

A songbird animal model for dissecting the genetic bases of autism spectrum disorder

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Abstract. The neural and genetic bases of human language development and associated neurodevelopmental disorders, including autism spectrum disorder (ASD), in which language impairment represents a core deficit, are poorly understood. Given that no single animal model can fully capture the behavioral and genetic complexity of ASD, work in songbird, an experimentally tractable animal model of vocal learning, can complement the valuable tool of rodent genetic models and contribute important insights to our understanding of the communication deficits observed in ASD. Like humans, but unlike traditional laboratory animals such as rodents or non-human primates, songbirds exhibit the capacity of vocal learning, a key subcomponent of language. Human speech and birdsong reveal important parallels, highlighting similar developmental critical periods, a homologous cortico-basal ganglia-thalamic circuitry, and a critical role for social influences in the learning of vocalizations. Here I highlight recent advances in using the songbird model to probe the cellular and molecular mechanisms underlying the formation and function of neural circuitry for birdsong and, by analogy, human language, with the ultimate goal of identifying any shared or human unique biological pathways underscoring language development and its disruption in ASD.

Keywords: Autism spectrum disorder, animal model, FOXP2, CNTNAP2, song circuitry, vocal learning

1. Introduction

Despite decades of research into the neurobiological basis of human language and communication disorders, we have an incomplete understanding of the molecular and cellular underpinnings of socially-learned vocal communication. Thanks to considerable progress in the field of human neurogenetics, it has become possible to map an increasing number of neural disorders onto abnormal genetic variants [1,2]. Of particular relevance to understanding the neurobiology of human speech and language are neurological syndromes with developmental onset in which language is affected, such as autism spectrum disorder (ASD). ASD encompasses a range of neurodevelopmental disorders of varying severity that involve impairments in three core social domains: language development and use, behavioral flexibility, and reciprocal social interaction. Within ASD, autism disorder (the most severe end of the spectrum) and pervasive developmental disorder – not otherwise specified represent the most common clinical

manifestations of the disease [3]. ASD has a strong genetic basis and robust heritability, with current prevalence estimates indicating that 1 in 166 children are diagnosed with the disorder each year [3]. In addition to the phenotypic variation associated with the degree of severity in the clinical presentation of the disease, a growing list of gene mutations and genetic structural variation linked to autism susceptibility [4,5] demonstrate that ASD is also a genetically heterogeneous disorder. Both rare and common genetic variants have been shown to modulate disease risk and it is currently estimated that no single genetic cause of autism may account for more than 1–2% of ASD cases [4,5]. However, the increasing realization that single gene lesions can be sufficient in causing ASD suggests that the search for rare mutations associated with ASD will remain a powerful tool in deciphering the etiology of the disease [4,5]. Given the behavioral and genetic complexity underlying ASD, the task of selecting appropriate animal models in which to study the disease can be particularly challenging. Appropriate selection of animal

models of ASD starts from the premise that core autism features such as the deficits of social interaction and impaired communication can be adequately assayed in animals and that these behavioral deficits represent a good approximation of the underlying neurobiological processes disrupted in ASD.

In this review, I outline the significance of a non-human vocal learner, the zebra finch songbird species, in exploring the neural and genetic mechanisms subserving the capacity for learned vocal communication that songbirds share with humans but not traditional laboratory animals such as rodents or non-human primates. Given the powerful analogy between birdsong and human speech, studies probing the link between autism-susceptibility and language-related genes, and vocal learning, in the experimentally tractable songbird model, hold tremendous promise in identifying any unique or shared neuromolecular pathways enabling learned vocalization. Uncovering basic biological mechanisms at play during vocal development has in turn implications for designing controlled molecular and cellular studies to shed light onto the biological pathways disrupted in communication disorders like ASD.

2. Modeling autism features in animals

2.1. Genetic models of ASD

Due to their genetic tractability, rodent animal models of ASD have traditionally proven useful in probing the individual contribution of ASD-related genetic variants to brain development and function. At the same time, gene knock-out and gene knock-in rodent models have provided the opportunity to isolate the effect of specific genetic lesions on the performance of species-typical social behavior, using behavioral testing paradigms relevant for screening for autistic-like deficits. This approach has been successful in the study of both heritable syndromic forms of autism like Fragile X or Rett syndrome and monogenic non-syndromic forms of ASD [8]. In the latter case, for example, mice bearing genetic lesions of *SHANK3* (SH3 and multiple ankyrin repeat domains 3), a gene that encodes a postsynaptic protein present at glutamatergic synapses, exhibit excessive, repetitive grooming and reduced social interactions [6]. *SHANK3* mutant animals also show morphological defects of striatal medium spiny neurons, as well as functional impairments at corticostriatal synapses [6]. Mice lacking *NEUROLIGIN-4*

which encodes a cell adhesion molecule similarly show selective deficits of reciprocal social interactions and impaired communication, quantified as a decrease in ultrasonic vocalizations (USVs) compared to wild-type animals [7].

There are, however, clear limitations in relying exclusively on mouse models in the study of ASD pathogenesis. It is, for instance, unclear to what extent the deficits in communication (e.g. reduced USVs) and reduced social interactions observed in mutant mice can be exclusively circumscribed to an autism-like phenotype, as clearly distinguishable from the behavioral abnormalities assayed in animal models of other neurodevelopmental disorders, for example schizophrenia [8]. At the same time, as illustrated by the studies referred to above, mouse models bearing mutations in autism-susceptibility genes may display some, typically two out of three, but not all, of the core deficits that represent the clinical hallmark of ASD. Finally, given that the disruption in the social function of USVs, which are not known to be learned, may not represent a good approximation of the language regression and linguistic deficits associated with ASD, alternative animal models need to be developed in which to better assay ASD-related vocal communication deficits.

2.2. The songbird model of socially-learned vocal communication

Towards the goal of selecting animal models better suited to assay the functional consequences of genetic lesions in inducing autistic-like vocal communication deficits, songbird researchers can turn to a model system that has important advantages over traditional laboratory animals. For several decades, the zebra finch songbird species has represented the only experimentally tractable animal model for investigating the neural substrate for the formation and function of a specialized, socially-sensitive neural circuitry for vocal learning [9–11]. Vocal learning, the capacity to learn to produce new sounds for communication, is a rare trait across animal groups, with humans and songbirds being the best characterized exemplars. Importantly, traditional laboratory animals such as rodents and non-human primates, while rich with communicative behaviors, are not thought to learn their vocalizations. In songbirds, vocal learning is part of species-characteristic behavior and, in some species such as the zebra finch, is sexually dimorphic: during post-hatch brain development, male, but not female, zebra finch, develop a discrete set of interconnected brain nuclei

dedicated to the learning and production of song [9, 10]. Thus, songbird provides a significant advantage over traditional animal models by enabling the study of an important subcomponent of language, vocal learning, in an experimentally tractable species, with the potential to illuminate how language learning processes and social influences on these developmental processes may become disrupted in ASD.

Song learning and human language acquisition share several key features [12,13]. In both songbirds and humans, vocal learning: 1) takes place during critical developmental phases comprised of partially overlapping stages of sensory acquisition and sensorimotor practice; 2) relies upon social interactions; 3) requires specialized brain circuitry dedicated to the development and production of the new sounds. Thus, in addition to its innate vocalizations, during song development a young songbird learns one or more songs from an adult tutor, usually its father. Initially, sensory exposure to a tutor song allows young birds to generate a brain ‘template’ of the adult tutor song that they attempt to match during the sensorimotor practice stage of song development. Both sensory and sensorimotor phases of song learning are critically dependent on auditory feedback. Like human infants, juvenile songbirds need to hear themselves in order to match their vocal motor output to the tutor template. The initial vocalizations (subsongs) of young birds are highly variable and unstable, akin to the babbling of human babies [12,13]. Through social interactions with their tutor, juvenile songbirds then refine these vocalizations and their subsong gradually evolves into a plastic song and eventually, as they approach sexual maturity, into crystallized (stable) adult song. Although the unchanging song of adult zebra finches appears to be in marked contrast to the relatively limitless capacity of human language to connect new sounds to meaning, several other songbird species are capable of learning to produce new songs throughout life (e.g. mockingbirds). Further, adult zebra finch song is actively maintained by auditory feedback throughout life and gradually deteriorates in males that are deafened [11,12]. The same is true with the human speech in individuals who become deaf as adults [14].

Zebra finches possess cortico-basal ganglia-thalamic circuitry analogous to that of the human, dedicated to the production and modification of fine sequential articulations known as song. In the zebra finch, the brain areas responsible for song have been well-characterized, facilitated by their sexual dimorphism, and are collectively referred to as the song circuit [9, 10]. Subregions within the cortical-like pallium, the

basal ganglia and thalamus are prominent and interconnected only in male zebra finches. These include the specialized subregions whose neurons are exclusively dedicated to song within each brain region. The song circuit is comprised of two interconnected pathways that underlie the development and production of learned song (Fig. 1). Both pathways stem from the cortical-like (pallial) HVC (acronym used as its proper name), homologous to association cortex in humans. In the first, known as the vocal motor pathway, necessary for song production [15], auditory inputs enter the circuit at HVC, which contains neurons that send axonal projections to another cortical-like area known as the robust nucleus of arcopallium (RA). RA, homologous to primary motor cortex, in turn, projects to brainstem motor neurons that control the syrinx or vocal organ and respiratory muscles used in singing [16]. The second pathway, known as the anterior forebrain pathway (AFP), analogous to the cortico-basal ganglia loop of mammals, is required for song learning and modification [17–19]. The AFP begins with area X (homologous to mammalian basal ganglia), a nucleus in the songbird striatum essential for song learning, which projects to the dorsolateral portion of the medial thalamus (DLM) and then to neurons in the lateral magnocellular nucleus of anterior nidopallium (LMAN), a frontal cortical-like nucleus involved in song modification. The AFP intersects vocal motor pathway via the projection of LMAN to RA and receives input from the latter through the projection of HVC to area X [20–22]. The similarity between mammalian cortico-striato-thalamo-cortical loops and song circuitry extends beyond neuroanatomical connectivity to neuromodulation: area X receives dense dopaminergic input from midbrain nuclei [23], as well as glutamatergic input from cortical-like HVC and LMAN [24]. Similar inputs to the mammalian striatum are critical for motor learning and reward [25,26], suggesting that similar mechanisms might operate in songbird circuitry to reinforce and guide song learning.

3. Genetic basis of socially-learned vocal communication

3.1. Language-related genes and vocal learning

Thanks to the recent advances in the field of human neurogenetics, a growing list of molecular targets disrupted in human communication disorders [1,2] has supplied important candidate molecules to be tested in

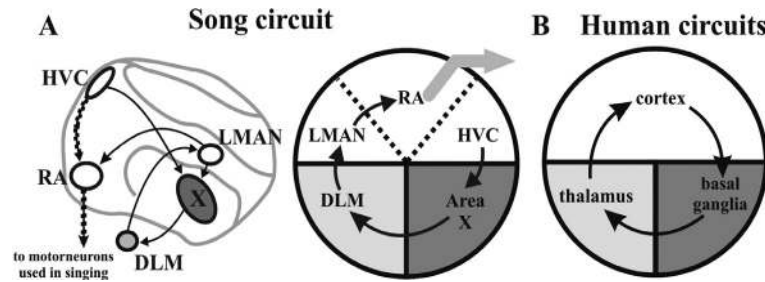


Fig. 1. Schematic comparison of connections within the song circuit and human cortico-basal ganglia-thalamo-cortical circuitry. (A) composite sagittal view of zebra finch telencephalon (left) and schematic view of the song circuit (right). (B) schematic view of the human cortico-basal ganglia-thalamo-cortical circuitry. The cortex and cortical-like pallium are shown in white, basal ganglia in dark gray, and thalamus in light gray. Auditory input (not shown) enters the song circuit at the HVC which relays it to the vocal motor pathway (stippled arrows) and the anterior forebrain pathway (AFP; plain arrows).

the songbird model, with the ultimate goal of revealing basic cellular and molecular underpinnings of human speech and language development, as well as relating these findings to key aspects of pathogenesis in ASD and related language disorders. As a first illustration of this principle, the discovery that a mutation in a single gene, encoding a transcription factor known as *FOXP2* (Forkhead box P2), can be directly linked to a severe speech and language disorder [27], has provided a unique opportunity to begin to dissect the genetic substrate of vocal learning using an experimentally tractable model system of socially-learned vocalization. Cross-species comparisons of gene expression between human and non-human vocal learners have indeed revealed that *FOXP2* has a remarkably similar pattern of distribution in the developing human and songbird brain, precisely within brain subregions specialized for the learning and production of vocalizations, including both cortical and striatal areas [28].

Importantly, as a transcription factor, *FOXP2* is expected to orchestrate the expression of many downstream gene targets, including those implicated in the patterning and functional connectivity of language-related areas. This prediction has been recently fulfilled by the discovery and validation of one of the first targets of *FOXP2* in human, contactin-associated protein-like 2 (*CNTNAP2*) [29–31], an autism susceptibility gene [32–34] that was previously shown to map within a language QTL interval [35]. Importantly, homozygous truncating mutations in *CNTNAP2* discovered in a group of Old Order Amish children diagnosed with cortical dysplasia-focal epilepsy (CDFE) syndrome were shown to result in severe deficits that included intractable seizures, language regression and other pervasive ASD-related developmental delays [36]. Subsequently, both common and rare genetic variants of *CNTNAP2* have been linked to ASD susceptibility [32–34],

including an association between *CNTNAP2* variation and a quantitative measure of language delay, age at first word [32]. In addition, *CNTNAP2* polymorphisms were significantly associated with nonsense-word repetition, a heritable marker of Specific Language Impairment [31], and copy number variants of *CNTNAP2* were shown to segregate with other complex psychiatric disorders such as schizophrenia [37].

The protein encoded by *CNTNAP2* is a member of the neuroligin superfamily of transmembrane proteins implicated in cell-cell communication in the nervous system. As a neuronal cell adhesion molecule, *CNTNAP2* is specifically implicated in neuronal-glia interaction via its well-described role in clustering Shaker-like voltage-gated potassium channels at the juxtaparanodal regions of myelinated axons [38–40]. Although its function in the peripheral nervous system is better understood, very little is known of the role of *CNTNAP2* in the central nervous system. Brain tissue resected from CDFE patients revealed abnormal patterns of neuronal migration and altered cytoarchitecture in cortical areas, consistent with a role in cortical histogenesis and laminar organization [36].

During development, *CNTNAP2* expression is enriched in human frontal cortex and basal ganglia comprising neural circuitry important for the development of language and other higher cognitive functions [41]. Surprisingly, cross-species comparisons among mammals demonstrated that *CNTNAP2* transcript distribution differs between vocal learners and non-vocal learners: its enrichment in language-related cortico-basal ganglia-thalamic circuitry in fetal human brain is in sharp contrast to a broad, non-punctuated distribution in developing mouse and rat brain at comparable embryonic stages [41]. This neuroanatomical specialization in humans is consistent with the genetic links to language, above. Together, these data provides strong

support for the hypothesis that *CNTNAP2* contributes to the regional cortical patterning and functional specialization of speech and language areas in the developing human brain. Importantly, the focal enrichment observed in human cortico-basal ganglia circuitry corresponds to a gestational stage prior to myelination, indicating that additional developmental roles of *CNTNAP2* in the central nervous system remain to be elucidated [41]. Consistent with the idea that *CNTNAP2* may play additional developmental roles, a recent study by Peñagarikano et al. [42] revealed that the autistic-like behavioral deficits of *CNTNAP2* knock-out mice, which included diminished social interactions and reduced USVs, are accompanied by defects in the patterns of neuronal migration of cortical projection neurons, as well as a reduction in the number of GABAergic interneurons.

3.2. *CNTNAP2* expression in songbird

Towards elucidating the role of *CNTNAP2* in the patterning of language-related brain areas and to investigate whether *CNTNAP2* is important for learned vocal communication in additional vocal learners, we performed a comprehensive study of *in situ* hybridization analyses to determine the distribution of *CNTNAP2* in songbird brain [43]. Using zebra finch-specific *in situ* riboprobes we determined that, within the male zebra finch brain, *CNTNAP2* transcript distribution punctuates the vocal learning circuitry, consistent with the sexual dimorphism of brain and song learning behavior [43].

In males, but not females, *CNTNAP2* transcript shows differential expression within song nuclei compared to surrounding regions which contain similar cell types [44,45]. *CNTNAP2* is markedly enriched in cortical-like (pallial) song nuclei LMAN, in the anterior forebrain, and RA, in the posterior forebrain. In striatal song nucleus area X, *CNTNAP2* transcript shows striking downregulation compared to surrounding tissue. The diminished expression in area X is particularly remarkable given that the cell packing density in this song nucleus is higher than in the outlying striatum [10]. Importantly, these changes in transcript levels are not observed in corresponding areas in the female striatum which lacks area X [9]. The differential expression of *CNTNAP2* in these brain subregions suggests that both upregulation and downregulation of transcript levels are important for the 'on-line' function of these specialized song-related areas in the adult male songbird.

During post-hatch development, the onset of sexually-dimorphic *CNTNAP2* expression takes place at 35 days in the case of song nucleus RA and \approx 40 days in the case of song nucleus LMAN [43]. The finding is remarkable given that these time points map onto the beginning of the sensorimotor practice phase of song learning, a stage when young male zebra finches begin to actively practice a tutor's song, suggesting that changes in *CNTNAP2* transcript abundance and distribution correlate with the formation and functional activation of these brain structures for song learning and production. Importantly, although in females *CNTNAP2* expression in song nuclei LMAN and RA shows a similar enrichment to that observed in males, after 35 days *CNTNAP2* transcript levels diminish dramatically in both of these female brain areas. In fact, the differential gene expression pattern observed between male and female brains after 35 days appears to closely track the divergent developmental trajectories of the song nuclei themselves which begin to regress in females precisely at the time (35 days) when they become synaptically connected in males [46,47]. Moreover, downregulation of transcript levels in area X which is observed throughout development and persists into adulthood, conversely indicates that low *CNTNAP2* transcript levels in this specialized striatal structure may enable its formation and participation in song learning processes. Together, these results provide support for the hypothesis that *CNTNAP2* is important both for the formation and functional specialization of song nuclei for vocal learning during post-hatch development in the male zebra finch. Finally, recent work from Condro et al. [48] consolidated these findings by revealing that the pattern of *CNTNAP2* protein distribution in the male songbird brain is remarkably similar to that established for mRNA, with high levels of protein detected in nucleus RA and low levels of protein detected in song nucleus area X throughout post-hatch development, demonstrating that the active form of the molecule is focally enriched or diminished, correspondingly, at identical key developmental time points during song learning [48].

3.3. *FMRP* expression in songbird

The relevance of the songbird model for probing the genetic substrate of human neural disorders of higher cognition and language has also been recently illustrated by a study by Winograd et al. [49] which focused on the expression of fragile X mental retardation protein (*FMRP*) in the zebra finch neural circuitry for song.

FMRP is encoded by *FMRI*, the gene mutated in fragile X syndrome (FXS), a common form of X-linked inherited mental retardation [50] that is associated with delays in speech and language development [51,52] and can show co-morbidity with autism [53]. Given the specific speech delays and deficits associated with FXS, the authors hypothesized that disruption of FMRP function is linked to impaired vocal learning and set out to characterize FMRP protein expression during vocal development and performance in songbird. Using a zebra finch specific antibody, the authors showed that FMRP was elevated at 30 days in male song nucleus RA, compared to surrounding telencephalic areas, and protein localization was restricted to neuronal cell types [49]. As in the case of *CNTNAP2*, the timing for these changes in FMRP abundance corresponds to the beginning of the sensorimotor practice stage of song learning, indicating a role for FMRP in the cellular and synaptic changes unfolding within premotor song nucleus RA at this key time point during song learning. The authors suggest that the pattern of FMRP expression during vocal development in songbird at a stage when song nucleus RA first becomes synaptically connected [46,47] indicates a role for FMRP in song circuit plasticity, with potential implications for understanding the mechanisms at play in the speech and cognitive deficits observed in patients with FXS. Finally, it is important to note that since the speech and language deficits are far better characterized for patients with FXS [51,52] than in the case of patients diagnosed with ASD, ongoing analyses of FMRP and *CNTNAP2* function in the songbird model may ultimately lead to complementary approaches in dissecting the molecular pathways underlying impaired vocal communication in the two language disorders.

3.4. From gene expression studies to *in vivo* functional investigations

Taken together, these findings demonstrate that comprehensive studies of gene and protein expression in the songbird model represent a fruitful approach in testing the involvement of language-related molecules in vocal learning. Because the processes of song learning can be easily manipulated in songbird, for example by manipulating the timing or length of exposure to a tutor, it becomes possible to tease out whether changes in gene expression are directly linked to the beginning or end of the critical period for song learning, and whether changes in transcript or protein distribution are responsive to social influences and behavioral state (e.g.

singing versus non-singing). In the case of *FOXP2*, Teramitsu and White [54] demonstrated that transcript levels can indeed be modulated ‘on-line’, such as when the adult birds sing, raising the possibility of ongoing, post-developmental roles for *FOXP2* in humans as well. In the case of *CNTNAP2*, the role of social influences and behavioral state in modulating transcript and protein levels in songbird neural circuitry for vocal learning, of potential relevance to understanding the social and communication deficits in ASD, remains to be investigated.

At the same time, by providing both key neuroanatomical sites and developmental time points for genetic intervention, determining the patterns of distribution of language-related candidate molecules in the songbird circuitry for learned vocalization can help guide functional *in vivo* analyses. Given that the formation of neural circuitry for song takes place during post-hatch development, targeted genetic intervention to manipulate gene expression of candidate molecules in select song nuclei is facilitated by the accessibility of these neural structures in the developing songbird brain. Moreover, following targeted genetic manipulation in song-related areas, ‘real-time’ functional assessment of the behavioral consequences of over- and underexpressing genetic constructs on vocal learning is enabled by reliable quantification of song vocalizations via dedicated sound analysis software.

Thus, despite the fact that songbirds are currently much less transgenically tractable than rodent models, controlled cellular and molecular studies relying on targeted genetic intervention are available in the zebra finch, and protocols for long-term transgene expression are currently being developed for songbirds [55]. As a first validation of these efforts, Haesler et al. [56] showed that lentivirally-mediated knockdown of *FOXP2* in area X of developing zebra finches prior to song learning results in incomplete and inaccurate imitation of tutor song. The authors demonstrated that virus-mediated RNA interference can be reliably used to alter gene levels in songbird circuitry at a critical time point during song learning.

Future functional *in vivo* approaches of genetic intervention in song circuitry are expected to similarly evaluate the cellular and behavioral consequences of targeted delivery of over- and underexpressing *CNTNAP2* genetic constructs during the formation of neural circuitry for song. As uncovered by the gene expression studies presented above, the targets for these genetic manipulations will include key nuclei specialized for song learning and production (LMAN, RA, area X).

Given that the cellular and synaptic changes that occur in these specialized brain areas during song learning are well-understood, targeted genetic intervention in these brain subregions should illuminate the role of *CNTNAP2* in key aspects of vocal development. Finally, it will be equally important to determine whether similar functional genetic links exist between *FOXP2* and *CNTNAP2* in songbird, as it has been shown in human, with *FOXP2* acting to dramatically repress *CNTNAP2* levels in human neuronal-like cell lines [31]. In particular, these latter functional analyses have the potential to ultimately uncover any human unique or shared neuromolecular pathways for speech and birdsong.

4. Conclusion

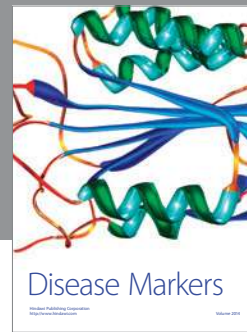
In summary, the songbird model offers an important advantage over traditional laboratory animals, in that it enables the study of vocal learning, a key sub-component of human language. Given its experimental tractability, the zebra finch songbird species is fully amenable to controlled molecular and cellular studies that are not ethical or practical in humans and that are expected to illuminate the biological pathways underscoring learned vocal communication. As a first test, the study of *FOXP2* and its transcriptional target in human, *CNTNAP2*, in the songbird model, revealed that the two language-related molecules are expressed at key locations and time points during the stages of song learning, suggesting a direct involvement in the sensorimotor processes underlying song learning and production. These findings now provide impetus for developing targeted functional investigations to probe the roles of these candidate molecules in the zebra finch neural circuitry for song. Moreover, the possibility that *FOXP2* and *CNTNAP2* may be functionally linked in songbird, as it has been shown in human, may ultimately reveal any unique or shared molecular and cellular pathways for speech and birdsong. Finally, controlled *in vivo* functional analyses probing the role of autism-susceptibility and language-related genes in the songbird circuitry for learned vocalization are expected to reveal basic molecular and cellular mechanisms at play in disorders of human speech and language such as ASD.

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