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Brain Hyperconnectivity in Children with Autism and its Links to Social Deficits

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SUMMARY

Autism spectrum disorder (ASD), a neurodevelopmental disorder affecting nearly 1 in 88 children, is thought to result from aberrant brain connectivity. Remarkably, there have been no systematic attempts to characterize whole-brain connectivity in children with ASD. Here, we use neuroimaging to show that there are more instances of greater functional connectivity in the brains of children with ASD in comparison to those of typically developing children. Hyperconnectivity in ASD was observed at the whole-brain and subsystems levels, across long- and short-range connections, and was associated with higher levels of fluctuations in regional brain signals. Brain hyperconnectivity predicted symptom severity in ASD, such that children with greater functional connectivity exhibited more severe social deficits. We replicated these findings in two additional independent cohorts, demonstrating again that at earlier ages, the brain of children with ASD is largely functionally hyperconnected in ways that contribute to social dysfunction. Our findings provide unique insights into brain mechanisms underlying childhood autism.

INTRODUCTION

Autism spectrum disorder (ASD), a neurodevelopmental disorder that affects nearly 1 in 88 children (Baio, 2012), is thought to affect multiple interconnected brain regions (Minshew and Williams, 2007). Knowledge of brain connectivity in ASD and its relation to core symptoms is therefore critical for understanding the neurobiology of ASD (Kennedy and Courchesne, 2008; Menon, 2011; Minshew and Williams, 2007; Monk et al., 2009; Vissers et al., 2012). Despite the early developmental origins of this disorder and its variable developmental trajectory, almost all of the current literature on brain connectivity has focused on adolescents and adults with ASD, rather than on children (Gotts et al., 2012; Kennedy and Courchesne, 2008).

Several previous studies in adults have reported that functional connectivity between brain areas engaged during cognitive tasks is weaker in ASD (Just et al., 2007; Kleinhans et al., 2008; Koshino et al., 2008), leading to the "underconnectivity theory" of autism (Just et al., 2012). Yet, empirical evidence in support of the underconnectivity theory comes primarily from analyses of a handful of regions of interest derived from task-based activation studies in adults, often with poor replication across studies because of variability in the choice of brain regions examined (Müller et al., 2011; Vissers et al., 2012).

Although very little is currently known about brain connectivity in childhood ASD, one of the earliest signs of autism is enlarged head circumference or macrocephaly (Lainhart et al., 1997). Infants and young children with ASD show signs of early brain overgrowth (Courchesne et al., 2003), and postmortem studies of children with ASD show that they have an overabundance or excess number of neurons in the prefrontal cortex (Courchesne et al., 2011). Animal models of autism have provided evidence for hyperconnectivity in intrinsic functional circuits at very early time points in development (Testa-Silva et al., 2012; Yizhar et al., 2011). These findings of macrocephaly and hyperconnectivity have yet to be reconciled with human neuroimaging studies. As a result, there is a profound inconsistency in the extant literature, arising both from the failure to adequately distinguish weak task-related modulation of functional connectivity from intrinsic functional brain connectivity, and from inadequate attention to childhood autism (Amaral, 2011). Additionally, a major weakness in the field has been limited sample sizes and, more importantly, the lack of replication of findings using identical analytic procedures (Vissers et al., 2012).

In this era of human brain connectomics, it is increasingly recognized that understanding complex brain function and dysfunction critically depends on accurate characterization of

connections between brain regions (Sporns, 2011). Comprehensive descriptions of whole-brain functional connectivity profiles in clinical disorders have begun to provide greater insights into the functional consequences of altered brain connectivity (Fornito et al., 2012; Supekar et al., 2008). Yet, very little is known about whole-brain functional connectivity in neurodevelopmental disorders such as ASD during childhood, and a mechanistic understanding of neural processing in ASD is completely absent.

Here, we address these critical gaps by using task-free fMRI (Greicius et al., 2003) to characterize whole-brain functional connectivity in three independent cohorts totaling 110 children aged 7–13 years with ASD and age-, gender-, and IQ-matched typically developing (TD) children. We test the hypothesis that childhood ASD is associated with altered intrinsic functional connectivity patterns that impact brain systems critical for social cognition. Critically, we replicate our key findings across the three cohorts and provide the most robust evidence for widespread functional brain hyperconnectivity in children with autism, demonstrating that at earlier ages, the brain in ASD is largely functionally hyperconnected. Extensive additional analyses confirmed our findings of intrinsic functional hyperconnectivity in childhood ASD. Finally, we demonstrate that this pattern of functional hyperconnectivity predicted autism symptoms such that children with greater functional connectivity exhibited more severe impairment in the social domain.

RESULTS

One cohort of 40 children (ASD = 20; TD = 20) was recruited at Stanford University; a second cohort of 40 children (ASD = 20; TD = 20) was recruited at Georgetown University and Children's National Medical Center (CNMC); a third cohort of 30 children (ASD = 15; TD = 15) was recruited at New York University (NYU) and obtained from the National Database of Autism Research (NDAR; http://ndar.nih.gov). Each cohort consisted of well-characterized children with ASD and a well-matched group of TD children. Task-free fMRI data were acquired from each child in the Stanford, Georgetown/CNMC, and NYU/NDAR cohorts (demographic data, data acquisition protocols, and data preprocessing procedures are described in Experimental Procedures). Here, we report findings from the Stanford cohort. Convergent findings from the Georgetown/CNMC and NYU/NDAR cohorts are described in the Supplemental Information.

Functional Brain Hyperconnectivity in ASD Children Compared to TD Children, at the Whole-Brain Level

We first examined differences in whole-brain functional connectivity patterns between children with ASD and TD children. Preprocessed fMRI data sets were parcellated into 90 cortical and subcortical regions using anatomical templates (Tzourio-Mazoyer et al., 2002). A time series was computed for each of the 90 regions by averaging all voxels within each region. Wavelet analysis of the extracted regional fMRI time series (Supekar et al., 2009) was used to compute interregional functional connectivity across the whole brain. Mean global connectivity (the average of wavelet correlation values across all possible pairwise functional connections) was higher in children with ASD compared to TD children ($p < 0.05$, Cohen effect size $d' = 0.67$, Figure 1B). Comparing wavelet correlation values of all possible pairwise functional connections (n = 4005), we found that there were more instances of greater connectivity in children with ASD (Figure 1A). Specifically, 588 pairs (15%) of anatomical regions showed higher correlations in the ASD group than the TD group ($p < 0.05$, corrected for multiple comparisons; Zalesky et al., 2010). No pairs of regions showed higher correlations in the TD compared to the ASD group. Additional analyses were conducted to determine the robustness of these results. First, we examined the potential effects of alternate region of interest (ROI) specification strategies on our results. We repeated our entire analysis using two additional ROI specification strategies: voxel-based, in which the regions of interest were cortical and subcortical voxels selected using the procedure described by van den Heuvel and colleagues (van den Heuvel et al., 2008), and functionally defined, in which the regions of interest were 264 putative functional brain areas selected using the procedure described by Power and colleagues (Power et al., 2011). These two strategies, along with our original automated anatomical labeling (AAL)-based ROI specification strategy, capture a wide range of ROI specification strategies, from random-voxel based to anatomically defined to functionally defined. Results from these additional analyses were consistent with the results from the original analysis (which used AAL-based ROIs), namely: compared to TD children, children with ASD showed significantly higher functional connectivity between pairwise ROIs. Second, we examined the potential effect of global signal regression on our results. We regressed out the global signal and repeated our functional connectivity analyses on the regressed out fMRI data. Results from these additional analyses were consistent with the results from the original analysis (which did not include global signal regression); namely, compared to TD children, children with ASD showed significantly higher functional connectivity. Taken together, results from these additional analyses further confirm the robustness of our findings of hyperconnectivity in children with ASD (see Supplemental Information for details).

Functional Brain Hyperconnectivity at the Subsystems Level in ASD Children Compared to TD Children

To investigate whether differences in functional brain connectivity span multiple functional subsystems, we used the parcellation scheme proposed by Mesulam (Mesulam, 1998) to examine functional connectivity in five functional subsystems: primary sensory, subcortical, limbic, paralimbic, and association areas. Each subsystem has a distinct cytoarchitectonic profile and subserves a unique set of functions, collectively mapping external sensory information to cognition (Mesulam, 1998). Higher mean functional connectivity in children with ASD, compared with TD children, was detected in the primary sensory, paralimbic, and association areas ($p < 0.05$, $d' > 0.7$; Figure 1C). Children with ASD also showed higher mean connectivity across subsystems: between primary sensory and paralimbic, primary sensory and association, and paralimbic and association ($p < 0.05$, $d' > 0.49$; Figure 1D). No links between or within any of these subsystems showed greater connectivity in the TD group compared with the ASD group. These results suggest that hyperconnectivity in ASD spans multiple functional subsystems of the human

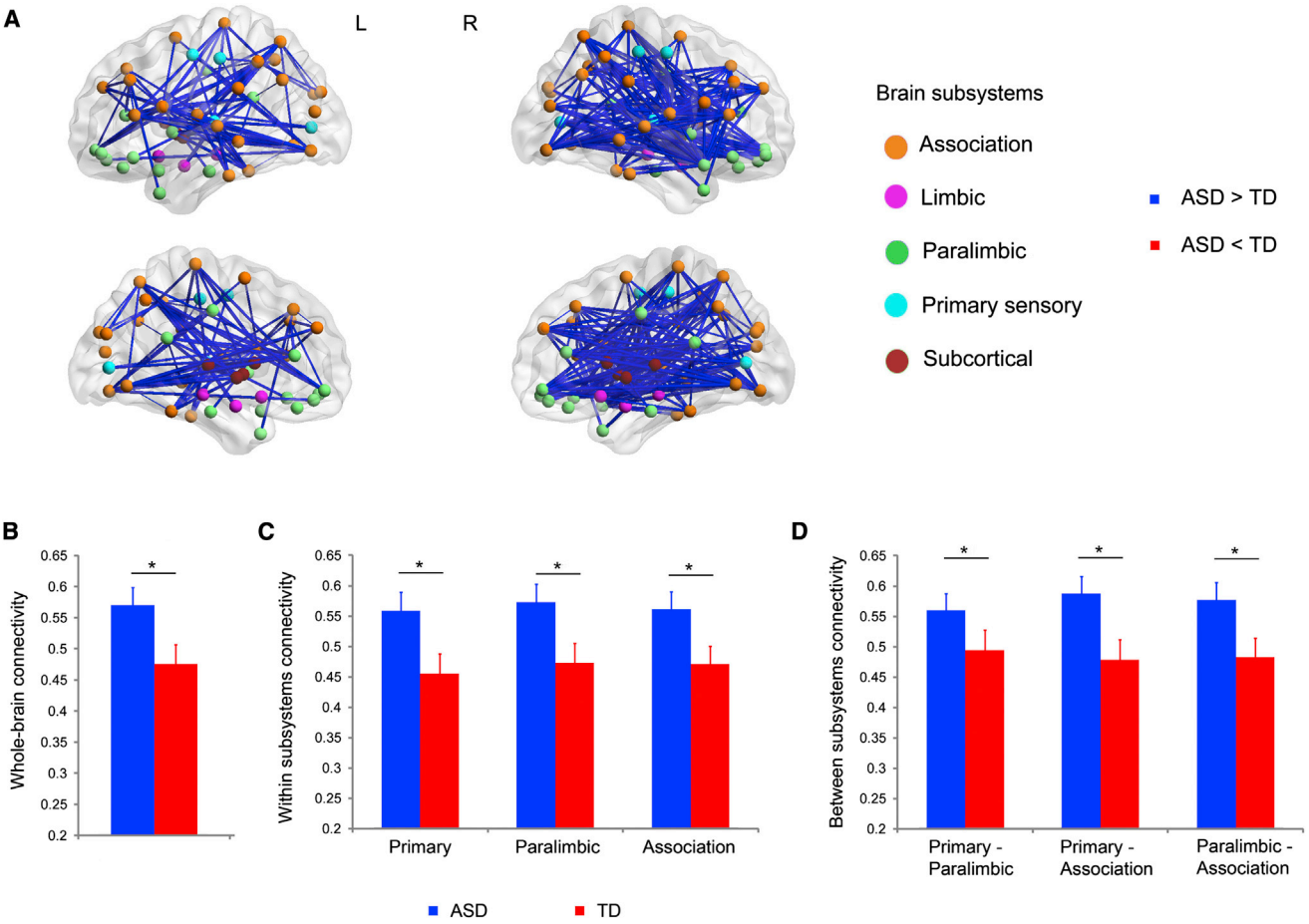

Figure 1. Functional Brain Hyperconnectivity in Children with ASD

(A) A total of 588 pairs (15%) of anatomical regions showed higher correlations in children with ASD compared to the TD group ($p < 0.05$, corrected for multiple comparisons). No pairs of regions showed higher correlations in the TD compared to the ASD group.

(B) Mean whole-brain connectivity was higher in children with ASD compared to TD children ($p < 0.05$, $d' = 0.67$).

(C) Within subsystems, mean connectivity was higher in children with ASD in primary sensory, paralimbic, and association areas ($p < 0.05$, $d' > 0.70$). A total of 25% of the total functional connections within primary sensory, 10% within paralimbic, and 19% within association areas showed greater functional connectivity in ASD than in TD.

(D) Across subsystems, mean functional connectivity between primary sensory and paralimbic, between primary sensory and association, and between paralimbic and association areas was greater in children with ASD ($p < 0.05$, $d' > 0.49$). A total of 18% of functional connections between primary sensory and paralimbic, 17% between primary sensory and association, and 17% between paralimbic and association areas were greater in children with ASD. No links, either within or between subsystems, showed greater connectivity in the TD group compared to the ASD group.

*$p < 0.05$. Error bars represent SEM. See also Figures S1 and S4.

brain. The paralimbic subsystem consists of the insula, anterior cingulate cortex, posterior cingulate cortex, and the orbitofrontal cortex, while association areas include the lateral frontal and parietal cortices.

Functional Brain Hyperconnectivity in ASD Children between Proximal and Distant Anatomical Regions

Reports in the literature suggest that short- and long-range connections may be differentially affected in ASD (Courchesne and Pierce, 2005). To examine whether both short- and long-range intrinsic functional connectivity is disrupted in children with ASD, we examined differences in regional functional connectivity in the two groups as a function of the interregional distance. The distance between two regions was computed by calculating the Euclidean distance between centroids of those regions (Fair et al., 2009; Supekar et al., 2009). We found that, compared to TD children, children with ASD showed higher functional connectivity across all distances examined (Figure 2). Thus, functional hyperconnectivity in ASD was observed between both proximal and distant anatomical regions.

Functional Brain Hyperconnectivity in ASD Children Is Associated with High Amplitude of Low-Frequency Fluctuations

To investigate potential node-level abnormalities contributing to global hyperconnectivity in children with ASD, we examined

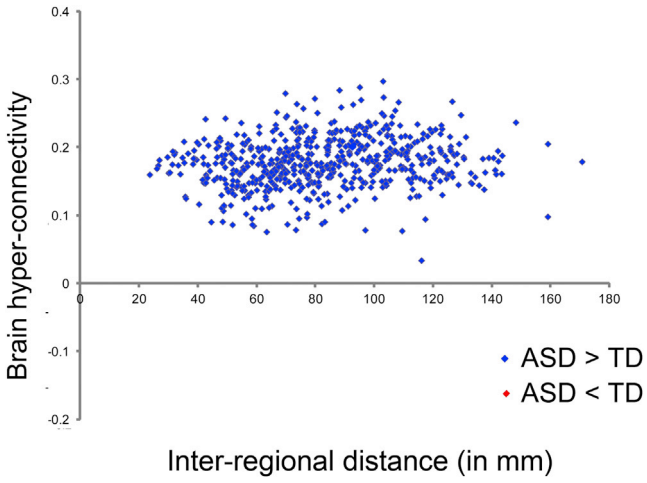

Figure 2. Functional Brain Hyperconnectivity in Children with ASD as a Function of Interregional Distance

Connections across all distances showed higher levels of hyperconnectivity in children with ASD, compared to TD children. Connectivity values that were stronger in children with ASD are shown in blue, and connections that were stronger in TD children are in red (none in this cohort). Interregional distance, *d*, was computed by calculating the Euclidean distance between region centroids. See also Figures S2 and S5.

the amplitude of low-frequency fluctuations (ALFFs) in the regional fMRI signal. Whereas functional connectivity provides an index of temporal synchrony between low-frequency fluctuations in regional fMRI signals, ALFF is a measure of regional changes in signal level (Yang et al., 2007). We computed ALFF values for each of the 90 anatomical regions of interest for each participant (see Experimental Procedures for details). We found that the global mean ALFF values were greater in the ASD group than the TD group ($p < 0.05$, $d' = 0.68$; Figures 3A and 3B). Furthermore, higher regional ALFF was associated with higher levels of whole-brain connectivity in children with ASD ($r = 0.54$, $p = 0.01$; Figure 3C). This empirical finding, together with neurophysiological modeling of the underlying neural mechanisms (see below), suggests that both local circuit abnormalities and interregional hyperconnectivity may contribute to atypical brain function in ASD (Yizhar et al., 2011).

Replication of Results in Two Additional Independent Cohorts of ASD and TD children

We repeated our entire fMRI analysis on the second group of children from the Georgetown/CNMC cohort as well as the third group of children from the NYU/NDAR cohort (Table S1). In spite of differences in geographical location (Northern California versus Washington DC versus New York), scanner (GE versus Siemens), fMRI pulse sequence (spiral in-out versus echo planar imaging), and other data acquisition protocols, results from both the Georgetown/CNMC and NYU/NDAR cohorts entirely replicated the Stanford-cohort findings of widespread functional hyperconnectivity, enhanced ALFF, and significant associations between ALFF and functional hyperconnectivity (see Supplemental Information for details).

Functional Brain Hyperconnectivity in ASD Children Predicts Symptom Severity

To investigate the extent to which widespread functional brain hyperconnectivity is associated with severity of symptoms in ASD, we examined the relationship between whole-brain functional connectivity and Autism Diagnostic Observation Schedule (ADOS) (Lord, 2000) and Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994) scores, using multivariate sparse regression analysis (Friedman et al., 2010) and nonparametric hypothesis testing (see Experimental Procedures for details). Functional brain hyperconnectivity predicted scores on the Social domain of the ADOS ($p = 0.002$) as well as the Social domain of the ADI-R ($p = 0.04$) such that children who showed greater levels of functional connectivity were more severely impaired in the social domain. This effect was independently replicated in the Georgetown/CNMC cohort for ADOS Social ($p = 0.001$) as well as ADI-R Social ($p = 0.03$) scores. Combining data from all 55 children with ASD across the three cohorts also replicated this effect for both the ADOS Social ($p = 0.001$) and the ADI-R Social ($p = 0.001$) domain scores, further demonstrating the robustness of our findings.

DISCUSSION

This study examines functional connectivity at the whole-brain level in children with ASD. We analyzed data from 110 children collected across three different sites, providing the largest sample of pediatric brain imaging data to date. Our findings from multiple cohorts provide convergent, robust, and replicable evidence for widespread functional brain hyperconnectivity in children with ASD. Our findings of functional hyperconnectivity are bolstered not only by the triple replication, but also by a link to clinical symptoms of ASD. Children with more severe impairment in the social domain exhibited greater functional connectivity. Our study reports a significant brain-behavior relationship linking aberrant whole-brain functional connectivity to one of the core symptoms of ASD.

Widespread Functional Brain Hyperconnectivity in Childhood ASD

We examined functional whole-brain connectivity in children with ASD and observed widespread hyperconnectivity in these children compared to TD children. Extensive additional analyses were conducted to determine the robustness of our findings. Specifically, we examined the potential effect of alternate ROI specification strategies as well as global signal regression on our findings. Results from these additional analyses confirmed our findings of widespread functional brain hyperconnectivity in ASD children. More importantly, to address the recent concerns about the effect of subject motion on functional connectivity findings (Deen and Pelphrey, 2012), (1) we used stringent motion inclusion criteria and matched the ASD groups and TD groups in terms of motion parameters; (2) we examined functional connectivity within a narrow frequency range (0.01–0.05 Hz; Scale 3), further removing physiological and motion-related artifacts (Cordes et al., 2001; Cordes et al., 2000); (3) we computed correlations between movement parameters and brain connectivity values and found that there was no significant

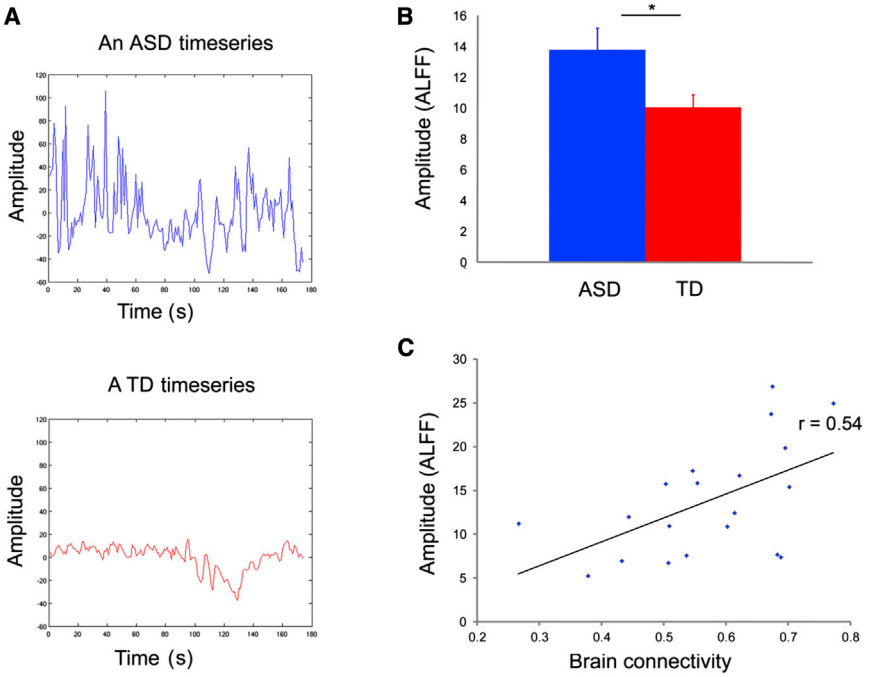

Figure 3. Higher Levels of Amplitude of BOLD Oscillations Are Associated with Functional Brain Hyperconnectivity in Children with ASD

(A) fMRI time series averaged across all gray-matter voxels in the brain from a representative child with ASD (blue) and a TD child (red), illustrating abnormally high amplitude fluctuations in children with ASD.

(B) Mean amplitude of fMRI BOLD oscillations (ALFF) was greater in ASD than in TD ($p < 0.05$, $d' = 0.68$).

(C) Higher regional ALFF was associated with higher levels of whole-brain connectivity in children with ASD ($r = 0.54$, $p = 0.01$).

*$p < 0.05$. Error bars represent SEM. See also Figures S3 and S6.

correlation between mean brain connectivity values and movement parameters; (4) we applied the revised data-scrubbing procedure described by Power and colleagues (Power et al., 2013) and found that our results remained unchanged after applying the scrubbing procedures; and (5) we performed imputation analysis that replaces volumes with relatively high motion proposed by Carp and colleagues (Carp, 2013) and found that our results remained unchanged after applying the imputation procedures. Critically, we replicated our main findings of brain hyperconnectivity in ASD across three independent cohorts, further providing robust evidence for widespread functional brain hyperconnectivity in children with autism.

Very few studies have examined functional connectivity in young children with ASD. A previous study reported weaker interhemispheric functional connectivity between temporal and prefrontal language areas in sleeping toddlers with ASD after regressing out auditory stimulus processing (Dinstein et al., 2011). The differential influence of connectivity changes in ASD during sleep is currently not well understood. Another study of 7- to 13-year-old children found increased connectivity of striatal systems in children with ASD compared to TD children (Di Martino et al., 2011). This report of "ectopic" hyperconnectivity of a specific system in children with ASD is in line with our current findings, and extends them to the whole brain level. Importantly, our study provides evidence for whole-brain hyperconnectivity in awake, nonsedated children with ASD. Additionally, subsystem analyses revealed hyperconnectivity across multiple functional subsystems, including sensory and association cortices, in children with ASD. These findings point to aberrant patterns of functional connectivity in brain systems related to cognitive, social, and affective processes (Mesulam, 1998). Examining the aberrant patterns of functional connectivity as a function of anatomical distance, we found functional hyperconnectivity in ASD between both proximal as well as distant anatomical regions, pointing to aberrant integration and segregation within both short- and long-range functional circuits in children with ASD (Sporns et al., 2000; Supekar et al., 2009). Taken together, these findings provide evidence for widespread functional brain hyperconnectivity in childhood ASD at both the whole-brain level and the level of major functional subsystems, for both short- and long-range anatomical connections.

Mechanisms Underlying Functional Brain Hyperconnectivity in Childhood ASD

To better characterize the neural mechanisms underlying functional hyperconnectivity in ASD, we then examined the amplitude of ALFFs in the regional fMRI signal. We found that abnormally high levels of fluctuations in regional fMRI signals were associated with higher levels of global functional hyperconnectivity in children with ASD. These results show that enhanced regional ALFF in children with ASD arising from an aberrant balance of excitation and inhibition in local neural circuits (Testa-Silva et al., 2012; Tuchman and Cuccaro, 2011; Yizhar et al., 2011) may be an important factor that contributes to interregional hyperconnectivity observed in our fMRI data as well as related electrophysiological data (Léveillé et al., 2010). This potential mechanistic underpinning of our results is consistent with an increasingly promising theory of autism, which postulates that the neurophysiological substrate underlying the disorder is an imbalance between excitation and inhibition (Rubenstein, 2010; Rubenstein and Merzenich, 2003; Vattikuti and Chow, 2010; Yizhar et al., 2011). Specifically, the theory suggests that the imbalance of excitation and inhibition in the local brain circuits subserving sensory, social, and affective processes, possibly caused by either increased synaptic excitation or decreased synaptic inhibition, could engender cognitive and behavioral deficits observed in ASD. Importantly, the proposed theory unifies multiple lines of experimental findings in ASD. Molecular studies have indicated gene-, receptor-, and enzyme-level deficits in inhibitory signaling pathways involving

gamma-aminobutyric acid (GABA) in ASD (Baroncelli et al., 2011; Gatto and Broadie, 2010; Pizzarelli and Cherubini, 2011). Findings from imaging studies have suggested that ASD is associated with hyperreactivity and high-frequency cortical oscillations (Gomot et al., 2008; Orekhova et al., 2007). Notably, epidemiological studies have consistently reported high levels of epilepsy in children with ASD (Tuchman and Cuccaro, 2011). Indeed, over one-third of children with ASD have comorbid epilepsy, and half of them have epileptiform EEGs (Clarke et al., 2005; Matsuo et al., 2010). We postulate that such abnormal neuronal discharges arising from excitation-inhibition imbalance caused by atypical underlying cellular/molecular circuitry might contribute to high levels of intrinsic ALFF responses and global hyperconnectivity. Based on evidence from animal studies (Rubenstein and Merzenich, 2003), we further propose that this hyperconnected brain state may make it more difficult for children with ASD to modulate brain activity levels in response to cognitive demands.

Taken together, empirical findings from three different cohorts of children provide insights into the mechanisms underlying widespread functional brain hyperconnectivity in childhood autism. More broadly, our findings provide brain-system-level empirical support for the unifying theory that autism arises from an imbalance of excitation and inhibition in developing neural systems (Rubenstein and Merzenich, 2003) and further suggest that local circuit hyperexcitability as a result of this imbalance likely contributes to aberrations in global brain connectivity in autism.

Developmental Account of Functional Brain Hyperconnectivity in Childhood ASD

The majority of published neuroimaging studies of ASD have focused on adolescents or adults with the disorder, leaving the question of brain connectivity in childhood ASD relatively open. The current results suggest that in children with ASD, unlike previously reported in adults with ASD (Assaf et al., 2010; Gotts et al., 2012; Kennedy and Courchesne, 2008; Monk et al., 2009; von dem Hagen et al., 2012), there are more instances of intrinsic functional hyperconnectivity than underconnectivity. This suggests that there may be a developmental trajectory in ASD that is altered from that of typical development. Structural neuroimaging studies of ASD provide evidence that brain volumetric differences observed in early childhood can be diminished or normalized or overcompensated with development (Amaral, 2011; Via et al., 2011).

Although the majority of studies of adolescent and adulthood ASD report intrinsic functional underconnectivity in the disorder (Assaf et al., 2010; Gotts et al., 2012; Kennedy and Courchesne, 2008; Monk et al., 2009; von dem Hagen et al., 2012), one of the few studies that has examined children younger than 12 years of age reported functional hyperconnectivity of the striatum in children with ASD (Di Martino et al., 2011). Longitudinal studies are needed to fully characterize age-related changes in brain connectivity associated with ASD. However, the findings here suggest that there may be a critical developmental shift, perhaps during the time of puberty (Peper et al., 2011), that differentially affects the maturation of connections in ASD (Uddin et al., 2013).

Behavioral Consequences of Functional Brain Hyperconnectivity in Childhood ASD

Our findings of widespread functional hyperconnectivity are strengthened not only by replication across the three cohorts, but also by a link to ASD symptoms. Brain hyperconnectivity predicted autism symptoms such that children with greater connectivity exhibited more severe impairment in the social domain. This brain-behavior relationship suggests that aberrant functional connectivity may underlie social deficits, which are the hallmark of ASD.

The relationships between functional brain hyperconnectivity and cognitive deficits in ASD we report here may provide a framework for understanding the complex behavioral manifestation of the disorder. Brain hyperconnectivity may result in isolation of neural systems involved in high-level cognitive processes, thus contributing to some of the core behavioral characteristics of the disorder, including deficits in navigating real-world social scenarios. Brain hyperconnectivity may limit flexible resource allocation, resulting in the rigidity and need for sameness that is often observed in individuals with ASD. The current findings provide a neural processing account of cognitive deficits in childhood ASD. At the same time, such hyperconnectivity might also contribute to "islets" of spared ability in autism, as have been described in the domains of visual search (Keehn et al., 2013) and mathematics (Baron-Cohen et al., 2001).

CONCLUSIONS

Understanding the neurobiology of ASD requires a critical examination of brain connectivity in children (Belmonte et al., 2004; Minshew and Keller, 2010; Uddin and Menon, 2009). This study addresses a critical and controversial question regarding the nature and extent of brain connectivity alterations in childhood ASD. Our findings not only provide direct evidence for hyperconnectivity at the whole-brain level spanning multiple functional subsystems, but also demonstrate a link to core clinical symptoms in school-age children with ASD. Our findings also provide insights into a link between enhanced local fluctuations and global aberrations in brain connectivity in school-age children with the disorder. More generally, this work challenges the notion of underconnectivity as the central neurobiological feature of ASD. Furthermore, our study highlights the importance of studying neurodevelopmental disorders closer to their onset, rather than in adulthood when a lifetime of compensatory mechanisms may have already taken place (Amaral, 2011).

EXPERIMENTAL PROCEDURES

Participants

Stanford Cohort

Twenty children with ASD and 20 age-, gender-, and IQ-matched TD children participated in this study after giving written, informed consent. For those subjects who were unable to give informed consent, written, informed consent was obtained from their legal guardian. The study protocol was approved by the Stanford University Institutional Review Board. The children with ASD (16 males, 4 females) ranged in age from 7 to 13 years (mean age: 10.1) with an IQ range of 78 to 142 (mean IQ: 113); the TD children (16 males, 4 females) ranged in age from 7 to 13 years (mean age: 10) with an IQ range of 79 to 136

(mean IQ: 111) (Table S1). Participants were recruited locally, from schools and clinics near Stanford University. All children were required to have a Full Scale IQ $\geq$ 70, as measured by the Wechsler Abbreviated Scale of Intelligence (WASI).

Children with ASD received a diagnosis based on scores from the Autism Diagnostic Interview-Revised (ADI-R) (Le Couteur et al., 1989; Lord et al., 1994) and/or the Autism Diagnostic Observation Schedule (ADOS) (Lord, 2000) following criteria established by the National Institute of Child Health & Human Development/National Institute of Deafness and Other Communication Disorders Collaborative Programs for Excellence in Autism (Lainhart, 2006). Children with ASD were screened through a parent phone interview and excluded if they had any history of known genetic, psychiatric, or neurological disorders (e.g., fragile X syndrome or Tourette syndrome) or were currently prescribed antipsychotic medications. TD children were screened and excluded if they or a first-degree relative had developmental, language, learning, neurological, or psychiatric disorders, or psychiatric medication usage, or if the child met the clinical criteria for a childhood disorder on the Child Symptom Inventory–Fourth Edition or Child and Adolescent Symptom Inventory. All participants underwent a battery of standardized neuropsychological assessments including WASI (Wechsler Intelligence Scale for Children–3rd Edition, Wechsler Intelligence Scale for Children–4th Edition, or WASI; The Psychological Corporation, 1999), and the Wechsler Individual Achievement Test (WIAT, 2nd edition). Full Scale IQ was determined from scores on the WASI.

Georgetown/CNMC Cohort

Twenty children with ASD and 20 age- and gender-matched TD children participated in this study after providing assent and parental consent according to guidelines of the Georgetown University institutional review board. The children with ASD (15 males, 5 females) ranged in age from 8 to 13 years (mean age: 11.04) with an IQ range of 85 to 138 (mean IQ: 114); the TD children (12 males, 8 females) ranged in age from 8 to 13 years (mean age: 10.83) with an IQ range of 99 to 138 (mean IQ: 123) (Table S1). Children were recruited through the local community via advertisements and a hospital's outpatient clinic specializing in ASD and neuropsychological assessment. All children were required to have a Full Scale IQ $\geq$ 70, as measured by WASI.

Children with ASD received a clinical diagnosis using criteria similar to the Stanford cohort. Additionally, they also received a clinical diagnosis based on *Diagnostic and Statistical Manual of Mental Disorders*, *Fourth Edition*, *Text Revision* criteria (American Psychiatric Association, 2000). The participant inclusion and exclusion criteria were identical to those of the Stanford cohort. All participants underwent a battery of standardized neuropsychological assessments including the WASI. Full Scale IQ was determined from scores on the WASI.

NYU/NDAR Cohort

Fifteen children with ASD and 15 age-, gender-, and IQ-matched TD children were included in this study. The subjects were identified from public domain research data repositories. Specifically, ASD subjects were identified by querying the National Database for Autism Research repository (NDAR; http://ndar.nih.gov). The query parameters were aged 7 to 13 years, phenotype ASD, and task-free fMRI data present. The age range was chosen to match that of the Stanford and Georgetown/CNMC cohorts. The query results yielded 15 children with ASD (11 males, 4 females) ranging in age from 7 to 13 years (mean age: 10.37) with an IQ range of 73 to 132 (mean IQ: 99), with task-free fMRI data. Notably, all of the subjects identified belong to one collection submitted by Francisco Castellanos at New York University (NYU). This collection did not include data from TD children. To address this issue, we queried the ADHD200 data set (http://fcon_1000.projects.nitrc.org/indi/adhd200/), which consists of task-free fMRI data from TD children and children with attention deficit/hyperactivity disorder (ADHD) across eight different sites, including Dr. Castellanos' lab at NYU. The query parameters were site NYU, age between 7 and 13, phenotype typically developing, task-free fMRI data present. The query results yielded 60 TD children. We used an in-house matching algorithm to select a subset of 15 TD children such that the mean age, mean IQ, and gender distribution was matched to the ASD group. The algorithm identified a well-matched subset of 15 TD children (11 males, 4 females) ranging in age from 7 to 13 years (mean age: 10.22) with an IQ range of 80 to 142 (mean IQ: 107) (Table S1).

fMRI

Data Acquisition and Preprocessing

For each subject in each of the three cohorts (Stanford, Georgetown/CNMC, and NYU/NDAR), a resting-state scan was acquired using protocols described in detail in the Supplemental Information. The acquired data from the three cohorts underwent identical preprocessing, as described in the Supplemental Information, and analytical processing, as described below.

Analysis of Whole-Brain Functional Connectivity

Preprocessed fMRI data sets were parcellated into 90 cortical and subcortical regions using previously published AAL anatomical template. Wavelet analysis of the extracted regional fMRI time series was used to compute interregional functional connectivity across the whole brain. We first examined differences in whole-brain functional connectivity patterns between the two groups. To demonstrate the robustness of our findings, these analyses were repeated using two alternate ROI specification strategies: *voxel based* (van den Heuvel et al., 2008) and *functionally defined* (Power et al., 2011), which provided convergent results (see Supplemental Information for details).

Next, to investigate whether differences in functional brain connectivity spans multiple functional subsystems, we used the parcellation scheme of Mesulam.

The aforementioned procedures are described in detail in the Supplemental Information.

Analysis of Amplitude of Low-Frequency Fluctuations

To further characterize functional connectivity, we computed ALFFs in the regional fMRI signal. While functional connectivity provides an index of temporal synchrony between low-frequency fluctuations in regional fMRI signals, ALFF is a measure of regional activity. ALFF has been previously used to quantify regional intrinsic activity (He et al., 2007; Zang et al., 2007) and has been suggested to reflect spontaneous neuronal activity (Zou et al., 2008). We computed ALFF values for each of the 90 anatomical ROIs and the mean of these 90 ALFF values for each subject. For each anatomical region of interest, ALFF was computed by (1) transforming the regional time series to the frequency domain, (2) calculating the power spectra of this transformed signal, and (3) averaging the square root of amplitude at each frequency component across 0.01–0.08 Hz (Zou et al., 2008).

Analysis of Differences in Functional Connectivity as a Function of Anatomical Distance

We next examined the relationship between differences in regional correlation values (connectivity) in the two groups and the interregional distance. The distance between two regions was computed by calculating the Euclidean distance between centroids of those regions.

Analysis of Functional Connectivity as a Function of Symptom Severity: Prediction Analysis

We next investigated whether regional connectivity in the ASD group predicted symptom severity. We used a multivariate sparse regression approach (Friedman et al., 2010), which models the relationship between the dependent variable (scores on ADOS or ADI-R Social domain) and the multiple independent variables (whole-brain interregional functional connectivity: 4,005 wavelet correlation values). An advantage of using a multivariate sparse regression approach, as opposed to traditional univariate correlation, is that it examines patterns of connectivity across the whole brain as opposed to a single average measure of whole-brain connectivity and is thus more sensitive. More importantly, such sparse methods are particularly elegant when the number of possible predictor variables is large and the number of observations is small, which is the case in our analysis. We used GLMnet (http://www-stat.stanford.edu/~tibs/glmnet-matlab), a state-of-the-art sparse regression algorithm that is widely used to examine multivariate relationships in large-scale genomic data (Friedman et al., 2010). GLMnet computes the model in such a way that the coefficients of independent variables that do not contribute to the prediction of the dependent variable are set to zero, thus producing sparse-interpretable solutions. L1-norm regularization is used to produce this sparse model. Nonparametric testing was used to assess the performance of the regression algorithm in predicting symptom severity. We first estimated R^2, the proportion of variance explained by the model, using a leave-one-out cross validation (LOOCV) procedure. In LOOCV, data are divided into N folds. A sparse regression model is built using

N − 1 folds, leaving out one sample. The left-out sample is then predicted using this model, and the predicted value is noted. The above procedure is repeated N times by leaving out one sample each time, and finally an R^2 is computed based on the observed and predicted values. This cross-validation procedure avoids overfitting that is likely to happen when the number of samples is low and the number of parameters in the models is large, which is the case in our analyses. Finally, the statistical significance of the sparse model was assessed using nonparametric analysis. The empirical null distribution of R^2 was estimated by generating 10,000 surrogate data sets under the null hypothesis that there was no association between scores on ADOS or ADI-R Social domain and whole-brain interregional functional connectivity patterns. Each surrogate data set D_i of size equal to the observed data set was generated by permuting the labels (scores on ADOS or ADI-R Social domain) on the observed data points. The sparse model computed on the observed data was used to predict labels of each surrogate data set D_i. R_i^2 was computed using the actual labels of D_i and predicted labels. This procedure produces a null distribution of R^2 of the sparse model. The statistical significance (p value) of the sparse model was then determined by counting the number of R_i^2 greater than R^2 and then dividing that count by the number of D_i (10,000 in our case).

Participant Motion Characterization

As reported here, we rigorously address the critical issue related to motion in resting-state functional connectivity analyses in several ways. First, we used stringent motion inclusion criteria, which is within the acceptable range of pediatric clinical neuroimaging studies. Second, the ASD group and TD groups at each of the three sites were very well matched in motion parameters. Third, we examined functional connectivity within a narrow frequency range (0.01 to 0.05 Hz; Scale 3). Several studies have now shown unambiguously that narrow-band-pass filtering in frequencies corresponding to Scale 3 is important for removing physiological and motion-related artifacts (Cordes et al., 2001; Cordes et al., 2000). Fourth, we computed correlations between movement parameters and brain connectivity values. We found that there was no significant correlation between mean brain connectivity values and movement parameters at each of three sites. Fifth, we applied data-scrubbing procedures using (1) the more stringent "revised data scrubbing" procedure by Power and colleagues (Power et al., 2013) and (2) "imputation" procedure by Carp and colleagues (Carp, 2013) and found that our results remained unchanged after applying these correction procedures.

The details of these analyses and the ensuing results are described in detail in the Supplemental Information. These results, and more importantly replication of our main findings of widespread brain hyperconnectivity in three independent cohorts comprised of children with varied movement parameters collected across three different scanners, confirm that our findings are robust against potential movement confounds.

Structural MRI

Data Acquisition

For each subject in each of the three cohorts (Stanford, Georgetown/CNMC, and NYU/NDAR), a structural MRI scan was acquired using protocols described in detail in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results and Discussion, Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.10.001.

AUTHOR CONTRIBUTIONS

K.S. and V.M. conceived and designed study; K.S. and V.M. analyzed data; K.S., L.Q.U., and V.M. wrote the paper; A.K. collected Stanford cohort imaging data; J.P. collected and analyzed Stanford cohort clinical data; and W.D.G., L.E.K, B.E.Y., and C.J.V. contributed Georgetown/CNMC cohort data and provided feedback on the paper.

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