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The Role of Candidate-Gene *CNTNAP2* in Childhood Apraxia of Speech and Specific Language Impairment

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Childhood apraxia of speech (CAS) is a debilitating pediatric speech disorder characterized by varying symptom profiles, comorbid deficits, and limited response to intervention. Specific Language Impairment (SLI) is an inherited pediatric language disorder characterized by delayed and/or disordered oral language skills including impaired semantics, syntax, and discourse. To date, the genes associated with CAS and SLI are not fully characterized. In the current study, we evaluated behavioral and genetic profiles of seven children with CAS and eight children with SLI, while ensuring all children were free of comorbid impairments. Deletions within *CNTNAP2* were found in two children with CAS but not in any of the children with SLI. These children exhibited average to high performance on language and word reading assessments in spite of poor articulation scores. These findings suggest that genetic variation within *CNTNAP2* may be related to speech production deficits.

Key words: gene variant; speech production; CAS; SLI

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INTRODUCTION

Childhood apraxia of speech (CAS) is a debilitating pediatric speech disorder that affects 1–2 children per thousand [Shriberg, Aram, & Kwiatkowski, 1997] and is often resistant to intervention [Lewis, Freebairn, Hansen, Iyengar, & Taylor, 2004; Teverovsky, Bickel, & Feldman, 2009]. CAS is characterized by a broad range of speech abnormalities that affect accuracy and consistency of speech sound production, as well as suprasegmental features such as prosody [Marquardt, 2004; Iuzzini, 2012], or intonation [American Speech-Language-Hearing, 2007]. Children with CAS may evidence concomitant deficits such as language impairment, dysarthria, or intellectual disability [American Speech-Language-Hearing, 2007]. Comorbid deficits contribute to the complex speech presentation observed in these children, which has led to challenges with early diagnosis and has motivated the search for reliable genetic markers. Specific Language Impairment (SLI) is a persistent developmental language impairment that is characterized by delayed and/or disordered oral language skills, including impaired discourse, syntax, and semantics. The high comorbidity between CAS and SLI [Shriberg, Tomblin, & McSweeny, 1999] makes it extremely difficult to determine which genes are related to the phenotypes of CAS and which are related to SLI, as opposed to which are associated with comorbid CAS-LI.

As part of a larger study on speech and language impairments, we evaluated the genetic profiles of children with a diagnosis of CAS or SLI. This sample was strictly controlled so that no children in the

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CAS group had comorbid reading, language, or cognitive impairments and no children in the SLI group had comorbid cognitive or articulation impairments. In this report, we discuss the speech and language phenotypes of two children with CAS who had deletions in the region of chromosome 7 that contains the neurexin gene *CNTNAP2* (7q35). *CNTNAP2* is located downstream from and is regulated by *FOXP2* (7q31), which has been linked to the occurrence of CAS in the oft-studied KE family [Lai, Fisher, Hurst, Vargha-Khadem, & Monaco, 2001; Vernes et al., 2008]. *CNTNAP2* is closely related to *FOXP2* and has been identified as a candidate gene for dyslexia, SLI, and autism [Laffin et al., 2012; Rodenas-Cuadrado, Ho, & Vernes, 2014]. To our knowledge, this is the first report to link variants in *CNTNAP2* to CAS without comorbid reading, language, and cognitive impairments, which indicates that *CNTNAP2* variants may be associated with deficits in speech production in the absence of comorbid reading, language, and cognitive impairments.

MATERIALS AND METHODS

Participants

Fifteen children ranging in age from 4;5–17;2 (years; months) participated as part of a larger study on the biological pathways of speech and language disorders. All procedures were approved by the Institutional Review Board of the University of Nebraska Medical Center and the University of Nebraska-Lincoln, and all participants were consented prior to participation. Participants underwent a series of commonly administered, age-appropriate speech, language, reading, and cognitive assessments including the *Goldman Fristoe Test of Articulation-2nd Edition* [*GFTA-2*; Goldman & Fristoe, 2000], the *Clinical Evaluation of Language Fundamentals-Fourth Edition* [*CELF-4*; Semel et al., 2003], *Reynolds Intellectual Assessment Scales* [*RIAS*; Reynolds & Kamphaus, 2003], and the *Woodcock Reading Mastery Test-Revised* [*WRMT-R*; Woodcock, 1998]. All participants were required to have normal cognition based on a standard score higher of 75 or higher on the *RIAS*.

Group Assignment

Participants in the CAS group were typically referred to the study with a history of CAS diagnosis and treatment by a clinician with expertise in CAS. The CAS diagnosis was confirmed if the participant evidenced at least 4 of 11 features associated with CAS [adapted from Shriberg, Potter, & Strand, 2011] during a standardized, norm-referenced articulation assessment [Goldman & Fristoe, 2000]. We reasoned that if a child with a history of CAS—who did not have comorbid language impairment, cognitive deficit, or dysarthria—produced a high number of features on simple test items, we could be more certain in confirming the CAS diagnosis rather than a different deficit (e.g., dyslexia), which could yield numerous errors on complex items [Catts, 1989]. Therefore, two trained raters, a speech language pathologist with expertise in CAS and a speech language pathology graduate student, independently blind-rated each child's responses on the *GFTA-2* using the operational definitions in Table III.

Participants in this group were also required to have an articulation test (*GFTA-2*) percentile score at or below the 16th percentile and a normal language standard score of 85 or higher on the *CELF-4*. None of the participants included in the CAS group in this study reported a history of diagnosis or treatment for language impairment.

Children were assigned to the SLI group based on *GFTA-2* percentile scores of 16 or higher, fewer than 4/11 CAS features, and a *CELF-4* standard score below 85. See Table I for inclusion criterion and Table II for assessment scores.

DNA Collection and Isolation

Buccal cell samples were collected from participants using the Isohelix DNA swab packs (Cell Projects, Ltd., Kent, United Kingdom), and DNA was extracted per manufacturer's recommendations using the QIACube (Qiagen, Valencia, CA). DNA quantity and quality were determined using the NanoDrop ND-1000® spectrophotometer (NanoDrop Technologies, Wilmington, DE) and agarose gel electrophoresis, respectively.

High-Resolution Genome-Wide Analysis

High-resolution genome-wide analysis was performed on genomic DNA using the CytoScanHD™ array (Affymetrix, Santa Clara, CA) according to manufacturer's instruction. This array contains more than 2.6 million markers for high-resolution whole-genome copy number analysis and 750,000 genotype-able single nucleotide polymorphisms (SNPs) for reliable detection of copy neutral loss of heterozygosity (CN-LOH). Data were visualized and analyzed with the Chromosome Analysis Suite (ChAS) software (Affymetrix) using the following filter parameters: (1) ≥25 markers and ≥5 kilobases (kb) for copy number variants (CNVs) and (2) ≥5 megabases (Mb) for CN-LOH.

Reliability of Perceptual Feature Ratings

To ensure confidence in group assignments inter-rater reliability of perceptual feature ratings was calculated on data from all participants. The intra-rater correlation coefficient with absolute error in

TABLE I. Inclusion Criterion for Both Groups

	Nonverbal IQ (standard score)	Speech production (percentile)	Language (standard score)	Word reading (standard score)	# of CAS features
CAS (N = 7)	>75	≤16th percentile	≥85	Any	≥4
SLI (N = 8)	>75	>16th percentile	<85	Any	<4

TABLE II. Behavioral Profiles for Both Groups. ***$p < 0.001$

	Nonverbal IQ (standard score)	Speech production (percentile)***	Language (standard score)***	Word reading (standard score)	# of CAS features***
CAS (N = 7)	110.43 (11.60)	3.14 (5.24) -percentile	109.57 (16.35)	101.57 (7.70)	7.14 (2.19)
SLI (N = 8)	102.33 (4.95)	34.44 (6.42) - percentile	73.67 (9.77)	97.89 (9.92)	1.44 (1.01)

parenthesis was .98 (.30 features), showing a high level of agreement between raters for perceptual feature rating using the operational definitions that are included in Table III.

RESULTS

CNTNAP2 Deletions in Children With CAS

In the current study, 2 of the 7 children with CAS exhibited deletions within the *CNTNAP2* gene, at 7q35, with array probes encompassing 6.77 kilo-bases (kb) in length. Deletion breakpoints were identical in both children spanning 147714709–147721486 bp (human GRCh37/hg19 assembly). The deletions were located in alternative intron 18 and were approximately 20 kb away from the nearest exon (Fig. 1). Unfortunately, we were unable to collect genetic samples from the participants' biological parents, so we could not determine if these deletions were inherited or de novo in nature. Child 1 was a 12-year-old female and child 2 was an 8-year-old male (see Table IV for assessment scores).

Behavioral Profiles of Children With CNTNAP2 Deletions

Nonverbal IQ. Both participants evidenced normal nonverbal intelligence. Child 1 exhibited average intelligence (SS = 95) and child 2 exhibited high intelligence (SS = 131), more than two standard deviations above the mean (Table IV).

TABLE III. Operational Definitions for CAS Characteristics

Characteristic	Definition
Vowel error	A vowel production error in which the vowel is substituted for another phoneme OR in which the vowel is recognizable as a specific phoneme but it is not produced exactly correctly (e.g., not a prototypical production, may sound like it's in between two vowels). It is not considered an error if the vowel is substituted with another phoneme that is consistent with an adult-like model (e.g., /hɑt dag/ /hɑt dɔg/)
Consonant distortion	A consonant production error in which a speech sound is recognizable as a specific phoneme but it is not produced exactly correctly (e.g., an /s/ that is produced with lateralization or dentalization).
Stress errors	An error in which the appropriate stress is not produced correctly. For example: conDUCT vs. CONduct have different stress patterns. It is considered an error if the stress is inappropriately equalized across syllables, or on the wrong syllable.
Syllable segregation	Brief or lengthy pause between syllables which is not appropriate.
Groping; prevocalic	(silent) articulatory searching prior to onset of phonation, possibly in an effort to improve the accuracy of the production. Video is needed to assess this feature.
Intrusive schwa (e.g. in clusters)	A schwa is added in between consonants. For example, it may be inserted in between the consonants in a cluster (e.g., /blu/ becomes /bə̲lu/). This NOT considered a "vowel error"
Voicing errors	A sound is produced as its voicing cognate (e.g., a /p/ that is produced as a /b/). In addition, this could also describe productions which appear to be in between voicing categories (e.g., blurring of voicing boundaries).
Slow rate	Speech rate is not typical. It is slower during production of part (e.g., zzziiiiiiper/zipper) or the whole word (e.g., tooommmmaaatoooo/tomato).
Increased difficulty with multisyllabic words	The participant has a disproportionately increased number of errors as the number of syllables increases (as compared to words with fewer syllables).
Resonance or nasality disturbance	Sounds either *hyponasal*: not enough airflow out of nose/"stuffy" OR *hypernasal*: too much airflow out of nose for non-nasal phonemes (e.g., plosives).
Difficulty achieving initial articulatory configurations or transitionary movement gestures	Initiation of utterance or initial speech sound may be difficult for child to produce and may sound lengthened or uncoordinated. Also, child may evidence lengthened or disrupted coarticulatory gestures or movement transitions from one sound to the next.

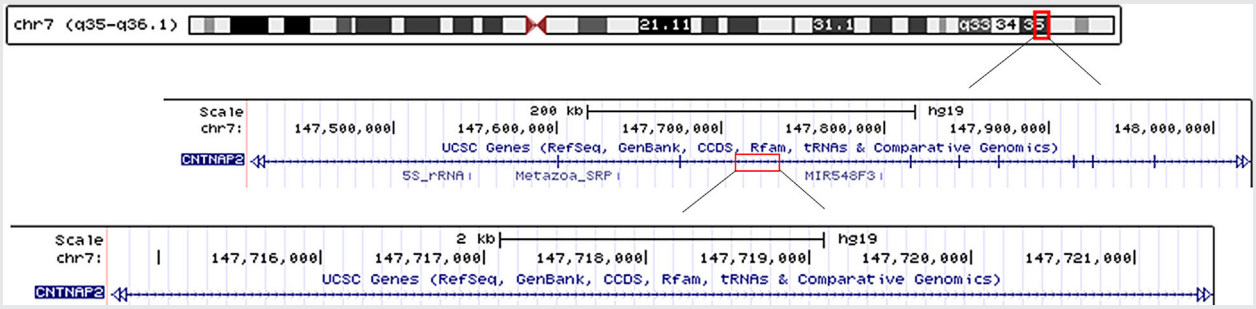

FIG. 1. Location of the deletion reported here within *CNTNAP2* in the context of *CNTNAP2's* location on chromosome 7. The deletion reported here was located in alternative intron 18 and was 20 kb from the nearest exon. Images were adapted from the UCSC genome database (https://genome.ucsc.edu/).

CAS features. The number of CAS features differed between the cases (Table IV). Child 1 had four features where participant 2 had nine features (out of a possible 11). The participants evidenced four CAS features in common, which included: vowel errors, consonant distortions, excessive or equal stress, and voicing errors. Participant 2 also evidenced syllable segregation, groping, slow rate, resonance disturbance, and difficulty achieving initial articulatory configuration or transitionary movement gestures.

Speech severity. Both children scored in the 1st percentile on the speech production assessment. This indicates that both children evidenced severe speech deficits relative to their same aged peers. (Table IV)

Language and literacy. Both children evidenced normal language and word reading ability. On the *CELF-4* core language test, child 1 had a standard score of 121 and child 2 a score of 111 indicating normal-to-high language ability for these participants (Table III). On the *WRMT* reading assessment, child 1 had a standard score of 105 and child 2 a score of 110 (Table IV). These results suggest that, in some children with CAS, deletions containing *CNTNAP2* may play a role in speech production without impacting language skills or reading ability.

Although it is possible that at the time of testing our participants evidenced remediated language impairments, their performance in the normal-to-high range on language and reading tests suggest that a history of language impairment is unlikely. Longitudinal research on children with language impairment [Stothard, Snowling, Bishop, Chipchase, & Kaplan, 1998] shows that children with a preschool history of language impairment who were retested during adolescence and scored in the normal range, still evidenced difficulty with phonological processing and literacy skills. Given that neither family reported difficulty with language or reading impairments, nor did either participant demonstrate performance at the lower end of normal on language or reading assessments, we feel confident that these children likely had CAS speech symptoms in the absence of language impairment.

Additional Genetics Findings

We note that both children had two additional CNVs that met repor5 criteria (Table V). Child 1 had deletions at 5q34 and 12p12.3. Previous studies have associated CNVs at 5q34 with facial abnormalities such as cleft lip, depressed nasal bridge, and microcephaly [Schafer et al., 2001; Chen et al., 2012]. Previous associations in this region included the genes *MSX2, NKX2-5*, and *NSD1*, none of which were included in the CNV seen in Child 1 (deletion was small and included only *ODZ2*). CNVs at 12p12.3 have been previously associated with language delay, dysmorphic features, and hypotonia [Gläser et al., 2003; Lamb et al., 2012]. These previous studies focused on nearby regions and the gene *SOX5*, which was not affected by the deletion seen in Child 1.

Child 2 had deletions at 1q25.1 and 15q21.2. Deletion at 1q25.1 has previously been linked to delayed language and expressive language impairment [Höglund, Jalkanen, Marttinen, & Alitalo, 2003], though the deletion previously reported was significantly

TABLE IV. Assessment Scores for Two Children With CNTNAP2 Deletions and CAS

	Nonverbal IQ (standard score)	Speech production (percentile)	Language (standard score)	Word reading (standard score)	# of CAS features
Child 1	95	1	121	105	4/11
Child 2	131	1	111	110	9/11

TABLE V. Summary of Additional CNVs in Two Children With CAS

	CNV identified	Type	Size (kbp)	Linear location	Previous associations
Child 1	5q34	Deletion	26.052	167061974-167088026	cleft lip, depressed nasal bridge, microcephaly (Chen et al., 2012; Schafer et al., 2001)
	12p12.3	Deletion	47.118	15826648-15873766	language delay, dysmorphic features and hypotonia (Gläser et al., 2003; Lamb et al., 2012)
Child 2	1q25.1	Deletion	224.684	174450384-174675068	delayed language and expressive language impairment (Höglund et al., 2003)
	15q21.2	Deletion	24.483	51800269 - 51824752	abnormal facial shape and muscular hypotonia (Moreno-De-Luca et al., 2011)

larger than the one seen in Child 2. Deletion at 15q21.2 has previously been associated with abnormal facial shape and muscular hypotonia [Moreno-De-Luca et al., 2011]. This previous deletion did not overlap with the deletion seen in Child 2. The differences between the deletions seen in our cases and CNVs reported in previous literature linking these regions to speech and language delay support the hypothesis that the deletion seen in the *CNTNAP2* gene in the current study may have a functional consequence on phenotype. Formal evaluation by a board-certified clinical geneticist was not performed for either participant. Additional studies will be needed to determine whether CNVs in these regions contribute to speech production deficits.

Lack of CNTNAP2 Variants in Children With SLI

Although *CNTNAP2* variants have predominantly been linked to the presence of SLI, we did not observe any CNVs at 7q35 in our sample of eight children with this disorder. The clinical significance of the CNVs identified in the children with SLI in other areas of the genome are currently being analyzed for a future report.

In Silico Analysis of the Deleted Region

Since the deletion is intronic, the putative mechanism for how this CNV could affect cellular function does not involve protein sequence changes. We examined public data sources for evidence that this region has regulatory potential [Karolchik et al., 2014]. Maunakea et al. [2010] showed two methylation peaks and two RNA-seq peaks within 2.2 kb in an adult human brain. There are two distinct 5′ SMART tags indicating two transcription start sites in opposite directions. These data are indicative of an enhancer RNA. Corroborating these data, the ENCODE database [Kellis et al., 2014] shows a DNaseI hypersensitive site in four cell lines (H9es, H1-hESC, lps, NT2-D1) and, overlapping this DNaseI hypersensitive site, ChIP-seq indicates binding of twelve transcription factors (NANOG, MAFK, CEBPB, EP300, BCL11A, JUND TEAD4, SP1, POU5F1, TCF12, SIN3A, HDAC2). Taken together, these data indicate that a bidirectional enhancer RNA is located within the 6.7 kb deletion. However, it is not possible to infer if the enhancer RNA acts in cis to affect *CNTNAP2* expression or in trans to regulate a different gene.

DISCUSSION

Summary of Results

In the current study, we describe two children with a strictly controlled diagnosis of childhood apraxia of speech (CAS) without comorbid reading, language, or cognitive impairments who had deletions in alternative intron 18 of *CNTNAP2* at 7q35. Both children with CAS evidenced normal language and word reading abilities. In contrast, we did not observe any variants in *CNTNAP2* in the eight children with SLI. These results suggest that in some children, deletions containing *CNTNAP2* may play a role in speech production in the presence of intact language-based processing skills.

Evaluation of Study Design

Strengths of the current study include absence of comorbid language and reading deficits and the precise speech, language, and reading phenotyping of each child, including the strict diagnostic criteria used to define CAS. In prior research, deletions containing *CNTNAP2* have been associated with a variety of diagnoses including autism, language impairment, speech delay, and dyslexia [Peter et al., 2011; Laffin et al., 2012]. The interpretation of these studies, however, is challenged by the high likelihood of comorbid speech motor problems in these cohorts. Our result that *CNTNAP2* variants occurred in multiple children with CAS in the absence of reading, language, and cognitive impairments raises the possibility that *CNTNAP2* variants are involved in the motor components of speech. One caveat of this study is that our sample size is small; therefore, we are cautious in making any definitive conclusions about these CNVs being causative for CAS.

Intron Versus Exon Deletion

Though the deletions reported here did not contain any exons, previous work in other disorders report intronic deletions in *CNTNAP2* that were associated with disorder phenotypes. For example, a recent paper described the case of a woman with epilepsy and schizophrenia who also had a small intron deletion in *CNTNAP2*, while her twin sister was free of both disorders and did not have this deletion [Friedman, Vrijenhoek, & Markx, 2007]. Other studies have linked intronic deletions in *CNTNAP2* to a

variety of features, including age of first word, receptive and expressive language impairment, and non-word repetition [Poot, Beyer, Schwaab, & Damatova, 2010; Peñagarikano & Geschwind, 2012]. Though larger sample sizes and functional studies are needed to more decisively determine the role of intronic CNVs on phenotype, our work supports previous reports that intronic deletions in *CNTNAP2* may have functional consequences on speech production abilities.

Prevalence of CNTNAP2 CNV

Though the sample size in the current study is small, recent work suggests the presence of this specific CNV in 2 of our 7 children with CAS may deviate from expectations relative to the general population. The prevalence of this CNV in our sample is 14.3%. To compare with two population-based samples, we considered only CNVs that overlap with the one described here, yet are still within the same intron (i.e., no exonic involvement). The first study found this variant in 0.349% of a Swiss European sample [N = 717; Vogler et al., 2010] and the second study found this variant in 1.74% of an Ontario population sample [30/873; Costain et al., 2013]. The difference in minor allele frequency is statistically significant across the Vogler and Costain studies (Fisher's exact test; $P < 0.001$) that could be due to a difference in probe density across array platforms. The Vogler study used the Affymetrix Genome-Wide Human SNP Array 6.0 while the Ontario study was CytoHD specific–the same technology as in our study. The difference could also be due to the fact that these populations were not strictly controlled for speech and language skills. The prevalence between our population and the Ontario study is different (28% vs. 1.74%; Fisher's exact test, $P = 0.024$), and in concert with the prevalence estimates of the disorder in the population [Shriberg, Aram, & Kwiatkoswki, 1997], suggest that this variant is more likely to occur in individuals with apraxia than by chance in the general population. Additional studies are needed to confirm that this CNV is more prevalent in children with CAS than a verified typically developing control population.

Biological Mechanisms of CNTNAP2 Variants

The downstream effect of this intronic CNV has not been developed in the literature. However, several studies define typical *CNTNAP2* regulation, and more recently, also define the effects of genetic variation on CNTNAP2 functioning in humans [Zeeland, 2010; Dennis & Jahanshad, 2011; Hohenberg & Wigand, 2013]. CNTNAP2 is a cell-cell adhesion molecular and member of the neurexin protein family [Poliak, Gollan, Martinez, & Custer, 1999]. CNTNAP2 mediates neuron-glia interactions at juxtaparanodal axonal regions and from that role is involved in myelination [Poliak & Gollan, 2001]. During neuronal differentiation, CNTNAP2 also localizes potassium channels giving it a dual role for propagation of action potentials within the juxtaparanodal region [Rasband, 2004]. Given this association with myelination and functioning of axons, studies have examined white matter in connection with *CNTNAP2* variation using in vivo tractography techniques. Thus far, these studies do observe significant difference between typically developing individuals carrying autism or language-impairment associated variants versus persons without those variants [Zeeland, 2010; Dennis & Jahanshad, 2011], and more recently a variant not previously associated with disease has also been found [Hohenberg & Wigand, 2013]. Interpreting these in vivo tractography measures in the context of neurobiological function is more challenging. Additionally, it is clear that CNTNAP2 is involved in other processes that will require additional study designs to fully elucidate. An example includes studying epigenetic regulation, where differences between humans and chimpanzees has been demonstrated [Schneider, Hajj, & Richter, 2014].

The Role of CNTNAP2 in Motor Impairments

Since the speech production-related gene *FOXP2* regulates *CNTNAP2,* one unexplored possibility is that the observed association between *CNTNAP2* and disorders such as dyslexia or autism is due to comorbid speech motor disturbances rather than to a reading or language impairment alone. Recent studies in animal models support this hypothesis. *CNTNAP2* [Condro & White, 2014] is important for song mimicking in birds, which is a process that is highly dependent on motor control. In addition, recent studies in individuals with dyslexia have reported associations between sequence variations in *CNTNAP2* and stuttering [Petrin et al., 2010] as well as difficulties with motor-heavy tasks such as rapid oral reading [Peter et al., 2011]. In addition, a recent study also reported an intron deletion in *CNTNAP2* in a child with CAS and a small insertion in two other children with the disorder [Worthey et al., 2013]. These findings support our finding that abnormalities in *CNTNAP2* can occur in persons with CAS without comorbid reading and language impairment. Finding *CNTNAP2* variants in children with CAS who do not have a language or reading impairment would indicate that *CNTNAP2* may adversely affect the pathway responsible for speech production, rather than the pathway responsible for language and reading, in these children.

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