental disorders, i.e. genome-wide array ‘is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies’ [Miller et al., 2010].

Their high frequency of detection notwithstanding, CNVs may by themselves not be sufficient to fully explain the patient’s phenotype, such as ASD [Marshall et al., 2008; Bucan et al., 2009; Salyakina et al., 2011; van Daalen et al., 2011]. Parents and siblings with transmitted CNVs have generally been considered healthy carriers. Yet, in families with ASD patient(s), testing of all siblings with the Social Responsiveness Scale has revealed that some of the individuals considered to be healthy carriers also showed subclinical behavioral impairments [van Daalen et al., 2011]. In these families, a CNV that cosegregates with such impairments should therefore be considered a ‘susceptibility factor’.

For SNVs, the situation is much less clear-cut. Some of the SNVs detected in early studies of cohorts of patients with neurodevelopmental and other disorders have also been detected in the NHLBI Exome Sequencing Project (ESP; <http://evs.gs.washington.edu/EVS/>) and the 1000 Genomes project of the Wellcome Trust (<http://www.1000genomes.org/>) [Bakkaloglu et al., 2008; O’Roak et al., 2011]. Nevertheless, gene-disrupting mutations such as nonsense, coding indels, and splice acceptor/donor site mutations, which lead to loss of gene function, have been found at significantly elevated frequencies in the genomes of patients with severe ID, epilepsy and ASD [Petrovski et al., 2013]. Among 1,000 participants of the NHLBI Exome Sequencing Project, 239 unique variants in 114 genes which were identified as disease-causing in the HGMD were investigated. Only few (~3.4% for participants of European descent and ~1.2% for African descent) of the high-penetrance actionable pathogenic or likely pathogenic variants were found among these well-phenotyped patients [Dorschner et al., 2013]. On the other hand, bioinformatic approaches to identify highly penetrant SNVs have thus far not allowed to classify each SNV as definitely pathogenic or not. An alternative approach may be to classify SNVs as ‘deleterious’ based on their probable reduction of organismal fitness, because they appear to have been depleted by natural selection [Kircher et al., 2014]. Since this is a novel approach, it will need validation before it can be incorporated into clinical decision making.

Once a CNV or a SNV affecting *CNTNAP2* has been discovered in a patient, genome-wide analyses should be performed to determine whether there are other contributing mutations [Poot et al., 2011]. For genome-wide analyses of CNVs, SNP arrays are the preferred tool since they may in addition to CNVs reveal possible uniparental disomy or regions of homozygosity, and whether a patient carries a risk allele identified by an association study of cohorts with the disorder of the patient (see above). A positive finding may point to potentially contributing risk alleles in the genome of the patient. In such a case re-sequencing part of or even the entire *CNTNAP2* genomic DNA may be considered. The latter may reveal mutations in, for instance, transcription factor binding or methylation sites, which would be missed if the re-sequencing effort was limited to exome sequencing only.

Once the full extent of disruptions and mutations in *CNTNAP2* and possibly other contributing genes and loci is known, medical geneticists face 2 types of questions. First, which of these findings should be disclosed to the patient or, if the patient is a minor, to his or her guard-

ian(s), and if so, what clinical significance should be attached to each of the disruptions and mutations [De Wolf et al., 2013]. The answer to these questions depends on the phenotype(s) of the patient, and on what model to explain these will be assumed. For instance, if the patient exhibits some form of language impairment, a *CNTNAP2* disruption or mutation can be considered to be of major impact [Poot et al., 2010]. In patients without language impairments, *CNTNAP2* disruptions or mutations may very well be of some clinical impact, as has recently been shown for *NPHP1* CNVs and mutations in patients with Bardet-Biedl syndrome [Lindstrand et al., 2014]. Although the debate on what to disclose or not is far from being completed, there is a growing consensus [Christenhusz et al., 2013; De Wolf et al., 2013]. First, findings with confirmed clinical utility where there is the possibility of treatment or prevention should be disclosed. Second, such disclosure should proceed with caution, especially if findings have been obtained with any of the new genetic technologies and genetic testing may involve minors. Third, the number of possible incidental findings should be limited even before genetic testing is considered. The latter is of particular importance to prevent confusion and unwarranted anxiety with the patients and their guardians. A still undecided issue is to what extent testing of carriers of susceptibility factors may affect family relationships and patterns of communication [Bailey et al.,

2014]. The latter question touches on the larger issue of informed decision-making within the nuclear and larger family surrounding patients with neurodevelopmental disorders.

Concluding Remarks

While a decade of research has revealed numerous novel and unexpected disruptions and mutations in *CNTNAP2*, one of the largest genes in the human genome, their possible impact on neurodevelopmental disorders remains to be elucidated. This challenge will open up new avenues in both neurobiological research and regarding the development of novel concepts to understand the myriad networks in which *CNTNAP2* may be involved. Finally, the wealth of data generated by the novel genomic technologies needs to be incorporated into clinical practice. This may turn out to be the greatest challenge that *CNTNAP2* will present to us in the near future.

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