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The neurogenetics of functional connectivity alterations in Autism: Insights from subtyping in 657 patients

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Short title: Neurogenetics of functional brain subtyping in Autism

Keywords: Autism, Subtyping, Excitation-Inhibition Balance, Hyper-connectivity, Allen Human Brain Atlas
Abstract

Background
There is little consensus and controversial evidence on anatomical alterations in the brain of patients with autism spectrum disorder (ASD), due in part to the large heterogeneity present in ASD, which in turn is a major drawback for developing therapies. One strategy to characterize this heterogeneity in ASD is to cluster large-scale functional brain connectivity profiles.

Methods
A subtyping approach based on consensus clustering of functional brain connectivity patterns was applied to a population of N=657 autistic patients with quality-assured neuroimaging data. We then used high-resolution gene transcriptomic data to characterize the molecular mechanism behind each subtype by performing enrichment analysis of the set of genes showing a high spatial similarity with the profiles of functional connectivity alterations between each subtype and the group of typically developing controls (TDC).

Results
Two major stable subtypes were found: Subtype 1 exhibited hypo-connectivity (less average connectivity than TDC) and subtype 2, hyper-connectivity. The two subtypes did not differ in structural imaging metrics in any of the analyzed regions (64 cortical and 14 subcortical), nor in any of the behavioral scores (including Intelligence Quotient, ADI and ADOS). Finally, only subtype 2, comprising about 42% of all patients, led to significant enrichments after multiple testing corrections. Notably, the dominant enrichment corresponded to excitation-inhibition (E/I) imbalance, a leading well-known primary mechanism in the pathophysiology of ASD.

Conclusions
Our results support a link between E/I imbalance and functional connectivity alterations, but only in one ASD subtype, overall characterized by brain hyper-connectivity and major alterations in somatomotor and default mode networks.
**Abbreviations:** ABIDE = Autism Brain Imaging Data Exchange, ADI-R = Autism Diagnostic Interview-Revised, ADOS-G = Autism Diagnostic Observation Schedule-Generic, AHBA = Allen Human Brain Atlas, AQ = Autism spectrum Questionnaire, ASD = Autism Spectrum Disorder, AUC = Area Under the Curve, CSF = cerebrospinal fluid, DSM-5 = The Diagnostic and Statistical Manual of Mental Disorders V, E/I = Excitation/Inhibition, FC = Functional Connectivity, FDR = False Discovery Rate, FIQ = Full Intelligence Quotient, GO = Gene Ontology, GSEA = Gene Set Enrichment Analysis, GWAS = Genome-Wide Association Studies, IQ = Intelligence Quotient, MDMR = Multivariate Distance Matrix Regression, MRI = Magnetic Resonance Imaging, NIMH = National Institute of Mental Health, NEG = Negative Associated, MLR = Multiple Linear Regression, PIQ = Performance Intelligence Quotient, POS = Positive Associated, QC = Quality Checks, ROI = Region Of Interest, SCQ = Social Communication Questionnaire, SFARI = Simons Foundation Autism Research Initiative, SRS = Social Responsiveness Scale, TDC = Typically Developing Control, VIQ = Verbal Intelligence Quotient.
Introduction

Autism encompasses multiple manifestations from impaired social communication and language to restricted or repetitive behavior patterns, interests, and activities(1–3). Due to the vast heterogeneity in behavior, and as recommended in The Diagnostic and Statistical Manual of Mental Disorders (DSM–5), this condition is referred to as autism spectrum disorder (ASD), in which the term "spectrum" emphasizes the variation in the type and severity of manifestations(4). ASD is thought to result from complex interactions during development between genetic, cellular, circuit, epigenetic and environmental factors(5–9). Several researchers have suggested that an excitation/inhibition (E/I) imbalance during development(10,11) may be an essential mechanism, yet specific factors driving the disease are not well understood. Therapeutic interventions aiming to restore the E/I balance in ASD are a major challenge(12).

Concerning neurobiology, heterogeneity in brain morphology(13) and brain networks have been found, e.g. in frontal, default mode and salience networks(14–19), as well as in the social network(20) – encompassing primary motor cortex, fusiform, amygdala, cerebellum, insula, somatosensory and anterior cingulate cortex(14,21,22). ASD is also heterogeneous in relation to network characteristics; less segregation and greater efficiency have been shown(23,24), and the opposite too(25) or a combination of both(26,27). Furthermore, ASD neuroanatomical correlates are not static but undergo changes throughout development(28–30), and the same seems to occur behaviorally in social functioning and communication(31). Altogether, accumulated evidence has shown high heterogeneity within ASD in the participation of functional brain networks and behavioral manifestations, and also, in the longitudinal trajectories at the single subject level.

Moreover, with neuroimaging studies, recent work has shown additional sources of heterogeneity due to variations in the diagnostic and inclusion criteria and differences in the processing neuroimaging pipeline(32,33). ASD is also a polygenic, highly heterogeneous condition, with 1010 genes associated with ASD as of July 8th 2022, according to the SFARI gene human database, see also(34). Of those, 213 have a relevance score of 1, meaning having maximum pathophysiological published evidence to ASD. This high genetic complexity is another manifestation of heterogeneity at several scales of this condition. Previous work has assessed the associations
between transcriptomics and brain morphology(35), showing that genes that are down-regulated and enriched for synaptic transmission in individuals with autism were associated with variations in cortical thickness.

Novel strategies for ASD subtyping are needed to overcome such multi-scale heterogeneity, which is the most significant drawback to the efficacy of therapies. Some studies have addressed the heterogeneity in ASD for better stratifying this condition(36–39). Previous work performed clustering pooling together ASD and TDC groups(37) and found two groups of hyper-connected and hypo-connected participants (each group containing both ASD and TDC participants). Stratification yields reduced inter-individual differences and, therefore, could complement - and even alleviate- the need for big sample sizes in autism-based biomarker discovery(40). Here, and following previous work(36,41,42), we looked at large-scale brain connectivity patterns common within groups of patients to deploy subtyping in ASD. In particular, we applied consensus clustering strategies to multivariate connectivity patterns of brain regions(43,44) for associating connectivity-based ASD subtypes with their neurogenetic profile. Following (45–52), we hypothesized different biological characterization underlying the neurodevelopmental and maturation brain connectivity profile for each subtype, unknown for this condition. For this, we used the Allen Human Brain Atlas (AHBA) of whole-brain transcriptional data(53) and performed subtyping on 657 autistic patients from the Autism Brain Imaging Data Exchange (ABIDE) repository(54), all of them having passed a very strict quality-assurance criterion of elimination of subjects by head movement during image acquisition, thus correcting a well-known spurious excess of functional connectivity driven by head movements, which is even more pronounced in the autistic condition. Moreover, to overcome inter-scanner variability in the functional connectivity values across different institutions, we applied rigorous harmonization strategies to transform heterogeneous data --and that comes from various institutions-- into equivalents(55–58).
Materials and Methods

Subjects

A total of N=2156 subjects from the ABIDE I(54) and ABIDE II(59) repositories were initially considered in this study, of which 1026 were ASD patients and 1130 were typically developing control (TDC) subjects. These data were collected across 35 different scanning cohorts. For each participant both anatomical and functional MRI data were used. Acquisition parameters for each scanning site are found at http://fcon_1000.projects.nitrc.org/indi/abide/. In addition, we extracted several composite scores from the Autism Diagnostic Observation Schedule-General (ADOS-G), Autism Diagnostic Interview-Revised (ADI-R), Vineland Adaptive Behavior Scales, Social Responsiveness Scale (SRS), Social Communication Questionnaire (SCQ) and Raw Score of the Autism Quotient (AQ), and the verbal, performance and full Intelligence Quotient (IQ) scores (respectively, VIQ, PIQ and FIQ) to address cognitive performance and disease severity information. After data quality-assurance (see Suppl. Information), the number of finally included subjects was 1541 (884 TDC, 657 ASD).

Statistical differences in brain morphology and behavior between ASD subtypes

We applied a multiple linear regression (MLR) to assess statistical differences between ASD subtypes in region-wise volume and thickness resulting from Freesurfer while controlling for age, sex and total intracranial volume; and a one-way ANOVA for differences in behavior. Multiple testing was corrected by controlling the False Discovery Rate (FDR).

Functional connectivity matrices

After neuroimaging preprocessing using state-of-the-art methodology (Suppl. Information), FreeSurfer v5.3.0 was used for brain segmentation and cortical parcellation. A total of 82 regions were generated from the Desikan-Killiany atlas, with 68 cortical regions (34 in each hemisphere) and 14 subcortical regions segmented from Freesurfer (left/right thalamus, caudate, putamen, pallidum, hippocampus, amygdala, accumbens). Different brain parcellations were also considered for the analyses (see Suppl. Information). For each subject, the parcellations were projected to the individual functional data and the mean functional time series of each region was obtained.
Finally, one connectivity matrix for each subject was built by Fisher Z-transforming the Pearson correlation coefficients between the region pairs of time series.

**Data Harmonization**

To harmonize our multi-institution functional connectivity data, and before performing subtyping, we used an in-house implementation of Combat (https://pypi.org/project/pycombat), adjusting these multi-institution batch effects by linear mixed modelling and the use of Empirical Bayes methods(56). We also included in this model the diagnosis label (TDC/ASD) as a biological variable of interest, so ensuring that group-level connectivity differences were preserved after harmonization. See Suppl. Information for details.

**ASD subtyping via consensus clustering**

Consensus clustering was applied to brain connectivity matrices(43,44). Since connectivity matrices may contain effects of no interest (e.g. age), prior to subtyping we regressed out age, sex and motion from each connectivity entry of the ASD subjects. To note, this regression-out step was only applied at this subtyping stage. In subsequent analyses, the harmonized connectivity matrices were used and the effect of these variables was controlled for using them as covariates. The stability of each subtype and the 95% confidence intervals of the estimated maximum modularity were assessed by bootstrapping(60).

**Association between subtypes and transcriptomics**

We computed the association between functional connectivity alterations represented by pseudo-R² maps (larger R² scores corresponding to greater alterations; see Suppl. Information) and brain transcriptomics maps using spatial autoregressive models, well-known to reduce the correlation bias produced by the similar transcriptomic expression in proximal brain regions(49). This analysis was implemented by means of the maximum-likelihood estimator routine (ML_Lag) from the Python Spatial Analysis Library (pysal)(61). As a result, for each gene we obtained one t-stat and one p-value, which allowed us to assess the association with the pseudo-R² maps while accounting for possible spatial autocorrelations. Among the significantly associated genes, we identified as relevant those genes included in the SFARI database (https://gene.sfari.org/) with a gene score equal to one. Several
post-hoc analyses tested the robustness of the significant association between the ASD subtypes and transcriptomics and whether our enrichment findings were specific to the ASD condition (see Suppl. Information).

**Gene set enrichment analysis and protein interaction analysis**

We only considered for the analyses such genes with p-FDR corrected value < 0.05 in each subtype. After that, we performed a Gene Set Enrichment Analysis (GSEA) using WebGestalt(62) (http://www.webgestalt.org/) introducing as the input the list of the corrected genes and the t-stat from the association analysis. We computed the GSEA for GO biological process(63) and Reactome pathways(64), and only considered enriched categories p-FDR corrected value < 0.05. We further applied an ensemble-based enrichment analysis, similar to the one developed in (65), to evaluate whether significant enrichment annotations were affected by inflation or false positive bias(65). First, we generated 10,000 surrogate brain maps with the same spatial autocorrelation as the original pseudo-R2 maps using BrainSMASH tool(66). For each of the surrogate maps we computed the association with brain transcriptome maps using spatial autoregressive models and used those genes with a p-FDR corrected value < 0.05 for computing enrichment analysis. For each of the annotations of interest we generated a distribution of the likelihood of that annotation being significant by these random maps. These distributions were used to compute a p-value to evaluate false positive bias. For the protein interaction analysis, we used the tool STRING v11.5(67) to generate a physical protein-protein interaction network for each subtype, with Experiments and Databases as interaction sources. These networks were after analyzed using Cytoscape v3.9.0.

**Results**

We obtained harmonized functional connectivity matrices from 657 ASD and 884 TDC subjects following a methodological sketch represented in Fig. S1. For subtyping, we first removed any effect from age, sex and head motion in the brain connectivity matrices of the ASD group and then applied a consensus clustering. We thus found two main subtypes\(^1\): the first one with 348 subjects (52.97% of all ASD subjects), and the second with 284 subjects (43.23%). In addition to these two subtypes, which were at the highest order in a hierarchy that broke down into

\(^1\)For subtyping, we only use connectivity matrices from subjects with ASD, although the association with brain transcriptomics was performed for functional connectivity alterations encoded in the pseudo-R2 maps.
smaller subtypes (Fig. S2), we also found two more residual subtypes of only 23 subjects (3.5%) and 2 subjects (0.3%) respectively. Our clustering solution, which exhibited modularity statistically different from zero (0.181, 95% [0.169-0.194]), provided for the two major subtypes a stability score of 0.953 and 0.819 respectively, suggesting high consistency and recovery after resampling (mean stability > 0.75, see(60)). In contrast, the residual subtypes were mostly not replicable during bootstrapping (mean stability < 0.5); as a result, they were ignored for further analysis. Furthermore, the robustness of the subtyping solution was assessed by two different strategies, namely, multi-resolution hierarchical clustering and cross-validation (Suppl. Information). As expected, given that their effects were removed prior to subtyping, none of the resulting subtypes were differentiated by age (absolute Cohen’s |d| = 0.04, t-test, p = 0.58), sex (Cramer’s V = 0.02, χ² test, p = 0.55) or head motion (absolute Cohen’s |d| = 0.04, t-test, p = 0.64).

With respect to cognitive and behavioral performance, the two subtypes were highly similar to each other since among 10 different scores compared, only two of them gave uncorrected statistical differences (ADOS TOTAL p=0.03, SRS TOTAL p=0.05), which became non-significant after correcting for multiple comparisons (ADOS TOTAL p-FDR=0.27, SRS TOTAL p-FDR=0.27). For further details on the subtype comparisons, see Table 1. Furthermore, no significant structural differences between subtypes 1 and 2 were found in region volume or thickness. Therefore, all the following analyses are based on differences in functional connectivity that each ASD subtype has in relation to TDC.

**Table 1: Behavioral characterization of ASD subtypes.** For each behavioral score, the number of observations in each subtype, their means and 95% confidence intervals, the uncorrected p-values, and the FDR-corrected p-values from a one-way ANOVA test to assess any statistical difference between them. For ABIDE data, we followed ADI_TOTAL = ADI_R_SOCIAL_TOTAL_A + ADI_R_VERBAL_TOTAL_BV, and ADOS_TOTAL = ADOS_COMM + ADOS_SOCIAL.
To assess the differences between groups in the overall connectivity per subject, defined here as the average positive correlation of the harmonized connectivity matrix (negative correlations were excluded due to the lack of consensus about their origin), we ran a MLR while controlling for age, sex and full intelligence quotient (FIQ) (Fig. 1). Subtype 1 showed significant hypo-connectivity to TDC ($\beta = -0.08, t(1227) = -14.86, p < 0.01$). The opposite was true for the subtype 2 ($\beta = 0.04, t(1163) = 6.91, p < 0.01$), thus corresponding to hyper-connectivity. Moreover, the difference in (absolute) $\beta$ coefficients provided for subtype 1 higher values than subtype 2,

<table>
<thead>
<tr>
<th>SCORE</th>
<th>SUBTYPE 1</th>
<th>SUBTYPE 2</th>
<th>P-VALUE</th>
<th>FDR-CORRECTED P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N&lt;sub&gt;subtype 1&lt;/sub&gt;/N&lt;sub&gt;subtype 2&lt;/sub&gt;)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIQ</td>
<td>105.23 [103.54 - 106.99]</td>
<td>106.81 [104.96 - 108.61]</td>
<td>0.25</td>
<td>0.49</td>
</tr>
<tr>
<td>VIQ</td>
<td>104.43 [102.28 - 106.51]</td>
<td>106.78 [104.44 - 108.98]</td>
<td>0.13</td>
<td>0.45</td>
</tr>
<tr>
<td>PIQ</td>
<td>105.0 [103.18 - 106.96]</td>
<td>105.4 [103.27 - 107.56]</td>
<td>0.80</td>
<td>0.97</td>
</tr>
<tr>
<td>ADI_TOTAL</td>
<td>35.21 [34.07 - 36.33]</td>
<td>34.07 [32.59 - 35.51]</td>
<td>0.21</td>
<td>0.49</td>
</tr>
<tr>
<td>ADOS_TOTAL</td>
<td>11.86 [11.37 - 12.36]</td>
<td>11.01 [10.46 - 11.58]</td>
<td>0.03</td>
<td>0.27</td>
</tr>
<tr>
<td>VINELAND_SUM_SCORES</td>
<td>248.98 [237.57 - 260.68]</td>
<td>254.84 [240.00 - 268.96]</td>
<td>0.54</td>
<td>0.77</td>
</tr>
<tr>
<td>VINELAND_ABC_STANDARD</td>
<td>79.43 [76.3 - 82.7]</td>
<td>79.16 [75.53 - 82.61]</td>
<td>0.91</td>
<td>0.97</td>
</tr>
<tr>
<td>SRS_RAW_TOTAL</td>
<td>94.45 [90.46 - 98.35]</td>
<td>88.49 [83.64 - 93.14]</td>
<td>0.05</td>
<td>0.27</td>
</tr>
<tr>
<td>SCQ_TOTAL</td>
<td>18.78 [17.22 - 20.38]</td>
<td>17.68 [16.14 - 19.23]</td>
<td>0.32</td>
<td>0.54</td>
</tr>
<tr>
<td>AQ_TOTAL</td>
<td>32.05 [29.27 - 34.55]</td>
<td>32.14 [27.81 - 36.19]</td>
<td>0.97</td>
<td>0.97</td>
</tr>
</tbody>
</table>
indicating a bigger separability in connectivity to TDC. Other metrics for defining overall connectivity per subject led to similar conclusions (see Suppl. Information).

Next, we assessed the differences in connectivity patterns between each ASD subtype and the TDC group, measured by region-wise normalized pseudo-R\(^2\) brain maps resulting from MDMR (Fig. 2, and Suppl. Information). The spatial similarity between these maps was very low (\(r(80)=0.09\), permutation-based \(p=0.67\), after using 5000 surrogates that preserved spatial autocorrelation), indicating that each subtype exhibited a distinct neurobiological profile of brain-wide connectivity pattern, as expected since the subtypes were obtained by clustering the functional connectivity profiles. Specifically, for subtype 1 higher differences as compared to TDC were found in the superior temporal gyrus, posterior cingulate cortex, and the insula, covering the functional networks of default mode and salience. For subtype 2 higher differences existed in the thalamus, similar to previous work(68), putamen, and precentral gyrus. Thus, alterations affecting the default-mode network were common to both subtypes, but one (subtype 1) also showed specific disruptions involving the salience network and the other in the somatomotor network (subtype 2).

(Figure 2)

For the biological characterization of each subtype, we set out to identify which genes had an expression across brain regions significantly associated (p-FDR < 0.05) with the differences in connectivity measured by the normalized R\(^2\) brain maps (Fig. 2, histograms), whereby larger R\(^2\) values correspond to bigger functional connectivity alterations. For subtype 1, a total of 195 negative-associated (NEG) genes and 364 positive-associated (POS) genes existed. Significant NEG-genes, also present in the SFARI gene human database with a relevance score of 1, were \textit{GFAP}, \textit{CHD7}, \textit{SKI}, \textit{SHANK3}, \textit{ANK3}, and \textit{CACNA1E}, while POS-genes were \textit{ASXL3}, \textit{MAP1A}, \textit{STXBP1}, \textit{DPYSL2}, \textit{KNCB1}, \textit{SCN8A}, \textit{RIMSI}, and \textit{CDKL5}. Similarly, for subtype 2, we found 142 NEG-genes, of which present in the SFARI list were \textit{GRIA2}, \textit{RFX3}, \textit{SHANK2}, \textit{GRIN2B}, \textit{DLG4}, \textit{LRRC4C}, \textit{ARX}, \textit{GABRB3}, and 180 POS-genes, including \textit{MAGEL2} and \textit{IQSEC2}. We next applied gene-enrichment to the list of significant genes within each subtype, finding no significant enrichment for subtype 1, the one with brain hypo-connectivity.
However, for subtype 2, the enrichment of the NEG-genes included GO Biological processes and Reactome pathways related to glutamate signaling (affecting both AMPA and NMDA receptors) and synapse organization in relation to the excitation-inhibition imbalance occurring during the development of brain circuits (Fig. 3A). We also assessed which NEG-genes participated in each biological process and pathway (Fig. 3B), finding that genes *DLG4, GRIN2B, GRIA2*, and *SHANK2* were participating in most of them; and in particular, the gene *DLG4* did it in all of them. In addition, the *DLG4* gene was the one with the highest degree in the protein interaction network.

We found a significant enrichment with biological processes related to E/I imbalance for subtype 2, but not for subtype 1. To test whether these findings suggested that the functional connectivity in subtype 1 was different from that in previous studies of ASD, we calculated the similarity of the connectivity profiles of our two subtypes with typical connectivity alterations in ASD, represented by brain maps in (69) and calculated from four different ASD databases of resting fMRI data. In particular, we calculated for each subtype the average spatial similarity across the four existing brain maps of connectivity alterations in (69). For subtype 1 the average similarity was not significant ($r(80) = 0.19, p = 0.45$), while for subtype 2 it was significant ($r(80) = 0.46, p = 0.02$), indicating that subtype 2 resembled more the typical connectivity alterations reported in ASD, which in fact is the subtype for which we found significant enrichment towards excitation-inhibition imbalance.

(Figure 3)

We also compared the transcriptomic-connectivity results from the generalized Louvain algorithm to those found by multiresolution clustering (Suppl. Information). As a measure of similarity between the two solutions, dice index values of the solutions were respectively 0.99 and 0.89, which indicated a high-level of reproducibility of the gene-expression association with brain alterations among the two clustering strategies.

In addition, we studied the effects of considering a different brain partition on the results of the transcription-connectivity association (Suppl. Information). Using the functionally defined Schaefer’s brain partition with 100 different regions, the association results obtained from the Desikan-Killiany atlas as compared to those from
Schaefer partition had very low similarity for subtype 1 ($r(1880) = -0.11$, $p < 0.001$), and slightly higher results were found for subtype 2 ($r(1880) = 0.40$, $p < 0.001$). By adding the same subcortical regions to the Schaefer partition (Suppl. Information), the gene association became very similar for the two brain partitions and for the two subtypes (Subtype 1, $r(1880) = 0.87$, $p < 0.001$; Subtype 2, $r(1880) = 0.92$, $p < 0.001$), suggesting a strong contribution of the subcortical alterations to the robustness of our association results. A description of the relevant genes related to subtype 2 is shown in Table S2. These results were obtained by using left-hemisphere transcription sites\(^2\), but the results were also preserved when we repeated the analysis for the two brain hemispheres (Fig. S3).

Finally, it is important to note that no significant enrichment was found for subtypes lower in the dendrogram level corresponding to the two subtypes described above (Fig. S2). The significant enrichment did not exist either after repeating the same analysis using the entire ASD group, indicating the need for subtyping first in the entire population to reveal our findings. To prove that our gene enrichment findings were specific to the ASD condition (Suppl. Information), we also repeated the same procedure but using only the TDC population in two matched subgroups of TDC subjects, one used for subtyping and the other for estimating the pseudo-$R^2$ maps. As a result, no gene survived by FDR correction in any subtype, thus concluding that the excitation-inhibition imbalance found in the hyper-connected autistic subtype is specific to the autistic condition. Likewise, although the subtyping performed in both ASD and TDC groups resulted in solutions with similar overall connectivity separation, resulting in two sets of hypo- and hyper-connected brains, an MDMR analysis applied to the functional correlation patterns showed that the hyper-connected subtype found in ASD was statistically different to that in TDC ($p < 0.001$). The hypo-connected subtypes in ASD and TDC were also different from each other ($p < 0.001$). This might explain why no similar findings in the enrichment were found for the hyper-connected TDC subgroup. Summing up, the significant association between excitation-inhibition imbalance and altered functional connectivity was observed when subtyping in ASD and only in the ASD group characterized by overall hyper-connectivity, demonstrating the specificity of the reported enrichment.

\(^2\)We focused on the left hemisphere as all donors provided sampling sites of genes in this hemisphere, and only two of the six donors from the AHBA dataset were sampled in both, left and right hemisphere.
Discussion

Two significant subtypes result from functional connectivity-based subtyping in a cohort of 657 autistic patients. The two are indistinguishable to behavioral scores, and also by morphometric comparisons based on structural neuroimaging, in agreement with recent results(40). Compared to TDC, the first subtype is characterized by hypo-connectivity, with major implications in the superior temporal gyrus, posterior cingulate cortex and insula, showing connectivity alterations in default mode and salience networks with no significant gene enrichment after correcting for multiple comparisons. The second subtype, representing 43% of subjects with autism, is characterized by hyper-connectivity, with major implications in the thalamus, putamen and precentral gyrus and showing network alterations in somatomotor and default mode networks. In a recent analysis linking genomics and resting functional connectivity in 32,726 individuals with psychiatric conditions, significant ASD contributions were shown in the thalamic and somatomotor networks(68), consistent with our results for subtype 2. Only subtype 2 had a significant gene enrichment towards glutamate signaling (affecting both AMPA and NMDA receptors), consistent with the excitation-inhibition imbalance that occurs during brain development, and one of the most accepted hypotheses in the pathophysiology of autism(70). Indeed, it is thought that in the development of ASD there is an increase in the ratio between excitation and inhibition, leading to hyper-excitability of cortical circuits(10). It is also possible that differential E/I alteration of selective brain circuits might result in an unaltered excitatory/inhibitory ratio at the network level(11). Our work maps patterns of functional connectivity alterations with genes that are involved in E/I balance. While it is true that perturbation in these genes in animal models strongly affects E/I imbalance in brain networks(71), however, the patients' data we have analyzed in this study does not directly address the E/I imbalance, and this is a limitation of our methodology. It is also important to emphasize that the E/I enrichment found in our study is specific to the ASD condition and as such does not occur in the TDC group. Moreover, the connectivity profile in the entire autistic population, i.e., if no subtyping is performed, does not have significant enrichment, evidencing the need of subtyping first to find the connection towards excitation-inhibition imbalance in one subtype of autistic patients.
Our subtyping approach was based on patterns of functional connectivity alterations. There are three major reasons supporting the non-use of structural features for our subtyping analysis. First, it would require a different clustering approach to the one adopted here, which is based on the consensus of connectivity patterns. Second, and based on recent data-driven results from an international autism imaging biomarker challenge (40) with more than 146 institutions submitting prediction algorithms, the 10 best-performance algorithms (with ASD prediction accuracies had AUC > 0.80) showed a dominant contribution of the functional modality, with a much higher discriminative power than the structural MRI data. Third, because our main goal was to study the origin of functional connectivity-based heterogeneity in autism, structural features (representing different brain aspects) give rise to a different kind of heterogeneity. As a result, the proper combination of these two diverse sources of heterogeneity would require a multimodal approach, different to the one developed here.

Our approach is unique in several ways. First, our study is based on a large cohort of patients with ASD (N = 657) from the ABIDE initiative, all of them having passed the rigorous criteria of motion removal, and it combines anatomical and functional neuroimaging data from 24 different institutions. Second, we have used Combat, a rigorous data harmonization method to eliminate the variability between MRI scans across the 24 institutions, one of the largest sources of variability when combining imaging data from multiple institutions (72). Third, our analysis of brain connectivity was carried out on a large-scale, where each brain region is represented by its connectivity pattern across the entire brain. Therefore, we do not consider a priori any brain region as more dominant or relevant than the others. Fourth, we made use of a consensus clustering approach we have developed (43,44), and that has been successfully tested by others (73), to group subjects in the same subtype if the connectivity profiles are similar across all the analyzed regions. Finally, we made use of the AHBA to describe the neurogenetic profiles of each subtype, which has been used before for morphometric information in ASD (35), but never for characterizing subtypes based on functional connectivity patterns of this condition.

Due to the large heterogeneity and diversity reported in ASD genetics, the use of AHBA may shed new light, as it provides information on the transcriptome across the brain in unprecedented detail, accounting for 3,702 sampling sites with transcription information of 20,500 genes as a specific signature for each anatomical region. Moreover,
the use of AHBA is complementary to other techniques, such as genome-wide association studies or GWAS(74), that simultaneously addresses genotype–phenotype associations from hundreds of thousands to millions of genetic variants in a data-driven manner. Indeed, GWAS has previously been used for ASD subtyping(75,76), yet using behavioral scores as traits and therefore, the subtypes obtained were more closely related to symptom severity and not to functional connectivity.

Our enrichment results for subtype 2 show that DLG4, a.k.a. PSD95, is the gene with major implication in the protein interaction network of subtype 2. DLG4 mediates NMDA and AMPA receptor clustering and function; it affects the glutamatergic transmission, and has been shown to have an aberrant function in ASD(77–81). DLG4 also influences the size and density of dendritic spines during brain development, having strong effects on synaptic connectivity and activity; e.g. reduced DLG4 activity leads to increased dendritic spine numbers(82).

Some limitations should be noted. First, our transcriptomic analysis was based on AHBA, which is derived from healthy donors and not from ASD patients’ brain tissues. Therefore, the relations studied here between ASD-dependent connectivity patterns and healthy transcriptomics highlight large-scale organization aspects of the connectivity alterations to gene expression. Future studies should confirm our findings using gene expression data from a pathologic cohort, which is not currently available. Second, the number of donors from the AHBA data is very limited (n=6) and the sampling sites available do not cover the full brain. Third, our subtyping method found two subtypes of patients that were hypo-connected and hyper-connected at the network level. The same classes of subtypes were found by subtyping the TDC group. However, when comparing first the hypo-connectivity subtypes between the ASD and TDC groups, and then the hyper-connectivity subtypes, connectivity patterns were significantly different in both cases, which justifies the significant enrichment found for the hyper-connectivity ASD subtype but not for TDC. Finally, our main neurogenetic finding in one ASD subtype, involving genes largely affecting the E/I imbalance, is just based on the statistical association between transcriptome activity and patterns of functional connectivity alterations. Future studies should explicitly test the causal link between E/I imbalance and functional connectivity alterations in ASD.
In summary, our novel approach, which includes data harmonization, multivariate distancing in large-scale functional connectivity patterns and transcriptome brain maps, reveals strong enrichment for glutamate signaling (affecting both AMPA and NMDA receptors) and synapse organization in one subgroup of autistic patients, reinforcing the hypothesis of an excitation-inhibition imbalance occurring during the brain development of such autistic patients.

**Data and code availability**

The data employed in this study belong to the ABIDE-I and ABIDE-II repositories. Their IDs can be found in [https://github.com/compneurobilbao/asd-subtyping-enrichment](https://github.com/compneurobilbao/asd-subtyping-enrichment), as well as the codes used for the analyses. The initial multicenter ABIDE dataset consisted of 35 different scanning cohorts and two groups of subjects, TDC (N=1130) and ASD (N=1026). After following our data quality-assurance, we obtained connectivity matrices of 884 TDC and 657 ASD, belonging to 24 institutions, which were ultimately used for the subtyping analysis. These matrices are available at [https://doi.org/10.6084/m9.figshare.21901821](https://doi.org/10.6084/m9.figshare.21901821).

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**Disclosures**
The authors report no biomedical financial interests or potential conflicts of interest.

References


**List of Figures:**

**Fig. 1. Two major stable ASD subtypes, one with hypo-connectivity and the other with hyper-connectivity.**

Histogram and box plots of the individual average connectivity values (measured as Fisher’s Z) for the TDC group (blue), the population of all ASD subjects without subtyping (brown) and the two ASD subtypes (pink and orange). Two other subtypes that were not stable after permutation testing are not depicted here and have been ignored for further analysis. The median value of the TDC group is marked as the connectivity baseline by a dashed vertical line. Values above the baseline correspond to overall hyper-connectivity and those below the baseline to overall hypo-connectivity. Subtype 1 is dominated by hypo-connectivity, and subtype 2 by hyper-connectivity. Additionally, for subtypes 1 and 2, we introduce two colors for subjects to show within-group connectivity differences, blue for hypo-connectivity, and red for hyper-connectivity.

**Fig. 2. Association between transcriptomics and connectivity patterns for each ASD subtype.** For subtypes 1 and 2, we calculated the pseudo-$R^2$ map taking into account the differences in the connectivity pattern that each subtype has to TDC. (Right) Brain maps of normalized pseudo-$R^2$. (Left) Histograms of association values between pseudo-$R^2$ and gene transcription activity (different values correspond to association with different genes). This procedure was repeated using the pseudo-$R^2$ map for each subtype. The tail of the negative (N) genes ($p$-FDR < 0.05 and t-stat < 0) is marked with a blue rectangle and the tail of the positive (P) genes ($p$-FDR < 0.05 and t-stat > 0) with a red one for both subtypes. Significance limits (t) is also shown. For each distribution tail, we also show the relevant genes present in the SFARI ASD genes with a score = 1.
Fig. 3. Excitation-inhibition imbalance enrichment only for one class of autistic subjects (Subtype 2). A: GSEA characterization of the FDR-significant genes in subtype 2, including the Gene Ontology (GO) Biological processes (dark gray) and Reactome pathways (light gray) enrichments. We further tested that the enrichment findings were not affected by their reporting rate in the literature, and for all cases reported here we obtained p-FDR < 0.05. B: Participation count that each gene has in the processes shown in A, ranging from 4 to 10 (corresponding to a participation in all processes in panel A, that only occurred for DLG4). C: Protein-protein interaction physical network from the list of FDR-significant genes. For ease of visualization, only sub-networks with a minimum of 10 genes are depicted. D: Node degree of the genes participating in the network shown in C. DLG4 is the gene with the highest degree. B, C, D: Bars corresponding to genes with SFARI score =1 are colored in red; SFARI score = 1S in dark red; SFARI score = 2 in orange; and SFARI score = 2S in dark orange, and the same color code was used in C and D for network nodes.
A  GSEA subtype 2 significant genes

- GO: Biological process
- Reactome pathways

- Neuronal system
- Glutamate binding, activation of AMPA receptors and synaptic plasticity
- Trafficking of AMPA receptors
- Glutamate receptor signaling pathway
- Transmission across Chemical Synapses
- Neurotransmitter receptors and postsynaptic signal transmission
- Synapse organization
- Protein-protein interactions at synapses
- Unblocking of NMDA receptors, glutamate binding and activation
- Activation of NMDA receptors and postsynaptic events

B  Gene-participation in GSEA

- SFARI genes
  - score = 1
  - score = 2
  - score = 2S

C  Protein interaction principal network

D  Protein interaction network degree