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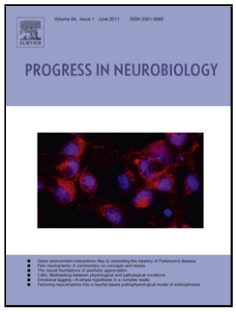

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Neurofilament-Lysosomal Genetic Intersections in the Cortical Network of Stuttering

Claudia Benito-Aragón[1,2#], Ricardo Gonzalez-Sarmiento[1,2#], Thomas Liddell[1,3], Ibai Diez[1,4], Federico d'Oleire Uquillas[5], Laura Ortiz-Terán[1,6], Elisenda Bueichekú[1,7], Ho Ming Chow[8,9], Soo-Eun Chang[8,10&], Jorge Sepulcre[1,11*&]

[1]Gordon Center for Medical Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

[2]University of Navarra School of Medicine, University of Navarra, Pamplona, Navarra, Spain

[3]University of Exeter, Exeter, England, UK

[4]Neurotechnology Laboratory, Tecnalia Health Department, Tecnalia, Derio, Basque Country, Spain

[5]Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

[6]Department of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

[7]Neuropsychology and Functional Neuroimaging Group, Department of Basic Psychology, Universitat Jaume I, Castellón, Spain

[8]Department of Psychiatry, University of Michigan, Michigan, USA

[9]Katzin Diagnostic and Research PET/MRI Center, Nemours/Alfred I. duPont Hospital for Children, Wilmington, DE, USA

[10]Cognitive Imaging Research Center, Department of Radiology, Michigan State University, East Lansing, MI, USA

[11]Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA.

*****Corresponding and Lead Author: Jorge Sepulcre. 149 13th St, Suite 5.209, Gordon Center for Medical Imaging, Department of Radiology, Massachusetts General Hospital, Charlestown, MA 02129. sepulcre@nmr.mgh.harvard.edu

[#]Shared first author contribution. [&]Shared last author contribution.

Highlights

- We identify the functional connectivity network that characterizes stuttering.
- We describe the topological similarity of the stuttering cortical network with genetic expression levels from the protein-coding transcriptome data of the Allen Human Brain Atlas.
- GNPTG significantly co-localizes with the stuttering cortical network.
- Our findings support that lysosomal-related genes, such as GNPTG, intersect with neurofilament-related genes, which may explain the intriguing link between lysosomal mutations and the presence of stuttering.

Abstract

The neurobiological underpinnings of stuttering, a speech disorder characterized by disrupted speech fluency, remain unclear. While recent developments in the field have afforded researchers with the ability to pinpoint several genetic profiles associated with stuttering, how these specific genetic backgrounds impact neuronal circuits and how they generate or facilitate the emergence of stuttered speech remains unknown. In this study we identified the large-scale cortical network that characterizes stuttering using functional connectivity MRI and graph theory. We performed a spatial similarity analysis that examines whether the topology of the stuttering cortical network intersects with genetic expression levels of previously reported genes for stuttering from the protein-coding transcriptome data of the Allen Human Brain Atlas. We found that GNPTG – a gene involved in the mannose-6-phosphate lysosomal targeting pathways –

was significantly co-localized with the stuttering cortical network. An enrichment analysis demonstrated that the genes identified with the stuttering cortical network shared a significantly overrepresented biological functionality of Neurofilament Cytoskeleton Organization (NEFH, NEFL and INA). The relationship between lysosomal pathways, cytoskeleton organization, and stuttering, was investigated by comparing the genetic interactome between GNPTG and the neurofilament genes implicated in the current study. We found that genes of the interactome network, including CDK5, SNCA, and ACTB, act as functional links between lysosomal and neurofilament genes. These findings support stuttering is due to a lysosomal dysfunction that impart deleterious effects on the neurofilament organization of the speech neuronal circuits. They help in solving the intriguing unsolved link between lysosomal mutations and the presence of stuttering.

Keywords: Stuttering, Genetics, Cortical Network, Lysosomal, Neurofilament.

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Introduction

Persistent developmental stuttering is a speech disorder characterized by disruption in speech production fluency that has a typical but not exclusive onset during early childhood (2-4 years old) (Craig-MCQuaide et al. 2014; Yairi and Ambrose, 2013). The incidence and prevalence of stuttering varies according to age group (Craig et al. 2002; for a review please see Yairi and Ambrose, 2013). Lifetime incidence was estimated in 5% by Andrews and Harris (1964) but more recent investigations have given higher figures ranging from 5% to 18% (Mänsson, 2000; Felsenfeld et al. 2000; Craig et al. 2002; Dworzynski et al. 2007; Reilly et al. 2009). In the recent literature, prevalence has been summarized to be around 1% (Bloodstein and Ratner, 2008). In relation to prevalence, Yairi and Ambrose (2013) highlight how differences in prevalence are found depending on the age-group with figures ranging between 0.3% and 5.6% (Okalidou and Kampanaros, 2001; McLeod and Harrison, 2009; Proctor et al. 2008; McKinnon et al. 2007; van Borsel et al. 2006; Craig et al. 2002). There is a wide range in recovery rates reported, from 50% to 94% (Mänsson, 2000; Ryan, 2001; Craig et al. 2002; Mänsson, 2005; Dworzynski et al. 2007; Howell and Davis, 2011). Primary speech symptoms of stuttering include interruptions in normal fluency and time patterning of speech. Repeated occurrences of the following appear: sound and syllable repetitions, sound prolongations, interjections, broken words, silent blocking, circumlocutions, speech accompanied by physical tension and/or monosyllabic whole-word repetitions (American Psychiatric Association, 2013).

There have been various theoretical and therapeutic hypotheses regarding the etiology of stuttering. Initially, clinicians searched for a relationship between anxiety and stuttering (Agnello, 1962; Santostefano, 1960). Consequently, some researchers delved into the possibility of psychiatric causes (Cantwell and Baker, 1977; Weber, 1965). With the advent of neuroimaging techniques, a paradigm shift arose implicating neuroanatomical factors and brain connectomic findings (Chang et al., 2015; Cieslak et al., 2015; De Nil et al., 2001; Wells and Moore, 1990; Brown et al., 2005). For instance, stuttering has been consistently associated with gray matter changes in the supplementary motor area (SMA), the primary motor area, the inferior frontal gyri, the pars opercularis (Brodmann area [BA] 44), the classical Broca and Wernicke areas, the superior temporal gyri, the subcentral area (BA 43), the insula, the precuneus, the basal ganglia-thalamo-cortical loop, the cerebellum and has more recently been associated with the default mode network, as well as changes in axonal tracts innervating motor,

auditory and perisylvian areas of the frontal and parietal lobes (Braun et al., 1997; Chang et al., 2015, 2018; Chang and Zhu, 2013; Fox, 2000; Fox et al., 1996; Ingham et al., 2012; Jiang et al., 2012; Lu et al., 2010, 2009; Neef et al., 2018; Neumann et al., 2005; Sakai et al., 2009; Wu et al., 1995). More recently, research has focused on a novel interpretation of this speech condition: the genetic foundations of stuttering (Drayna and Kang, 2011; Kang et al., 2010; Raza et al., 2016). Speech and language development has been associated to genetic expression of genes such as FOXP2, ROBO1, CNTNAP2, KIAA0319, DCDC2, SLC6A3/DAT, DRD2, AP4E1 and ELKS/ERC1, of which mutations relate to developmental verbal dyspraxia, specific language impairment, dyslexia, speech sound disorder, and attention deficit hyperactivity disorder (Bates et al., 2011; Chen et al., 2014; Deriziotis and Fisher, 2013; Lai et al., 2001; Lan et al., 2009; Morgan et al., 2016; Pennington and Bishop, 2009; Petrin et al., 2010; Raza et al., 2015; Eising et al., 2018). Importantly, recent breakthroughs describe that specific genetic variants in GNPTAB, GNPTG and NAGPA – all related to lysosomal processes and known to cause mucolipidosis type II and III autosomal recessive homozygous mutations – are specifically linked to cases of stuttering (Frigerio-Domingues and Drayna, 2017; Kang et al., 2010; Kang and Drayna, 2012; Raza et al., 2016). Taken together, the underpinnings of stuttering may involve key intersections between specific genetic backgrounds, such as lysosomal pathways, and brain connectomic changes. However, how stuttering-related genes influence those neuronal circuits in order to generate stuttering speech is still not fully understood.

Our study had two goals. Firstly, we aimed to describe the large-scale cortical network that characterizes people who stutter (PWS) using functional connectivity MRI and novel approaches based on graph theory. Secondly, we examined how the cortical network of PWS relates to genetic expression patterns in the human brain, using previously reported stuttering-related gene sets, the protein-coding transcriptome data of the Allen Human Brain Atlas (AHBA; Shen et al., 2012), and genetic enrichment and interactome analyses. We hypothesized that connectivity features defining the stuttering network co-localize with genetic expression levels of genes conferring risk and vulnerability for neuronal dysfunctionality during speech production. In other words, we postulate that a high overlap between the stuttering brain network and the topology distribution of certain genes could suggest a plausible contribution to brain-circuit vulnerabilities in stuttering. Overall, answering these questions may help bridge the gap

in understanding the relationship between lysosomal metabolic pathways and brain connectivity changes underlying stuttered speech.

Methods

Meta-Analysis for Regions of Interest Identification

We used an integrated meta-analysis strategy to accomplish our aim of characterizing the cortical network of PWS based on available research data. This approach allowed us to obtain reliable evidence of stuttering related areas without introducing bias toward the arbitrary inclusion or exclusion of specific regions of the human brain. Using Activation Likelihood Estimation (GingerALE 2.3.6; Eickhoff et al., 2012, 2009; Turkeltaub et al., 2012), we performed a meta-analysis of previous literature that used functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) to examine differences in brain activation between PWS and normally fluent controls (NFC). GingerALE is a meta-analytic technique that reveals foci of concordant results among a wide range of brain imaging studies by taking the maximum activation probabilities and testing them versus a null hypothesis of spatially independent activations using a random-effects analysis (Eickhoff et al., 2009). All the studies included in the meta-analysis were searched in PubMed and Web of Science using the terms "stutter + task + fMRI" and "stutter + task + PET" (January, 2017). Inclusion criteria included: (i) published in a peer-reviewed scientific journal; (ii) use of fMRI or PET techniques to measure activation; (iii) comparison of task activation between PWS and NFC groups; and (iv) reporting of coordinate-based data in standard stereotaxic space (whether Talairach or MNI). We did not include single-subject studies, and both activation changes, increased or decreased activations, were included. Out of 36 potential studies, 11 conformed to all inclusion criteria (**Supplementary Table 1**; De Nil et al., 2000; Stager et al., 2003; Preibisch et al., 2003; De Nil et al., 2008; Watkins et al., 2008; Chang et al., 2009; Ingham et al., 2012; Liu et al., 2014; Lu et al., 2016; Harrewijn et al., 2017; Yang et al., 2017). The studies comprised of 20 different experiments, with 430 total participants (214 cases and 216 controls; smallest sample size of a single study was 26, with an average of 39 subjects per study across all 20 experiments). In the fMRI category, 8 studies were used (15 tasks in total), while in the PET category, 3 studies (5 tasks in total) were used. To customize our exploratory detection toward candidate regions of interest related to stuttering, we used a liberal threshold of $p<0.001$ in GingerALE (Eickhoff et al., 2012, 2009; Turkeltaub et al., 2012).

Participants and Functional Connectivity Imaging

We included several sets of participants for the characterization of the stuttering network via functional connectivity MRI: (1) a healthy adults dataset (n=100 from the Brain Genomic Superstruct Project (Holmes et al., 2015); mean age of 21.2; 51% male; all right handed), (2) a child developmental stuttering dataset (N=31; mean age of 6.45; 52% male; all with persistent stuttering), and (3) two control samples matched to the child stuttering sample (n=39 and n=24; mean age of 6.24; 41% male). Healthy adults subjects provided written informed consent in accordance with the Helsinki Declaration and guidelines set by institutional review boards of Harvard University, Partners Healthcare, and Michigan State University. Please find in **Supplementary Materials** the details regarding MRI data acquisition and data pre-processing for all the sets of participants included in the study.

Stuttering Network Characterization: Strategy 1 and 2

To characterize the functional connectivity network associated with stuttering regions of interest, we implement a whole-brain graph theory approach (diagram in **Fig. 1-I**). For all participants in the study, we first computed Pearson's r correlation coefficients between all pairs of voxels across the brain using the time course of low-frequency blood oxygen level-dependent (BOLD) fluctuations in a brain mask of 4652 (n) voxels (n x n association matrix) (Laura Ortiz-Terán et al., 2017). Pearson's r correlation association matrices were corrected using a false discovery rate threshold (FDR) at a q level of 0.001 (Benjamini and Hochberg, 1995) to discard false positive connections from the data. Finally, we applied two complementary graph theory strategies to the association matrices focused on the regions of interest ascertained in our meta-analysis. Specifically, we calculated all functional connections of brain voxels that reach the stuttering-related regions of interest (called targets in diagram of **Fig. 1-I**). In graph-theory strategy 1, we computed the weighted degree centrality of all brain voxels by summing their functional connections that reach any target voxel (Equation 1). In graph-theory strategy 2, we detected brain voxels (called interconnectors in diagram of **Fig. 1-I**) for which functional connections reach multiple targets, from 100% to 0% if they reach all or none of the stuttering regions of interest (Equation 2). Please note that strategy 1 is tantamount to the zero % condition in strategy 2. Both approaches generated cortical maps that detected cortical regions characterized by different levels of connections to stuttering-related areas. Importantly, we used these

two strategies to reveal the underlying network associated with stuttering in NFC, and to later assess connectivity alterations in PWS compared to matched controls.

Equation 1:

$$WD_i = \sum_{s=1}^{m} FC(i, s)$$

Where i is the specific voxel to compute the weighted degree, s represents the target's index, m the total number of targets and FC the functional connectivity matrix FDR-corrected for multiple comparisons.

Equation 2:

$$WD_i = \sum_{s=1}^{m} FC(i, s) \quad \left[\frac{(\sum_{s=1}^{m} FC_b(i, s))}{m} \geq thr\right]$$

Where FC_b is the binarized version of FC, and thr is the threshold value ranging from 0 to 1 to define the specific number of targets reached by the voxel's connectivity (0%-100% of targets or stuttering-related regions of interest). A thr of 0 is equivalent to the initial WD condition in Equation 1 and a value of 1 is equivalent to voxels that are connected to all targets simultaneously.

Overlap Between Stuttering and Language Networks

We investigated the correspondence between the stuttering-related cortical network with other language-related networks (Sepulcre, 2015). Particularly, we spatially compared the topological distribution of the connectivity map obtained from our graph theory strategy 1 with five cortical masks, namely, from auditory-motor integration, Wernicke's, Broca's F3 opercularis, Broca's F3 triangularis, and Broca's F3 orbitalis networks (Sepulcre, 2015). We obtained an overlap index of the stuttering connectivity map versus the rest of the language-related connectivity maps by extracting the intensity of the weighted degree centrality score in the stuttering network divided by the size of the corresponding mask.

Brain Co-Localization Between the Stuttering Network and Gene Expression

We used aprioristic knowledge of previously described genes linked to stuttering cases (Frigerio-Domingues and Drayna, 2017; Kang et al., 2010; Kang and Drayna, 2012; Raza et al., 2016), as well as data-driven approaches based on the full genome-

wide (protein-coding) transcriptome of the AHBA, to search for cortical genetic profiles underlying the neurobiological basis of stuttering [based on approaches developed in (Diez and Sepulcre, 2018; L. Ortiz-Terán et al., 2017; Sepulcre et al., 2018)]. Firstly, we investigated genes that were strongly associated to stuttering, such as CNTNAP2, GNPTAB, GNPTG and NAGPA, as well as genes that were strongly associated to speech and language development, such as FOXP2, ROBO1. Reference genetic expression levels were obtained from the AHBA (French and Paus, 2015) (diagram in **Fig. 1-II**). Furthermore, we used an anatomical transformation of the AHBA transcription profiles (20,737 protein-coding genes, based on 58,692 measurements of gene expression in 3,702 brain samples from 6 adult human participants) in 68 pre-specified brain regions of the Desikan-Killiany atlas (Desikan et al., 2006) covering the entire cortex (diagram in **Fig. 1-II**). We investigated the spatial topological similarity between stuttering-related and language-related genetic expression data by computing the Euclidean distance between corresponding vectors (see Euclidean distance formula in **Fig. 1-III**), where n is each of the 68 Desikan atlas regions, vector g represents the 68 gene expression values for each gene, and vector s is the stuttering network in the form of mean connectivity values of all voxels in each of these 68 regions. We used a hierarchical clustering approach and Silhouette criterion to assess the optimal cluster number and cluster organization between stuttering-related and language-related genes (clustergram function in MATLAB with Euclidean-distance and average-linkage settings). Secondly, the Desikan-Killiany atlas was used to convert the stuttering network map into the same space as the AHBA data. Each voxel of the stuttering network map –connectivity-derived data- was assigned to a region of the Desikan-Killiany atlas (68 regions), later, the mean value for each region was calculated. Then, we analyzed the spatial topological similarity between the stuttering imaging phenotype and the entire transcriptome of AHBA data (cortical gene expression levels of 20,737 genes). Thus, we built a null hypothesis distribution based on the entire protein-coding transcriptome in which we computed the p-value of similarity between the stuttering imaging phenotype and specific genes. We considered two standard deviations above the transcriptome mean as the statistically significance level (Diez and Sepulcre, 2018; L. Ortiz-Terán et al., 2017; Sepulcre et al., 2018). This approach also served to obtain the set of genes for data-driven genetic functionality and gene ontology assessments.

Gene Ontology Analysis and Interactome

We used an over-representation analysis to gain knowledge-guided insight into the possible biological processes or cellular components associated with the stuttering connectomic-genetics interactome. To that end, we employed the data-driven gene set obtained from the previous step and Gene Ontology (GO; Ashburner et al., 2000); by introducing the list of genes in the online GO software (http://geneontology.org). We used GO binomial tests to describe the genetic annotation-based functionality of associated biological processes or cellular components, specifying the "homo sapiens" as the reference list and the data-driven gene list from the previous step as the analyzed list (FWE correction at $p<0.05$ level, and >10-fold over-representation). Moreover, we used an interactome analysis to investigate genetic interactions among the identified set of genes in order to investigate evidences of genetic functional relationships and interactions beyond their spatial co-localization in the cortex (based on Genemania software; http://www.genemania.org; Mostafavi et al., 2008; and Cytoscape software; www.cytoscape.org; Lopes et al., 2011). Weight of genetic associations were based on interaction profiles from co-expressions, co-localizations, genetic interactions, pathways, predicted physical interactions, and shared protein domains (Mostafavi et al., 2008). Finally, we performed a node-level betweenness centrality assessment to identify the specific roles of genes of interest (Seeds/Goals of interest in **Fig. 5**; GNPTG, INA, NEFL, NEFH; please see Results sections for details). Betweenness centrality was computed using the next formula:

$$b(v) = \sum_{i \neq v \neq j} \frac{\sigma_{ij}(v)}{\sigma_{ij}}$$

Where σ_{st} is the total number of shortest paths from i to j and σ_{ij} (v) total number of shortest paths passing through node v.

Visualization

We used Caret v5.65 software to represent the results in a three dimensional Population-Average Landmark and Surface-based (PALS) surface (PALS-B12) using the "enclosing voxel algorithm" and "fiducial and flat mapping" settings (Van Essen et al., 2001). We used Cytoscape software for network visualization of the interactome analysis (Lopes et al., 2011).

Results

Cortical Network Underlying Stuttering

Our imaging meta-analysis identified regions of interest related to stuttering in several candidate locations in the cortex (see binary map, **Fig. 2-I**, top), namely, in the bilateral precentral gyrus, left superior temporal gyrus, bilateral middle temporal gyrus, bilateral medial frontal gyrus, right anterior cingulate cortex, left insula and bilateral inferior parietal lobe. We also found two clusters outside of the cortical mantle, one in the right putamen, and the other one in the right VIII region of the cerebellum (these two regions are not visualized in the cortical maps of **Fig. 2** but are included in all analyses).

Next, we characterized the underlying network connectivity of stuttering-related regions of interest in NFC, both adults and children. We found a high degree of significant connectivity between stuttering-related regions in bilateral auditory, motor and perisylvian areas (Strategy 1 in **Fig. 2-I**, bottom left and right), particularly in the frontal and parietal operculums, ventral precentral and postcentral gyri, superior temporal gyri, anterior insula, and Heschl's gyri, as well as midline regions such as the supplementary motor area and middle cingulum. Importantly, several of these regions have roles of interconnectors between stuttering-related areas (Strategy 2 in **Fig. 2-I**, bottom left and right), particularly frontal and parietal operculum regions such as OP4 (see inset; Strategy 2 in **Fig. 2-I**, bottom left and right). To confirm these findings, we used an independent analysis in which, in this case, we compared the Strategy 1 voxel-level weighted degree between children who stutter (CWS) and NFC groups (**Fig. 2-II**). Congruently with the findings from the meta-analysis derived characterization (**Fig. 2-I**), we found that connectivity among stuttering-related regions is disrupted in the aforementioned areas (CWS<NFC, corrected $p<0.05$; **Fig. 2-II**). The CWS>NFC contrast did not yield any significant results. We thus avoided the inclusion of any a priori network potentially involved in speech production. **Supplementary Figure 1** shows an additional comparison between CWS and NFC using voxel-level weighted degree centrality values without any region of interest or meta-analysis derived areas of interest. This data-driven strategy yielded converging results with meta-analysis based findings.

The stuttering connectivity network shared cortical topologies with other large-scale language-related networks (**Fig. 3-I** and **3-II**). Auditory-motor integration areas,

Broca's F3 opercularis, and Wernicke's area, highly overlap with the stuttering network (**Fig. 3-III**).

Stuttering Network Topology Intersects with Cerebral GNPTG Gene Expression

Our analysis of cortical similarity between gene expression levels of stuttering and language-related genes revealed that NAGPA, GNPTG, GNPTAB and CNTNAP2 – particularly NAGPA, GNPTAB and CNTNAP2 – displayed a high resemblance (dark squares in co-expression matrix; **Fig. 4-I**), while ROBO1 and FOXP2 formed a separate cluster (grey squares in co-expression matrix; **Fig. 4-I**). Importantly, when all transcriptome data was taken into account for the similarity assessment of the stuttering connectivity map, only GNPTG reached statistical significance among the stuttering- and language-related genes (see GNPTG in the null hypothesis histogram; **Fig. 4-II**). **Supplementary Figure 2** displays the gene expression levels of GNPTG and histograms of similarity scores between the stuttering network and individual transcriptome data of all AHBA donors. GNPTG gene expression levels showed a high distribution of similarity with the stuttering connectivity map (**Fig. 4-II** and **4-III**), particularly in auditory-motor integration areas (star symbols in **Fig. 4-III**).

The Stuttering Network Relates to Lysosomal and Neurofilament Functionality

The cortical similarity analysis also revealed that other genes (**Supplementary Table 2**), beyond GNPTG, displayed statistically significant similarities with the stuttering connectivity map (read line in x axis; **Fig. 4-II**). In order to assess whether an overrepresentation of genetic functionalities existed in this gene set, we performed a GO analysis targeting biological processes. We found that the genetic expression co-localized with the stuttering cortical network was highly enriched not only in lysosomal related functions, such as protein localization to lysosome (green highlight in **Fig. 4-IV**), but also in neurofilament cytoskeletal organization (red highlight in **Fig. 4-IV**), specifically involving NEFH, NEFL and INA genes. Other biological functions related to general cellular processes were found as well, such as mitochondrial related transport, protein-membrane targeting, and glycolysis (see the complete list of biological processes in **Fig. 4-IV**).

As our main findings showed that a genetic profile related to lysosomal and neurofilament genes may be involved in the brain pathophysiology of stuttering, we explored whether GNPTG, NEFH, NEFL and INA genes displayed genetic interactions

beyond the spatial cortical domain using an interactome analysis. We found that GNPTG (green node in **Fig. 5-I**) interlinks with NEFH, NEFL and INA (grey nodes in **Fig. 5-I**) through specific genetic inter-players (red and orange nodes in **Fig. 5-I**). Of relevance, CDK5 and SNCA showed a high betweenness centrality supporting genetic influences between GNPTG and neurofilament organizational genes (**Fig. 5-I** and **5-II**).

Discussion

Language and speech form the pillars of human spoken communication, and with the advent of modern neuroimaging technology, recent studies have provided enough novel insights into the neurobiological basis of stuttering to form a foundation for the study of brain anatomical and functional variants underlying this condition (Chang and Zhu, 2013; Fox, 2000; Fox et al., 1996; Ingham et al., 2012; Jiang et al., 2012; Lu et al., 2010; Neef et al., 2018; Wu et al., 1995). Most recently, specific genetic traits have been linked to cases of stuttering, offering new opportunities to better understand the neurobiological basis of this speech condition (Kang et al., 2010; Raza et al., 2016). In this work, we provide a substantial advancement in the understanding of stuttering by integrating neuroimaging and genetic research approaches. Specifically, we combined stuttering-related connectomic findings with gene expression levels in the human cortex, and found that alterations in functional connectivity network organization of stuttering-related brain regions were spatially co-localized with cortical expression levels of a lysosomal gene, GNPTG, whose mutations have been linked to stuttering.

To date, convergent findings of functional and structural neural anomalies across both adults and CWS have been localized to left hemisphere perisylvian structures and connectivities among them, including those affecting auditory-motor integration for speech control (Cai et al., 2014; Chang et al., 2018; Neef et al., 2017). The stuttering neural network identified through the graph theory approach taken in this study corroborate these previous results. Differences that do exist between adults and CWS have mostly been reported in right hemisphere homologues- that is, hyperactivity and increased structural volume in perisylvian areas of the right hemisphere have been found in adults who stutter (Foundas et al., 2004; Belyk et al., 2015), and are not consistently reported in CWS (Chang et al., 2008; Chow and Chang, 2017). The literature on the neural bases of childhood stuttering is extremely limited due to the lack of research in this area, although examining children close to symptom onset is recognized as a critical need to better understand vulnerable neural networks associated with stuttering. The current analyses thus focused on examining a relatively large dataset acquired from young CWS. Thus, the stuttering neural network identified here is thought to better represent neural networks that are closely associated with stuttering pathophysiology and less influenced by compensatory processes that are likely present as a result of decades of stuttering in adults who stutter.

Our characterization of the stuttering network shows that connectivity in perisylvian areas are of key relevance to the stuttering condition. In the past, it has been hypothesized that structural connectivity disruption along the arcuate fasciculus, which links the Broca and Wernicke areas and lays beneath perisylvian areas, would explain stuttering (Chang et al., 2015; Cieslak et al., 2015). Auditory-motor integration dysfunctions have also been commonly attributed to stuttering. Our analysis on brain connectivity found that the stuttering network substantially overlaps with the Broca's F3 opercularis and Wernicke's networks, as well as the auditory-motor integration network. Importantly, we found that the stuttering network also significantly co-localizes with the distribution of expression of the GNPTG gene, most notably within the auditory-motor integration areas. We postulate that co-localization between an imaging phenotype, the stuttering functional connectivity network, and genetic expression levels of a given gene, indicates possible causal relationships between the two. In other words, mutations in a specific gene that is embedded within the same cortical topology of a cerebral network modulates the functionality of that network. Our findings suggest that the auditory-motor integration network, predominantly in the fronto-parietal operculum regions (such as the OP4 region), may be highly vulnerable to neuronal circuit dysfunctions associated with GNPTG-lysosomal malfunctioning.

Prior investigations have revealed that genes involved in the mannose-6-phosphate lysosomal targeting pathway, namely GNPTG, GNPTAB, and NAGPA, are likely related to etiological causes of stuttering (Kang et al., 2010; Kang and Drayna, 2012; Raza et al., 2016). GNPTG, located on chromosome 16, encodes for the gamma subunit N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) enzyme, while GNPTAB-located on chromosome 12-encodes the alpha and beta subunits of the same enzyme. NAGPA-located on chromosome 16-encodes for a second enzyme in the mannose-6-phosphate lysosomal targeting pathway called the GlcNAc-1-phosphodiester-N-acetylglucosaminidase enzyme. Mutations in GNPTAB were thought to cause stuttering, but such a relationship was only evident in around 10% of cases (Drayna and Kang, 2011). Further investigations concluded that mutations in GNPTAB, GNPTG and NAGPA together could only account for 16% of persistent stuttering cases (Raza et al., 2016). They also hypothesized that variations in GNPTAB and GNPTG involved in stuttering were at different sites than those mutations causing mucolipidosis type II (alpha-beta) or type III (alpha-beta-gamma). Recent studies using homozygosity mapping and Sanger sequencing have found 3 variations that co-segregate with

stuttering in the families studied (Kazemi et al., 2018). These variations included two variants of GNPTAB – one, which had never been accounted for in stuttering but had been reported in mucolipidosis type II in homozygosity – and a variation of GNPTG. In our study, we found that genetic expression levels of mannose-6-phosphate lysosomal targeting pathway genes are closely distributed across the human cortex. GNPTG, GNPTAB, and NAGPA, as well as CNTNAP2, a gene encoding a neuronal trans-membrane protein of the neurexin superfamily involved in neural-glia interactions and potassium channels in myelinated axons, were co-expressed in similar brain locations, while other language-related genes co-expressed together, such as FOXP2 and ROBO1, but not with the lysosomal genes or CNTNAP2.

Despite a link between speech development and specific gene expression being suggested as early as in the 1960s, the FOXP2 gene commonly associated with speech and language development was not discovered in the form we know until 2001 (Lai et al., 2001). Since, research has revealed a more detailed understanding of the involvement of FOXP2 in speech and language development and its relationship to language disorders (Morgan et al., 2016). This initial proof of a genetic link to speech paved the way for further genetic influences to be hypothesized, such as the ROBO1 gene (Bates et al., 2011) which is involved in infant speech development, an insufficiency of which may be related to dyslexia (a disorder often associated with language development impediments; Hannula-Jouppi et al., 2005). The CNTNAP2 gene, associated to language impairment and dyslexia, is also thought to be a gene of interest with regard to stuttering (Petrin et al., 2010). However, conflicting studies suggest that CNTNAP2, along with the FOXP2 gene, may have little impact on persistent stuttering (Han et al., 2014). Based on our co-localization results, our findings support the latter view. We could confirm a significant association between GNPTG and the stuttering network among all the previously described candidate genes, and no language-related FOXP2, ROBO1, CNTNAP2 nor other lysosomal genes (other than GNPTG) were corroborated in our study. Further research is thus needed in this regard.

Following our main assumption that co-expression of genes across the cortex relates to functional network connectivity, we used a data-driven approach to investigate whether expression of a large gene set shares a similar spatial distribution to a connectivity network related to stuttering. This gene set showed an overrepresented biological functionality in two relevant domains for stuttering: 1) protein localization to lysosome, and 2) neurofilament cytoskeleton organization.

In the past, researchers struggled with how to interpret a lysosomal dysfunctionality that creates impairment in the fluency of speech. Our findings support the premise that mutations in the lysosomal processing pathway may induce alterations in other genetic functionalities dwelling in the same cortical areas, the most important being neurofilament organization. Thus, we believe that a lysosomal dysfunctionality directly influences neuronal circuits via a deleterious effect on neurofilaments, which in turn would be responsible for impaired functional connectivity between stuttering-related regions. Moreover, after our genetic interactome analysis between GNPTG and the neurofilament genes (NEFH, NEFL and INA), our study suggests that two important intermediaries, CDK5 and SNCA, may play critical roles in the damaging relationship between lysosomal dysfunction and neurofilaments. CDK5 phosphorylates KSPXK motifs; neurofilament heavy (NF-H – product of NEFH) contains 34 repeats of this motif, making it a great substrate for CDK5. This phosphorylation of KSP repeats reveals a transport regulation mechanism, those with fewer phosphorylated motifs correlated with faster transport (Grant et al., 2001; Sun et al., 1996). Moreover, GNPTG is involved in the mannose-6-phosphate lysosomal targeting pathway, whose role is to tag lysosomal enzymes with a mannose, directing them towards endosomes and further on to lysosomes. An enzyme that is delivered to lysosomes through this route is cathepsin D (CTSD). CTSD is a protease in charge of degrading old proteins including alpha-synuclein, a product of the SNCA gene and one of the intermediary genes found in the current study (Bourdenx et al., 2014; Miura et al., 2014). We speculate that interference in the mannose-6-phosphate pathway might predispose to abnormal alpha-synuclein degradation and possible accumulation.

Exploring the relationship between SNCA and neurofilament integrity, recent studies revealed that injection of aggregated alpha-synuclein induced inclusions of the neurofilament light (NF-L – product of the NEFL gene), and some even proposed the increase of NF-L as a biomarker in cerebrospinal fluid and blood plasma for various diseases, including alpha-synucleinopathies (Bacioglu et al., 2016; Sacino et al., 2014). Therefore, studying the functional alterations related to CDK5 and SNCA may help explain how lysosomal dysfunction of GNPTG induces aberrant effects in neurofilaments of the stuttering network.

In conclusion, we report novel findings that help bridge between functional neural networks and gene mutations previously linked to stuttering. Based on combined analysis of functional connectivity MRI data from both children and adults who stutter

and gene expression maps, we report that stuttering-related functional connectivity networks co-localized with gene expression of the lysosomal trafficking gene GNTPG. Mutations of this gene and other similar genes embedded within the same cortical topology of cerebral networks suggest that these mutations could modulate the function of these networks. Our findings point to the auditory-motor integration network as highly vulnerable to neuronal circuit dysfunctions associated with GNPTG-lysosomal malfunctioning. These results provide first evidence of possible causal links between gene mutations and aberrant brain connectivity in stuttering, and further, suggest biological pathways associated to neurofilaments that may help explain the neural mechanisms resulting in persistent developmental stuttering.

Limitations

The evidences found here are constrained to available descriptions of the genetic profiles from six donors of the AHBA atlas, and their topological similarities with independent neuroimaging samples of NFS and PWS. However, data supporting the genetic foundations of the organization of the human cortex, as well as the connectomic-genetics of stuttering is growing at a rapid pace. Therefore, the rise of new evidence and in the percentage of stuttering symptoms accounted by genes already related to this disorder is expected to dramatically increase in the next few decades. Although we believe the AHBA is an excellent resource to build the groundwork of genetics-neuroimaging interactions not yet seen in the field, we must consider that this information comes from only six adult participants. To the extent that these resources are improved (i.e., the genetic profile of PWS is fully characterized or more brain atlases including genetic information are available), the description of the neurobiological bases underlying stuttering will be more precise and complete. Until similar transcriptome data become available for stuttering cases, we believe the combination of neuroimaging and genetic analyses, as the one provided in this study, help investigate how specific stuttering-related genes might be linked to biological processes underlying speech production in humans.

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Figure 1. Diagram of Graph Theory Metrics and Connectomics-Genetics Similarity Approach. The stuttering network was characterized using 1) regions of interest identified from a meta-analysis of fMRI studies, 2) whole-brain low-frequency BOLD fluctuations and 3) two graph theory strategies (**I**). In graph-theory strategy 1, we calculated the functional connectivity patterns of brain voxels (light blue nodes in **I**) that connect to stuttering-related regions of interest (dark nodes or targets in **I**). In graph-theory strategy 2, we calculated the functional connectivity patterns of brain voxels (orange and red nodes, interconnectors in **I**) that reach a percentage of stuttering-related regions of interest simultaneously. Genetic expression data of stuttering- and language-related genes (**II**) were analyzed and compared with the stuttering network using a Euclidean distance approach (**III**).

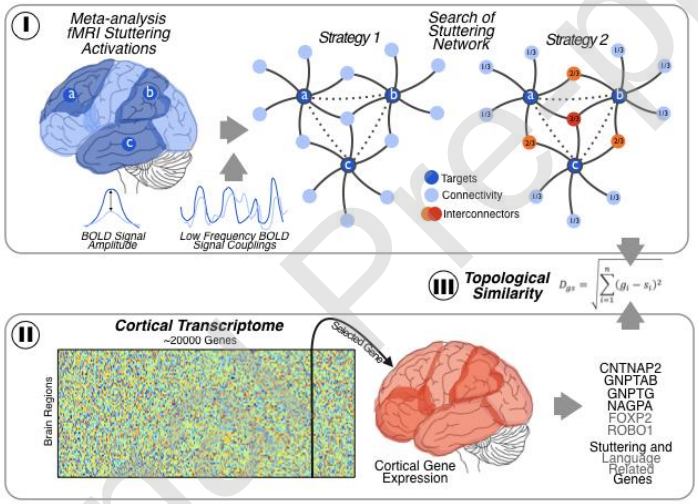

Figure 1

Figure 2. Cortical Network Underlying Stuttering. Regions of interest obtained from a meta-analysis of fMRI and PET activation studies in stuttering (**I**). Stuttering Network characterization based on functional connectivity of normally fluent controls (NFC) in adults (left) and children (right) samples (from graph theory Strategy 1 and 2 (20% to 70% visualization); **II**) and group contrast between children who stutter (CWS) and NFC (**III**). Color scale in **I** represents the z-score transformation of the weighted degree centrality score (minimum = 0SD and maximum = 2SD). Color scale in **II** represents the whole spectrum of permutation-based corrected t-test values. R: right. L: left.

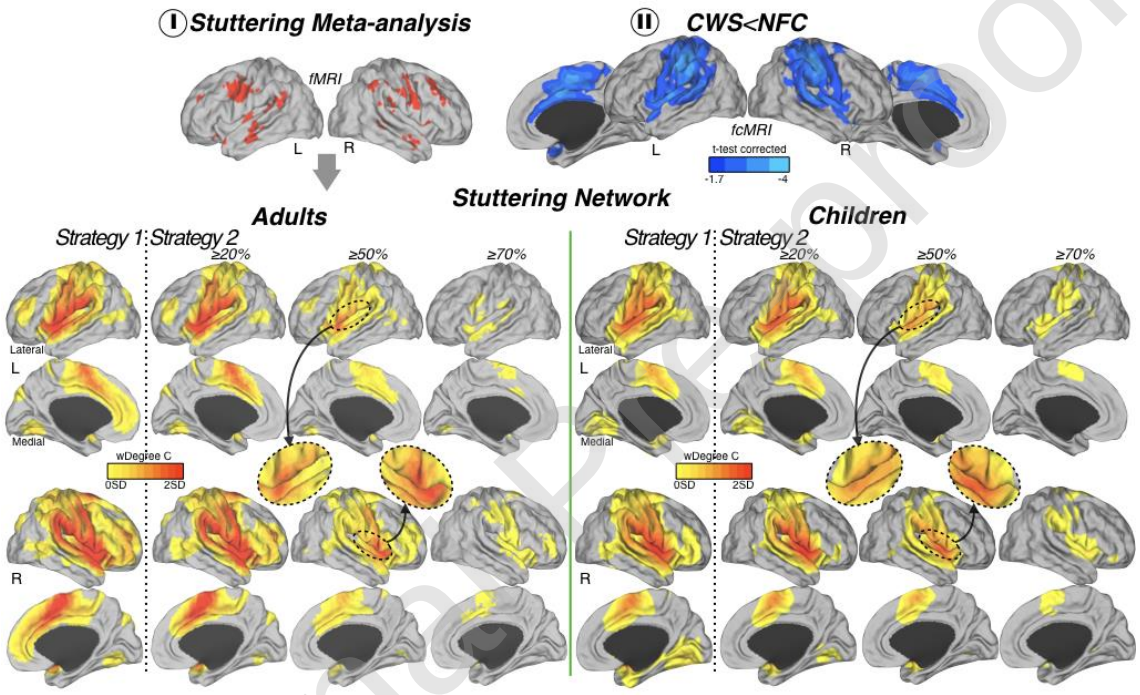

Figure 2

Figure 3. Stuttering Network versus Other Language-Related Networks. Cortical templates of the auditory-motor integration network, Wernicke's network, Broca's F3 opercularis, Broca's F3 triangularis, and Broca's F3 orbitalis, are represented in **I** (adapted from Sepulcre, 2013). Overlap between these language-related networks and the stuttering network in flat projections (**II**) and bar graph (**III**). Color scale in **II** represents the z-score transformation of the weighted degree centrality score (minimum = 0SD and maximum = 2SD).

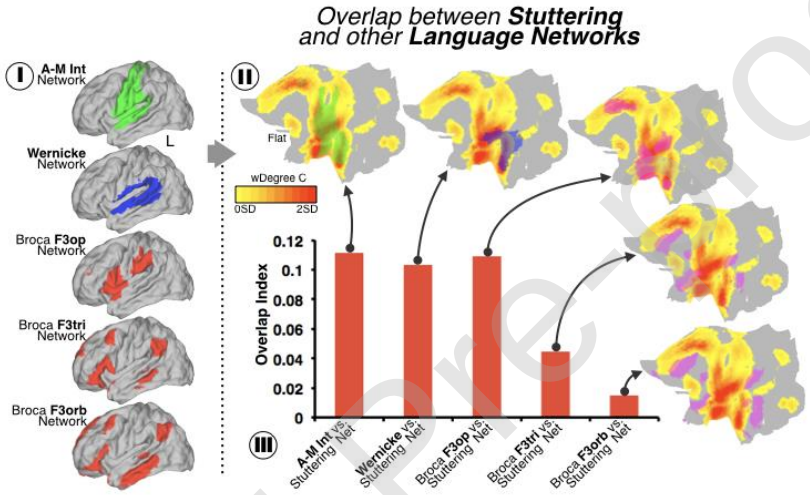

Figure 3

Figure 4. Stuttering Network Topology and Genetic Expression Levels of the Human Cortex. Spatial similarity (or co-expression pattern) between genes previously described as stuttering and language-related (matrix and hierarchical clustering of Euclidean distances; **I**). Distribution of all similarity scores between the stuttering network and the entire transcriptome data from the Allen Human Brain Atlas (histogram of Euclidean distances; **II**). Comparative topology of cortical projections (regular and flat) between the stuttering network (left) and gene expression levels of GNPTG (right) in Desikan-Killiany atlas space (**III**). Gene Ontology Overrepresentation analysis of genes displaying statistically significant similarity scores with the stuttering network (red horizontal in histogram in **II** and **III**). Color scale in **III** represents the z-score transformation of the weighted degree centrality score (minimum = -2SD and maximum = 2SD) and the AHBA score of GNPTG transcripts (minimum = 2% and maximum = 98%).

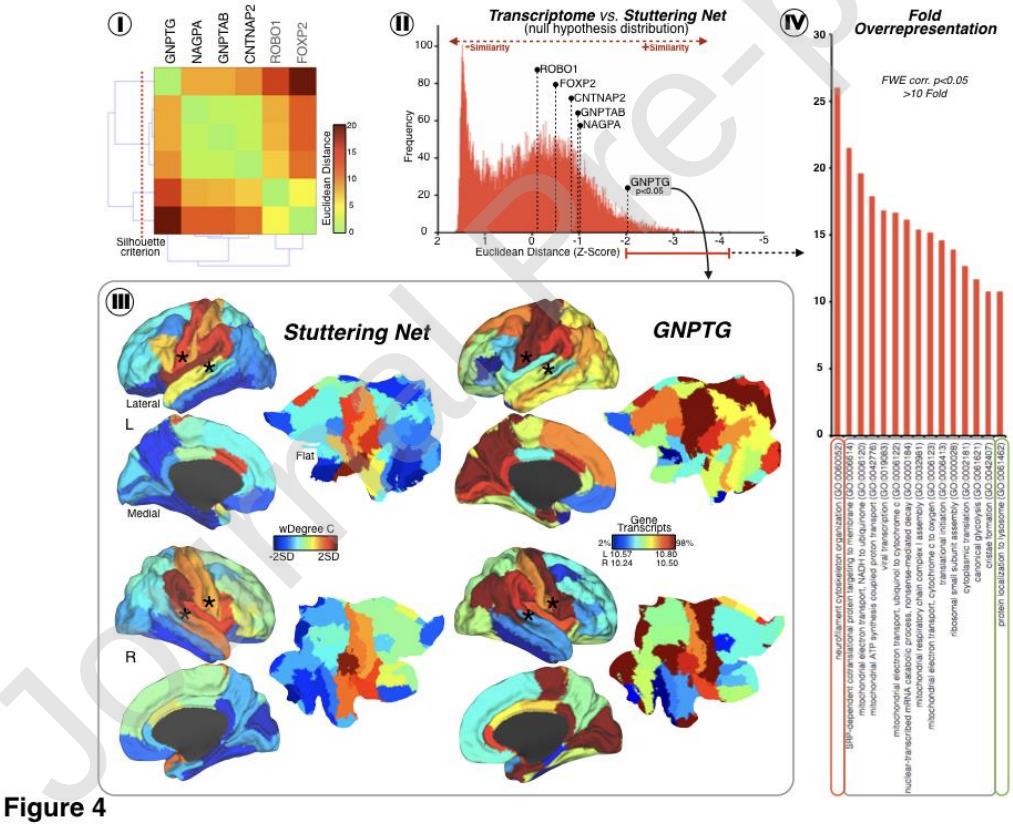

Figure 4

Figure 5. Genetic Interactome Analysis Between GNPTG and Neurofilament Genes. Genetic network (non-brain tissue based) and betweenness centrality of the interactions between GNPTG and neurofilament genes (NEFH, NEFL and INA).

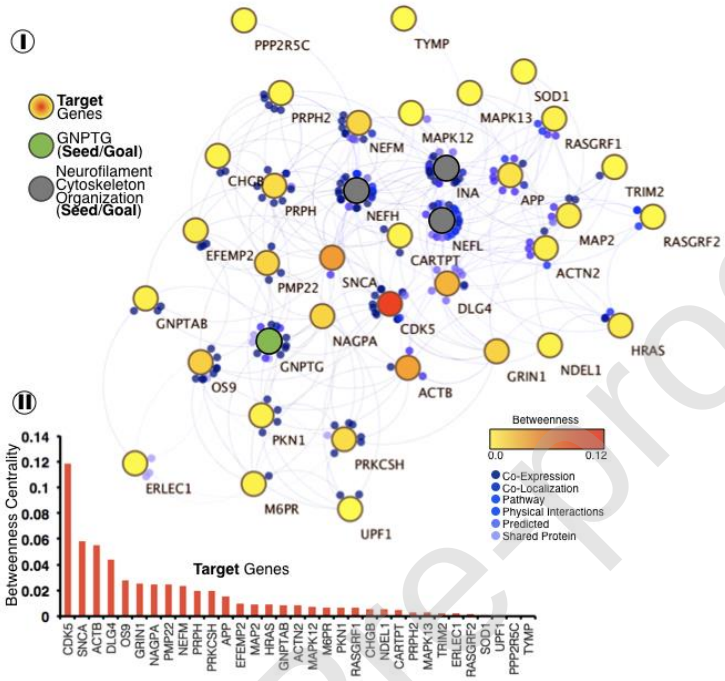

Figure 5