

Functional Connectivity of Affective Face Processing Networks in Autism

by

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Abstract:

Autism is a behaviorally defined neurodevelopmental disorder with deficits in the social, communicative, and behavioral domains. We hypothesized that dysfunctional subcortico-cortical and cortico-cortical networks of facial affect processing may contribute to the impairment in autism social behavior. Using a facial emotion discrimination task, functional magnetic resonance imaging (fMRI), and functional connectivity analyses, we quantified the integrity of functional amygdala and interhemispheric circuitry and assessed its association with behavioral measures of task performance and autism clinical severity.

The first study quantified the integrity of functional amygdala connectivity when processing facial affect and identified significant associations between functional connectivity and behavior that distinguished autism from controls. In the second and third studies, we respectively isolated functional amygdala and interhemispheric connectivity networks specific to a given facial affect in autism and controls. Using partial correlation analysis, we quantified the integrity of connectivity in resting networks and emotional and neutral face processing networks and tested their association with behavioral measures of task performance and clinical autism severity. Additionally, in the third study, we quantified the association of affective connectivity dynamics with density measures of the corpus callosum, a fiber bundle connecting the hemispheres. In the fourth study, we used neuroinformatic techniques including graph theory, agglomerative hierarchical clustering, and multidimensional scaling to test whether signatures of interhemispheric connectivity could distinguish autism from controls. In the fifth study,

we developed open-source software to test the hypothesis that the temporal derivative of pulse oximetry signals collected during fMRI acquisition would be useful for future fMRI studies.

Our studies extended autism investigations from altered activation in specific brain regions to dysfunctional patterns of interconnectivity between brain regions. We identified subcortico-cortical and cortico-cortical facial affect processing networks in autism with overconnected and underconnected circuits and showed that the integrity of specific functional circuits in some networks were associated with callosum density and behavioral measures that distinguished groups. Based on altered affective brain connectivity with the amygdala and among interhemispheric homologues, we propose that impaired behavioral catalysts, reduced metavariability, reduced metastability, and altered connectivity signatures are useful network constructs to characterize deficits of affective network dynamics in the autism connectome.

Chapter 1:
Introduction

Chapter 1: Introduction

Autism

Autism is a behaviorally defined neurodevelopmental disorder with a classic triad of deficits in the social, communicative, and behavioral domains (Rapin 1997; Lord, Cook et al. 2000). The term “autism” was originally coined by Eugen Bleuler in 1911 (Bleuler 1911/1951) to describe schizophrenia behavior as a “detachment from reality with the relative and absolute predominance of the inner life” (Parnas, Bovet et al. 2002). In 1943, Leo Kanner defined a disorder of “affective contact” as early infantile autism (Kanner and Eisenberg 1957) based on a cohort of eleven children (age 2-8) with an “innate inability to form the usual, biologically provided affect with people” (Kanner 1943) that continued into adulthood (Kanner 1971). Interestingly, Asperger independently used the term autism in 1944 to describe an “autistic psychopathy” (Asperger 1944; Asperger 1944/1991) in children with impaired social interaction that now bears his name (Wing 1981). Although Kanner first thought that autism behaviors were “inborn” errors that occurred with otherwise normal development, autistic disorder, also known as Kanner’s autism or early infantile autism, is now classified as part of a spectrum of neurodevelopment disorders (Wing 1996; Volkmar and Klin 2005).

According to classification by the Diagnostic and Statistical Manual (DSM) Text Revision (DSM-IV-TR) (American Psychiatric Association. 2000), the pervasive developmental disorders (PDDs) include childhood disintegrative disorder, Rett’s disorder, autistic disorder, Asperger’s disorder, and pervasive developmental disorder-not otherwise specified (PDD-NOS). Current estimates of PDD rates range from 30-

60/10,000 which is a large increase from the 1966 measure of 4/10,000 (Rutter 2005), 9-11/10,000 for autistic disorder (male/female ratio of 4.3:1), and about 2.5/10,000 for Asperger's disorder (Fombonne 2003). Individuals with autistic and Asperger's disorder have developmental impairments in social, communicative, and behavioral domains and their diagnoses are unique in the DSM-IV-TR (American Psychiatric Association. 2000). Diagnostic criteria of autistic disorder symptoms in the DSM is largely based on Wing's triad (Wing and Gould 1979) which includes impairment in social interaction, in verbal and nonverbal communication, and in imagination, associated with repetitive stereotyped behaviors (Happé and Frith 1996; Wing 1996). Asperger's disorder is distinguishable from autistic disorder by a lack of significant cognitive and language developmental delays. Autistic and Asperger's disorders are sometimes classified with PDD-NOS to comprise the Autism Spectrum Disorders (ASDs) (Committee on Children With Disabilities 2001). Through the Autism and Developmental Disabilities Monitoring (ADDM) Network, the Centers for Disease Control and Prevention (CDC) estimates the United States prevalence of ASDs to be 1 in 150 among 8-year-olds (CDC 2007; CDC 2007; CDC 2007). Both the terms ASD and autism, as distinguished from autistic disorder, may refer to PDDs (Rapin 1997; Committee on Children With Disabilities 2001; Rapin 2002). The discussion which follows uses the term autism in the context of ASDs to guide our hypothesis of functional affective face processing circuitry alterations in patient-volunteers with autistic and Asperger's disorder.

Functional Connectivity and Autism Social Behavior

Affective face processing is a task of affect recognition or affective intent (Davidson and Slagter 2000) and social cognition (Abdi and Sharma 2004) known to involve a distributed network (Haxby, Hoffman et al. 2002) composed of several circuits that include the amygdala, hippocampus, and prefrontal regions (Adolphs 2002). Affective face processing impairment is one component of social behavioral deficits in autism. Several neuropathological and neuropsychological models of autism indicate that the distinctive affective face processing networks in autism (Hobson, Ouston et al. 1988; Critchley, Daly et al. 2000; Dalton, Nacewicz et al. 2005) are directly related to neuronal alterations with consequences to system level communication between brain regions, or functional connectivity, that can be studied using advanced imaging techniques (Ramnani and Penny 2004).

Functional MRI Connectivity

In the early 1990's, functional magnetic resonance imaging (fMRI), a non-invasive imaging modality, was first applied to humans to detect changes in brain activation using the endogenous blood oxygenation level dependence (BOLD) contrast (Bandettini, Wong et al. 1992). To measure activation, BOLD contrast signal can be modeled as a convolution of neural activity with an impulse response function to account for hemodynamic delay in T2* weighted signal (Bandettini, Jesmanowicz et al. 1993; Rabe-Hesketh, Bullmore et al. 1997). A few years later, fMRI was used to quantify the

integrity of functional interactivity, or functional connectivity, in the motor cortex of resting human brain (Biswal, Yetkin et al. 1995; Biswal, Van et al. 1997).

Functional connectivity, the “temporal correlations between remote physiological events” (Friston 1994) is a means by which information integrated between and within brain regions can be detected using fMRI. Functional connectivity between nodes is necessary to form functional circuits in cognitive and affective networks. Unlike effective connectivity, “the influence one neuronal system exerts over another” (Friston, Frith et al. 1993), functional connectivity neither presumes a causal connection between regions nor explains the driving force of interregional correlations. The integrity of functional connectivity between nodes determines the extent of integration of circuits in the functional network. Functional connectivity analysis has several application in cognitive and affective neuroscience including investigations of cocaine administration (Li, Biswal et al. 2000), Alzheimer’s disease (Li, Li et al. 2002), depression (Irwin, Anderle et al. 2004), and intraregional connectivity (Johansen-Berg, Behrens et al. 2004) to name a few. Brain communication within the affective face processing network can be examined using fMRI, a facial affect processing task, and a functional connectivity analytical approach.

Functional Connectivity of Affective Face Processing Networks in Controls

The functional connectivity of affective face processing circuitry has already been reported in human controls. Functional connectivity seeded by the amygdala during an fMRI attentional face-processing task was able to identify regions of connectivity

(V1/V2) not previously identified as task-induced activation (Pessoa, Kastner et al. 2002). Because the activation of some nodes in the affective face processing network vary with facial affect (Posamentier and Abdi 2003), changes in the integrity of functional connectivity with these nodes may vary as a function of facial affect. In a PET study, psychophysiological interaction analysis revealed that the integrity of connectivity between the amygdala and extrastriate cortex varied with the emotion expressed in the face (fear vs. happy) and suggested that the amygdala-extrastriate pathway is a dynamic circuit in affective face processing (Morris, Friston et al. 1998). The processing of fearful and angry emotional face stimuli in controls is distributed across a network of regions in communication with the amygdala including the insula, parahippocampal gyrus, orbitofrontal cortex, and the subgenual, posterior, and supragenual cingulate (Stein, Wiedholz et al. 2007). The integrity of functional interhemispheric and amygdala connectivity in the network of affective face processing remains to be shown in autism networks.

Neuropathological and Neuropsychological Models of Autism

Altered connectivity in autism is thought to occur at the neuronal level instigating systemic alterations in structure and function (For review, see Pardo and Eberhart 2007). Several neuroregulatory proteins which can impact neuronal connectivity show altered levels in autism. For example, reelin, an extracellular matrix glycoprotein with a role in neuron guidance, neurotransmission, and dendritic spine plasticity (Rice and Curran 2001), is reduced in autism brain (Fatemi 2002; Fatemi 2004). The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) gene codes for a phosphatase that

inhibits the phosphoinositide 3-kinase pathway that regulates neuronal circuit formation through downstream effects on neuronal growth and migration (van Diepen and Eickholt 2008). PTEN mutations are rare in autism, but in the 20% of autism individuals with macrocephaly defined as a head circumference greater than two standard deviations from the mean, PTEN mutations occur with a frequency of about 1 in 88 and are strongly associated with macrocephaly (Buxbaum, Cai et al. 2007). Mutations to synaptic structural proteins may affect synaptic communication between neurons in autism.

Neuroligins comprise a family of postsynaptic cell adhesion proteins that are important for functional synapses (Varoqueaux, Aramuni et al. 2006). Mutations in the neuroligin 3 and neuroligin 4 gene were susceptibility factors associated with autism (Jamain, Quach et al. 2003). Mutations to SHANK3, a scaffolding protein in the postsynaptic density of excitatory synapses that can bind neuroligins, are also associated with autism (Durand, Betancur et al. 2007). Screens of genes associated with predisposition to autism have implicated cAMP signaling in the autism phenotype. Variants in the cAMP guanine nucleotide exchange factor II (cAMP-GEFII) gene, which codes for a regulator of G proteins that is functionally dependent on cAMP levels and is involved in neuronal development and exocytotic processes, are associated with the autism phenotype (Bacchelli, Blasi et al. 2003). Variants in the mGluR8 gene (GRM8), which codes for the mGluR8 receptor that has an inhibitory effect on cAMP signaling, is associated with an increased susceptibility to autism (Serajee, Zhong et al. 2003). For discussion of cAMP alterations in autism and fragile X, the most common genetic cause of autism, see Chapter 8.1. The signaling of hepatocyte growth factor through the MET tyrosine kinase receptor contributes to dendrite formation and the development of functional neuronal

circuits (Gutierrez, Dolcet et al. 2004). A post-mortem brain study of temporal neocortex identified a reduction of MET protein levels in autism (Campbell, D'Oronzio et al. 2007). Based on histopathological cellular studies, individuals with autism have an increased number of smaller minicolumns, the basic functional unit of cortex (Buxhoeveden and Casanova 2002), that are less compact relative to controls in prefrontal cortex and in temporal regions. This anatomical configuration may enhance cortical noise in these regions and reduce the signal to noise ratio of information transfer (Casanova, Buxhoeveden et al. 2002; Belmonte, Cook et al. 2004). With intrinsic connectivity in the amygdala required for proper information transfer (Bonda 2000; Amaral and Corbett 2003), altered neuronal packing in the autistic amygdala (Bauman and Kemper 1985) may influence intraamygdala connectivity that would impact ectoamygdala communication. Bilaterally enlarged amygdalae noted in autism may be due to a neuronal pruning defect (Howard, Cowell et al. 2000), although the results of autism volumetric analyses of amygdalae vary in the literature (Amaral and Corbett 2003; Herbert 2004).

Aberrant myelination may reduce the efficiency of neurotransmission in autism and the synchrony of brain communication (Reiss, Feinstein et al. 1986; Dong and Greenough 2004; Akshoomoff 2005; Koul 2005). Anatomical alterations to corpus callosum (Chung, Dalton et al. 2004) may contribute to altered interhemispheric functional networks in autism (Cherkassky, Kana et al. 2006; Just, Cherkassky et al. 2007). Diffusion tensor imaging studies also provide a basis for altered communication in the autism brain (Alexander, Lee et al. 2007). A diffusion tensor imaging study of autism revealed decreases of fractional anisotropy (FA), an index of neuronal integrity, in regions

associated with affective processing including the prefrontal cortex and amygdala that have direct implications for autism deficits in socio-affective behavior (Barnea-Goraly, Kwon et al. 2004).

Several neuropsychological models of autism (Robbins 2004) support the view that the maldeveloped integrity of autism brain circuitry seems to hinder the proper integration of information across functionally segregated regions (Belmonte, Cook et al. 2004). As an explanation of autism behavior, the “weak central coherence” theory proposed by Frith (Frith 1989) suggests the autism brain favors local to global processing. Gustafsson (Gustafsson 1997) proposed that excessive lateral inhibition resulting in weak central coherence may impair the ability to form feature maps in autism which would prevent the normal processing of sensory information such as emotional faces. The amygdala is a central node of connectivity in the fronto-limbic system (Young, Scannell et al. 1994) and its dysfunction in autism contributes to deficits in social behavior according to the amygdala theory of autism (Baron-Cohen, Ring et al. 2000; Howard, Cowell et al. 2000). The “temporal binding deficit” theory suggests that weak central coherence in autism is due to a lack of functional integration; and, this integrative binding deficit could be investigated by observing interregional coherence patterns (Brock, Brown et al. 2002). The underconnectivity hypothesis posits that functional brain connectivity is reduced in autism cortico-cortical networks (Just, Cherkassky et al. 2004). Autism theories involving altered signaling through the cyclic AMP cascade, such as the opiate theory of autism, can be found in Chapter 8.1. These autism neuropathological and neuropsychological models provide a basis for system level investigations of functional connectivity.

Functional Connectivity in Autism

System based studies of autism functional connectivity have been briefly reviewed (Herbert 2004; Wickelgren 2005). Horowitz was the first to examine functional interactions in autism and found frontoparietal underconnectivity in a PET study of glucose metabolism (Horwitz, Rumsey et al. 1988). A PET study using social animations identified reduced connectivity in patient-volunteers with autism between extrastriate cortex and superior temporal regions (Castelli, Frith et al. 2002). An fMRI sentence comprehension study supported the underconnectivity hypothesis of autism with reduced regional integration of cortical language areas (Just, Cherkassky et al. 2004). Patterns of fMRI connectivity between the amygdala and parahippocampal gyrus among patient-volunteers with Asperger's disorder, a high functioning form of autism on the spectrum of autism disorders, are increased relative to controls during a block-design fearful face processing task (Welchew, Ashwin et al. 2005). An fMRI study seeded by the right fusiform identified reduced functional connectivity with the left amygdala during face processing which was negatively associated with social autism severity scores (Kleinhaus, Richards et al. 2008). The frontal and temporal lobes are implicated in the autism behavioral phenotype (Damasio and Maurer 1978; Maurer and Damasio 1982). Dysfunction of circuitry at the junction of the amygdala and hippocampus was implicated in a block-design fMRI study of autism facial emotion processing (Critchley, Daly et al. 2000). The cerebello-limbic circuitry is also central to the autism behavioral, cognitive, and emotional deficits (Lee, Lopez-Alberola et al. 2003). Lesions of rat cerebellar vermis produce behavioral patterns mimicking autism (Bobee, Mariette et al. 2000). Event-

related potential studies of autism suggest that the cerebellum modulates frontal and parietal networks of attention (Townsend, Westerfield et al. 2001) which may impact emotional face processing (Pessoa, Kastner et al. 2002). Taken together, the balance between node segregation and integration into functionally connected circuits may be disrupted in the autism social face processing network.

Although currently the leading functional connectivity framework for autism networks, the underconnectivity hypothesis (Just, Cherkassky et al. 2004) is controversial and may not be generalizable to other tasks and to other networks (Mizuno, Villalobos et al. 2006; Muller 2007). Both subcortico-cortical and cortico-cortical functional connectivity studies have reported overconnectivity in the autism connectivity phenotype (For review, see Muller 2007). A potential biological mechanism in autism is that an increased ratio of excitation to inhibition through respective glutamatergic and GABAergic signaling in sensory, social, and emotional systems could increase functional connectivity by reducing the signal to noise (SNR) ratio of information transfer (Rubenstein and Merzenich 2003). Either underconnectivity or overconnectivity may be associated with the reduced information content in low SNR neural circuitry either through reductions in signal or through increases in noise (Belmonte, Cook et al. 2004). Based on histopathological cellular studies, individuals with autism have an increased number of smaller minicolumns (Buxhoeveden and Casanova 2002) that may increase intracortical signaling (Casanova 2004), reduce lateral inhibition, and cause terminal fields of subcortical afferents to synapse on multiple minicolumns unintentionally enhancing cortical noise in these regions (Casanova, Buxhoeveden et al. 2002) and thereby reducing the signal to

noise ratio of information transfer (Belmonte, Cook et al. 2004). The directionality of pathofunctional connectivity integrity deserves further study in autism.

Specific Aims

The intent of this thesis is to characterize functional connectivity networks of affective face processing that distinguish autism and controls and to test whether the integrity of functional connectivity is associated with behavioral measures of task performance and autism clinical severity. In so doing, we could determine whether the underconnectivity hypothesis was generalizable to an affective face processing task and to both subcortico-cortical and cortico-cortical networks or whether an alternative network framework would be needed to characterize the autism connectivity phenotype and its impact on behavior (Mizuno, Villalobos et al. 2006; Muller 2007). The first study (Chapter 2) quantified the integrity of amygdala connectivity when processing facial affect and identified significant associations between functional connectivity and behavior that distinguished autism from controls. In the second study, the isolation of networks specific to a given facial affect required a novel statistical connectivity approach to assess functional amygdala connectivity networks (Chapter 3). We used partial correlation analysis to separately quantify the integrity of amygdala connectivity in resting networks, emotional face processing networks, and neutral face processing networks in autism and controls and tested the association of functional amygdala circuitry with behavior. The third study quantified the interhemispheric connectivity of resting networks, emotional face processing networks, and neutral face processing networks for autism and controls and the association of affective connectivity dynamics with corpus callosum density and

with behavioral measures of task performance and clinical autism severity (Chapter 4). The fourth study used neuroinformatic techniques including graph theory, agglomerative hierarchical clustering, and multidimensional scaling to test whether patterns, or functional connectivity signatures, in the interhemispheric connectivity measures could distinguish autism from controls (Chapter 5). The fifth study involved development of software to test the hypothesis that the temporal derivative of pulse oximetry signals collected during fMRI acquisition could improve R-wave estimation and therefore provide a useful method to improve functional connectivity analyses (Chapter 6).

Appendix

The appendix contains a review of the cyclic AMP phenotype in autism and controls (Chapter 8.1); a study examining the levels of cyclic AMP in mouse blood and brain, fly heads, and differentiated human neural cells (Chapter 8.2); a study comparing cAMP levels in neural progenitors and differentiated cells that has implications for cAMP therapy in autism (Chapter 8.3); a journal club review of cortical development using diffusion tensor imaging (Chapter 8.4); a statistical review of functional connectivity approaches (Chapter 8.5); a study of learning gains in the UW-Madison Ways of Knowing Biology course using an open source method to make virtual computer images of human brain and Phineas Gage's skull a physical reality through stereolithograph 3D printing (Chapter 8.6); a study examining student learning gains and course content in the Neuroimaging of Cognitive Disorders Course at UW-Madison (Chapter 8.7); and a study of learning gains examining the utility of themes from the NIH Roadmap initiative to educate undergraduate biology students about imaging (Chapter 8.8).

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Chapter 2:

Abnormalities of amygdala connectivity in autism predict gaze behavior and deficits in the decoding of emotional faces

Classification:

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Title:

Abnormalities of amygdala connectivity in autism predict gaze behavior and deficits in the decoding of emotional faces

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Abstract:

The processing of emotional faces requires a network of communication among brain regions including the amygdala. We report here that differential patterns of connectivity with the amygdala are present in autism, including a dorsal-ventral stream imbalance, and that autism behavioral performance and gaze behavior is predicted by the strength of amygdala connectivity alterations. Intracerebral connectivity may serve as an endophenotypic marker of this disorder.

Introduction:

Autism is a behaviorally defined neurodevelopmental disorder with a characteristic triad of deficient diagnostic domains: social, behavioral, and communicative. Deficits in the domain of social interaction are evidenced in autism by atypical emotional face processing ability (1) and distinctive patterns of brain activity (2). Facial emotions communicate social information and their processing requires a syncytium of segregated brain regions to integrate into a functional network (3, 4). The amygdala, as a central node of connectivity in the emotional face processing network, is theorized to contribute to social behavior deficits in autism (5, 6) and may do so either through disruptions in focal activity or in relation to dysfunctional patterns of connectivity with other brain regions. While prior autism studies have documented alterations of focal amygdala activity in this network (7), we sought to determine whether altered connectivity in limbic-cortical emotion processing circuitry contributes to impaired social interactions characteristic of autism by examining the dependence of emotional face processing performance on the integrity of amygdala interactivity with

other brain regions. An inability to form a finely tuned network would impair the processing of information from perception to response and implicate abnormalities of cerebral connectivity as a core feature of autism that could provide a unifying mechanism for the triad of domains deficient in autism.

Evidence from anatomical, behavioral, and functional studies support a connectivity (8) framework as a basis for investigating the emotional face processing network of autism. Neuroimaging and histopathologic studies provide evidence that altered connectivity in autism begins at the neuronal level, reflecting systemic alterations in structure and function (9-11). Several neuroregulatory proteins like reelin, a glycoprotein with a role in neural guidance and dendritic spine plasticity, can impact neuronal connections in autism (12). Altered neuronal packing may contribute to the reduced size of cortical minicolumns (13) and volumetric changes of the amygdala (14-16). Aberrant myelination noted in autism would reduce efficiency of neurotransmission and alter the connectivity in key neural circuits (17-19). A recent diffusion tensor imaging study of autism revealed decreases of fractional anisotropy, an index of neuronal integrity, in regions associated with affective processing including the prefrontal cortex and amygdala (20).

Several neuropsychological models (21) of behavior in autism support the view that the maldeveloped integrity of autistic brain circuitry hinders the proper integration of information across functionally segregated regions (22). Herbert (15) has argued that social and emotional processing abnormalities in autism are a result of systems disruptions that likely arise from dysfunctional connectivity. Although such hypothesized patterns of abnormal connectivity have been documented (10, 15, 23-26),

their functional consequences have not been studied. Such connectional disruptions can be investigated by relating functional patterns of interregional connectivity measures to objectively measured indices of social and emotional behavior. Studies of autism using functional connectivity (10, 15), a correlative measure by which information integrated between brain regions can be detected using neuroimaging techniques (27), lend credence to the notion of intraencephalon dyscommunication in autism. Horwitz was the first to examine functional interactions in autism and found frontoparietal underconnectivity in a resting state PET study of glucose metabolism (23). Another PET study using social animations identified functional underconnectivity between extrastriate cortex and superior temporal regions in autism (24). An fMRI sentence comprehension study supports an “underconnectivity hypothesis” of autism with reduced regional integration of cortical language areas (25). Using multidimensional scaling analysis, an fMRI study of fearful face processing revealed functional amygdala-parahippocampal overconnectivity in patient-volunteers with Asperger’s syndrome (26).

Based on prior work in this laboratory which determined that gaze behavior in autism depends on the extent to which focal regions in a network of gaze processing circuitry are activated (7), we hypothesized that connectivity in the limbic-cortical emotion processing circuitry is altered in autism and contributes to impaired performance on measures of social and emotional perception. In addition, we hypothesized a priori that amygdala-superior temporal gyrus (STG) connectivity is altered in autism and contributes to aberrant eye gaze behavior based on multiple lines of evidence based on: (1) the role of the STA, which we define here to include the the superior temporal sulcus (STS) region and the superior temporal gyrus (STG) (28, 29), in

facial emotion processing (30), in gaze perception (31), and in integration of an observer's gaze direction with gaze processing (29); (2) reduced grey matter (32) and reduced diffusion tensor imaging measures of fractional anisotropy (20) in autism STA; (3) the known anatomical connections between the amygdala and STA (33, 34); (4) the role of the amygdala in gaze processing(35); and, (5) the heightened amygdala activity during eye fixation in autism(7).

To test this hypothesis, twelve controls and twelve individuals with autism participated in a 3.0 Tesla functional MRI study using an emotional face processing task which required subjects to discriminate neutral from emotional faces. We quantified the strength of the functional interaction with the amygdala using a seed voxel correlation approach based on each individual's anatomically defined amygdala region of interest. We then assessed the relationship between behavioral performance and the connectivity of functional amygdala circuitry in autism and controls.

Results:

Behavioral Measures

Compared to controls, subjects with autism produced slower responses to emotional faces [$t(20) = 2.32$, $P = 0.031$], comparable response times to neutral faces [$t(20) = 0.680$, $P = 0.50$], and performed the task less accurately [$t(22) = -3.065$, $P = 0.006$]. Controls (mean $\pm$ SD = 1111 $\pm$ 182 ms) produced significantly faster responses than subjects with autism (mean $\pm$ SD = 1310 $\pm$ 221 ms) when viewing emotional faces [$t(20) = 2.32$, $P = 0.031$]. The autism (1394 $\pm$ 351 ms) response time to neutral faces was comparable to controls (1290 $\pm$ 362ms; $t(20) = 0.680$, $P = 0.50$). Subjects

with autism correctly identified significantly fewer (mean $\pm$ SD = 25.3 $\pm$ 15.9) facial emotions compared to controls (mean $\pm$ SD = 39.4 $\pm$ 0.79; $t(22) = -3.065$, $P = 0.006$). Two individuals with autism who were nonverbal and performed the task at chance level were included in the connectivity analysis to include observations that span the broad range of autism presentations. After removing the two non-responders from the autism group (mean $\pm$ SD = 30.4 $\pm$ 11.7), significant group differences in accuracy remained [$t(20) = 2.66$, $P = 0.015$]. There was not a speed-accuracy tradeoff for either group when regressing reaction time on the number of correct responses (autism: [$F(1,8) = 1.66$; $P = 0.23$]; controls: [$F(1,10) = 1.07$; $P = 0.33$]). The group difference in the eye to face gaze ratio was marginally significant [$t(21) = -1.84$; $p = 0.08$; autism mean $\pm$ SD: 0.179 $\pm$ 0.16; control mean $\pm$ SD: 0.304 $\pm$ 0.16]. The relationship between response accuracy and the eye to face gaze ratio was significant in autism [$r(10) = 0.617$; $P = 0.03$], not significant in controls [$r(9) = -0.075$; $P = 0.83$], and was marginally significant between groups [$z = 1.64$; $P = 0.10$]. The relationship between emotional face response times and the eye to face gaze ratio was significant in autism [$r(8) = -0.700$; $P = 0.02$], not significant in controls [$r(9) = 0.367$; $P = 0.30$], and significantly different between groups [$z = -2.28$; $P = 0.02$]. The relationship between neutral face response times and the eye to face gaze ratio was not significant in autism [$r(8) = -0.040$; $P = 0.91$], not significant in controls [$r(9) = 0.322$; $P = 0.33$], and not significantly different between groups [$z = -0.722$; $P = 0.47$]. Because the control group performed near the ceiling (98.5%), correlations with accuracy measures for the controls are constrained by the truncated range.

Functional Amygdala Connectivity with Whole Brain

Group Main Effect

Using a mixed effect ANOVA, we spatially mapped group differences in functional connectivity between the amygdala and all other brain regions (Figure 1, Supplementary Figure 1a). The group main effect analysis identified three significant [$F(1,22) \geq 9.26$; $P = 0.006$; volume $\geq 560 \mu\text{L}$; group alpha = 0.05] clusters of altered connectivity with the amygdalae (Figure 1a). These clusters were localized to the right parahippocampal gyrus [$F(1,22) = 23.42$; $P = 0.000$], left parahippocampal gyrus [$F(1,22) = 14.45$; $P = 0.001$], and the right superior parietal lobule [$F(1,22) = 21.37$; $P = 0.000$]. The group main effect clusters were used as regions of interest to quantify between group protected t-scores (2-tailed, unpaired) and connectivity values from each subject's whole brain functional connectivity maps (Supplementary Figure 3 a-d). Compared to controls, the amygdalae of individuals with autism exhibited greater connectivity with the parahippocampal regions bilaterally and reduced connectivity with the right superior parietal region (Figure 1 a-b, Group Main Effect). The mean connectivity values for each group for each cluster are displayed in Figure 1b.

Group x Hemisphere Interaction

The Group x Hemisphere interaction analysis identified three clusters of significant [$F(1,22) \geq 9.26$; $P = 0.006$; volume $\geq 560 \mu\text{L}$; group alpha = 0.05] connectivity that differed between groups and showed differential amygdala connectivity dependent on the hemispheric location of the amygdala seed. These clusters were localized to the right middle frontal gyrus [$F(1,22) = 18.36$; $P = 0.000$], left insula [$F(1,22) = 34.65$; $P = 0.000$], and the left parahippocampus [$F(1,22) = 26.97$; $P \leq$

0.000]. The centroid of the left parahippocampal cluster for the Group x Hemisphere and group main effect analyses were different and considered to be unique clusters. The right amygdala shows group differences with all three clusters, whereas the left amygdala shows none. The Group x Hemisphere clusters (Supplementary Figure 2a) were used as regions of interest to quantify between group protected t-statistics ($P = 0.05$, 2-tailed). The right amygdala of individuals with autism displayed greater connectivity than controls with both the left insula and left parahippocampus, and reduced connectivity with the right middle frontal gyrus (Supplementary Figure 2, Group x Hemisphere Interaction).

Functional Amygdala Connectivity Associations with Emotional Face Performance

Group Main Effect

The group main effect functional connectivity ROI cluster averages were used in bivariate correlation analyses with behavioral performance (See Figure 2). Among individuals with autism, those with increased connectivity between the right amygdala and both the left and right parahippocampal group main effect clusters exhibited slower response times to the emotional faces ($r = 0.79$ for left parahippocampus; $r = 0.66$ for right parahippocampus) while among the controls, connectivity between these regions was not significantly associated with performance ($r = 0.19$ for left parahippocampus; $r = 0.47$ for right parahippocampus); and, after controlling for neutral face reaction times, the association of autism emotional face reaction times with right amygdala connectivity was significant for the left parahippocampus [$r = 0.902$, $t(7) = 5.54$, $P = 0.001$] and significantly different between groups [$z = 2.70$; $P = 0.007$] (Supplementary Figure 3c).

The correlation between mean right amygdala-right superior parietal lobule underconnectivity and the number of correctly identified facial emotions was marginally significant in autism against a null correlation (autism $r = -0.546$, 2-tailed $P = 0.066$, $n = 12$) and showed significant group differences with controls [control $r = 0.545$, 2-tailed $P = 0.067$, $n=12$; $z = -2.60$; $P = 0.009$]. However, this group difference must be interpreted in the context of near ceiling control performance limiting the range of fitted values. The association of emotional face response time with connectivity between the left amygdala and right parahippocampus was significant in autism [$r = 0.758$; $t(8)=3.29$; $P = 0.011$]; and remained significant after controlling for neutral face response times [$r = 0.759$; $t(7)=3.08$; $P = 0.018$].

After controlling for neutral face reaction times, autism associations of emotional face reaction times with right amygdala-group main effect cluster connectivity were significant for the left parahippocampus ($r = 0.902$, $t(7) = 5.54$, $P = 0.001$), were marginally significant for the right parahippocampus ($r = 0.655$, $t(7) = 2.29$, $P = 0.056$), and those associations for controls remained insignificant ($r = 0.0265$, $t(9) = 0.0795$, $P = 0.94$ for left parahippocampus; $r = 0.193$, $t(9) = 0.591$, $P = 0.57$ for right parahippocampus). The group difference in the association between emotional face reaction times with right amygdala connectivity to the right parahippocampus was not significant [$z'(\text{autism-control}) = 0.553$; $P = 0.581$] and to the left parahippocampus was marginally significant [$z = 1.77$; $P = 0.077$]; and, after controlling for neutral face response times, the right parahippocampal association remained insignificant [$z = 1.09$; $P = 0.28$] but the group difference in the left parahippocampal association was significant [$z = 2.70$; $P = 0.007$].

The association of emotional face response time with connectivity between the left amygdala and right parahippocampus was significant in autism [$r = 0.758$; $t(8)=3.29$; $P = 0.011$] and controls [$r = 0.627$; $t(10) = 2.55$, $P = 0.029$] but showed no group differences [$z = 0.506$; $P = 0.61$]. After controlling for neutral face response times, this association was significant in autism [$r = 0.759$; $t(7)=3.08$; $P = 0.018$], was marginally significant in controls [$r = 0.565$; $t(9) = 2.05$, $P = 0.070$], but showed no group differences [$z = 0.654$; $P = 0.51$].

The association of emotional response time to left amygdala-left parahippocampal (group main effect cluster) connectivity was marginally significant in controls [$r = 0.563$; $t(10) = 2.15$; $P = 0.057$], was not significant in autism [$r=0.374$; $t(8) = 1.14$; $P = 0.29$], and showed no significant group differences [$z = -0.484$; $P = 0.63$]. After controlling for neutral face reaction times, these associations were not significant in controls [$r = 0.0472$; $t(9)=0.142$; $P = 0.89$] or autism [$r = 0.426$; $t(7)= 1.25$; $P = 0.25$] and showed no group differences [$z = 0.755$; $P = 0.45$].

Group x Hemisphere Interaction

The Group x Hemisphere functional connectivity ROI cluster averages were used in a bivariate correlation analysis with behavioral measures (Supplementary Figure 3 e-f). Among individuals with autism, those with increased connectivity between the right amygdala and both the parahippocampus and insula showed evidence of poorer or slower performance on the emotional face discrimination task. Poorer accuracy performance in autism was significantly associated with right amygdala-left insula overconnectivity (right amygdala: autism $r = -0.598$, 2-tailed $P = 0.040$, $n = 12$) while increased

connectivity between the right amygdala and left parahippocampus was associated with slower response times in subjects with autism ($r = 0.88$) (Supplementary Figure 3).

Among controls, correlations of connectivity between these regions and accuracy ($r=0.12$) or response time ($r = 0.31$) were considerably lower. There was a significant group difference in the correlation of emotional face response time with connectivity between the right amygdala and the left parahippocampus before [$z = 2.08$; $P = 0.037$] and after [$z = 2.34$; $P = 0.019$] partialling out the effect of neutral face response times [autism $r = 0.908$, $t(7) = 5.74$, $P = 0.001$; control $r = 0.248$, $t(9) = 0.769$, $P = 0.461$] (Figure 1c).

The association of emotional face response times with left amygdala-Group x Hemisphere cluster connectivity was marginally significant for the left parahippocampus [controls $r = 0.553$, $t(10) = 2.10$, $P = 0.062$; autism $r = 0.386$, $t(8) = 1.18$, $P = 0.27$] and for the right middle frontal gyrus [controls $r = -0.300$, $t(10) = -0.992$, $P = 0.34$; autism $r = -0.553$, $t(8) = -1.88$, $P = 0.097$]; was not significantly related after accounting for neutral face reaction times [left parahippocampus autism $r = 0.479$, $t(7) = 1.44$, $P = 0.19$; control $r = 0.445$, $t(9) = 1.49$, $P = 0.17$; right middle frontal gyrus autism $r = -0.558$, $t(7) = -1.78$, $P = 0.12$; control $r = -0.200$, $t(9) = -0.611$, $P = 0.56$]; and showed no group differences [left parahippocampus $z = 0.0794$, $P = 0.937$; right middle frontal gyrus $z = -0.792$, $P = 0.43$].

Functional Amygdala Connectivity Associations with Neutral Face Performance

No significant group differences in connectivity-neutral face response time associations were identified after controlling for emotional face response times. A relationship of potential interest was localized to the left amygdala-left parahippocampus

(group main effect) circuit and was significant in controls [$r = 0.67$; $t(10)=2.85$; $P = 0.017$], was not significant in autism [$r = -0.20$, $t(8)= -0.588$, $P = 0.57$], and showed significant group differences [$z = -2.02$; $P = 0.044$]; however, after controlling for emotional face response time in each group, these partial associations were not significant in controls [$r = 0.440$, $t(9) = 1.47$; $P = 0.176$] or autism [$r = -0.296$; $t(7)=-0.821$; $P = 0.439$] and did not show group differences [$z = -1.44$; $P = 0.15$]. Another potential relationship was tested between neutral face response times and connectivity of the right amygdala with the left parahippocampus (group main effect cluster) which was not significant in autism [$r = -0.297$; $t(8) = -0.880$; $P = 0.405$], or controls [$r = 0.217$; $t(10) = 0.704$; $P = 0.497$], and showed no group differences [$z = -1.05$; $P = 0.295$]. After accounting for emotional face response times, this connectivity association with neutral face performance was significant in autism [$r = -0.735$; $t(7) = -2.86$; $P = 0.024$], not significant in controls [$r = 0.108$; $t(9) = 0.327$; $P = 0.75$], and showed marginally significant group differences [$z = 2.70$; $P = 0.052$].

Amygdala Connectivity with Superior Temporal Gyrus (STG)

Using a voxelwise analysis (Supplementary Figure 1b), we identified group differences in functional connectivity of the amygdalae with the STG (Figure 2 a-b, Group Main Effect). The amygdalae-right STG circuit was functionally overconnected in autism (Figure 2a-b, Group Main Effect) and the extent of this overconnectivity predicted the fraction of time that individuals with autism spent fixating the eyes when gazing upon a face (left amygdala [autism: $t(10)=-3.24$, $p=0.009$; control: $t(9)=1.27$, $p=0.236$], Figure 2c; right amygdala [autism: $t(10)=-4.81$, $p=0.001$; control: $t(9)=-0.407$, $p=0.694$],

Supplementary Figure 4). This relationship is significantly different between groups for right STG connectivity with the left [$t(19)=-2.95$, $p=0.008$], but not the right [$t(19)=-1.828$, $p=0.083$], amygdala.

Discussion:

In this study, an inability to form a finely tuned emotional face processing network impedes and impairs information processing(22) from perception to response(36) and offers evidence from the social domain that cerebral connectivity may provide a useful endophenotypic framework to unify the seemingly disparate triad of deficient domains(15). The observation that eye avoidance behavior is associated with overcommunication between nodes involved in gaze processing (STG) and emotional face processing (amygdala) is consistent with our prior findings showing that individuals with autism exhibit hyperactivation in the amygdala in response to this task and modulation of amygdala activity when subjects avert their gaze away from the eye region of faces (7). Given that a specific subset of autism individuals displaying amygdala overconnectivity with the parahippocampus and insula are those who are most impaired on behavioral measures of facial emotion discrimination, our findings indicate that individuals with a dysconnectivity endophenotype are particularly susceptible to dysintegration when processing the emotional content communicated by faces.

Our study corroborates a prior finding of amygdala-parahippocampal overconnectivity (26) and extends these results to include amygdala underconnectivity with prefrontal and parietal regions, as well as overconnectivity with insula and parahippocampal regions. We determined the functional consequences of these abnormal

patterns of amygdalar connectivity in autism by examining relations between indices of connectivity and behavioral performance. Although group differences in connectivity between right amygdala and right middle frontal gyrus were evident, there were no group differences in the prediction of performance from these connectivity indices. However, amygdala dysconnectivity with other brain regions did implicate dysintegration as a functional feature of emotional face processing behavior in autism. Amygdala overconnectivity with the parahippocampal and insula regions predicted poorer performance in subjects with autism significantly more strongly than in controls. No significant group differences in associations between connectivity and neutral face response times were present after accounting for response times to emotional faces. It may well be that these are the subjects in whom increased electrodermal responding to emotional stimuli might also be found (37). In contrast to overconnectivity with emotion processing regions, it was found that underconnectivity between the right amygdala and the right superior parietal lobule significantly more strongly predicted worse performance in subjects with autism compared with controls. Superior parietal dysynchrony with the amygdala, a key modulatory node in the ventral visual stream (38), implicates a functional imbalance between dorsal and ventral visual stream activity consistent with prior reports in autism (39-42). In support of a dysintegration hypothesis, emotional face processing produces a skewed balance of amygdala integration with functional underconnectivity to attentional nodes (frontopolar, parietal) and overconnectivity to affective nodes (parahippocampus, insula). The fact that this dysintegrated pattern of amygdala connectivity occurs simultaneously leads us to hypothesize that overconnectivity of the amygdala with regions processing the affective intent of facial

emotions impairs amygdala integration with attentional and gaze networks, but carefully designed studies will be necessary to determine the effective directionality and causality of this interaction.

Connectional dysintegration within the affective circuitry of the brain may be a core defect that could explain the triad of domains deficient in autism (15). By employing a facial emotion discrimination task that probes the social domain of autistic behavior, we were able to identify functional amygdala connectivity alterations that impair the flow of emotional information and may directly handicap social behavior in autism. The pattern of amygdala connectivity supports our hypothesis of cerebral dysintegration in which the processing and integration of emotional information (22) is impaired and impacts behavior as indicated by the integrity of functional connectivity measures and behavioral performance on the emotional face processing task (36). Future studies will need to address the degree to which glia (43), pathologic hemodynamics (44), or eye gaze behavior (7) may have contributed to group functional connectivity differences, as well as the potential for hormonal (45) or pharmacotherapies to normalize functional connectivity alterations and improve behavior.

Methods:

Study design and task methodology have been reported elsewhere (7). Briefly, twelve male control subjects with a mean age ($\pm$ SD) of 17.0 ($\pm$ 2.86) and twelve males with autism or Asperger's disorder aged 16.75 ($\pm$ 4.52) years were recruited from a list of available autism volunteers maintained at the University of Wisconsin-Madison Waisman Center. Ages were not significantly different [$t(22) = -0.162$, $P =$

0.87] between groups. We recorded eye position during the scan session with the iView infrared eye tracking system. The fraction of time that subjects spent fixating the eyes when gazing upon the face (gaze ratio of eye to face) (46) was calculated for each subject except one control subject. Using AFNI (47) to conduct a voxelwise whole brain functional connectivity analysis seeded by individually parcellated amygdalae (46) and a mixed effect Group x Hemisphere of Amygdala Seed x Subject ANOVA (Supplementary Methods), we examined the integrity of functional amygdala interactions with both the whole brain and the STG in particular and their relation to behavioral performance and eye gaze behavior, respectively (Supplementary Note 4). Criteria for diagnosing autism and Asperger's Disorder included DSM-IV and confirmation with the Autism Diagnostic Interview Revised (ADI-R). Subjects participated in an event-related fMRI study using a facial emotion recognition task. In total 40 faces, 16 neutral and 24 emotional (8 happy, 8 fear, 8 anger), from the Karolinska Directed Emotional Faces Set (48) were presented for 3 seconds each with a jittered inter-trial interval ranging from 5 to 7 seconds. Subjects were instructed to decide if the face displayed an emotion (happy, fear, anger) or was neutral and to indicate their choice with a button box press. Both accuracy (percent correct) and reaction times were measured. Image acquisition consisted of 409 gradient recalled echo-planar images using BOLD contrast (TE = 30 ms; TR = 2 sec; FOV = 240x240 mm, 64x64 matrix, voxel size= 3.75 x 3.75 x 5mm) acquired sagittally (30 slices, 4mm thick with 1mm gap) with a 3.0 Tesla GE SIGNA Scanner (Waukesha, WI). Axial T1-weighted 3D SPGR images were also acquired (TE = 8 ms, TR = 35 ms, FOV = 240x240 mm, 256x192 matrix, 124 axial slices, slice thickness = 1.1-1.2 mm, NEX = 1, flip angle = 30 degrees). Analyses were carried out using AFNI(47) and software

developed in house. Echoplanar images were filtered in the spatial frequency domain, reconstructed, motion corrected, and registered to anatomical data. Voxelwise whole brain functional connectivity maps seeded by each amygdala served as a metric for the spatial extent and degree of functional connectivity, or functional integrity, between a given region and the amygdala. Two whole brain functional connectivity maps, one map per amygdala, were generated for each subject. Whole brain correlation maps were entered into a three-way ANOVA (Group x Hemisphere x Subject) with Group and Hemisphere fixed and Subjects nested and randomized within Group. Using individual voxel probability thresholding in combination with cluster volumes, we corrected the whole brain [F(1,22) $\geq$ 9.26; P = 0.006; volume $\geq$ 560 μ L; group alpha = 0.05] and amygdala-STG analyses [F(1,22) $\geq$ 8.84; P = 0.007; volume $\geq$ 350 μ L; group alpha = 0.05]. A Talarach-based bilateral STG ROI available in AFNI was used to constrain the analysis. Subsequent protected two-tailed t-tests were used to determine directionality of functional connectivity effects. Group differences in connectivity correlations with performance were assessed using the standardized difference (autism-controls) in z' scores produced by Fisher's r to z' transformation. Partial correlations were used to increase the precision of connectivity relations with either emotional or neutral face reaction times by controlling for reaction times to neutral or emotional faces, respectively. Coordinates x,y, and z are reported in the RAI convention. We also conducted an amygdala-STG connectivity analysis (Supplementary Figure 1b) and tested for significant group differences in the relation of connectivity in this circuit with eye-gaze behavior. Regression and correlation analyses were conducted in R (version 2.1.1) and SPSS 12.0 (SPSS Inc., Chicago).

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Figure Legends

Figure 1: Amygdala Connectivity with Whole Brain.

a) Group Main Effect Clusters of Significance. Corrected for Multiple Comparisons: (I) Right Parahippocampal Gyrus: $x = -15.7$, $y = 13.4$, $z = -20.6$, volume = 1344 μL , $t(22) = -4.834$ (II) Left Parahippocampal Gyrus: $x = 20.0$, $y = 7.7$, $z = -19.4$, volume = 614 μL , $t(22) = -3.801$ (III) Right Superior Parietal Lobule: $x = -32.4$, $y = 70.4$, $z = 51.3$, volume = 578 μL , $t(22) = 4.623$ b) Mean Connectivity versus Group Main Effect Cluster Location. Connectivity of the Right or Left Amygdala with the (I) Right Parahippocampal Gyrus, (II) the Left Parahippocampal Gyrus, and (III) the Right Superior Parietal Lobule. Error bars are SEM. c) Right Amygdala Connectivity with Left Parahippocampus (Group x Hemisphere Cluster) versus Response Time (ms) to Emotional Faces [control $r = 0.311$, 2-tailed $P = 0.33$, $n = 12$; autism $r = 0.879$, 2-tailed $P = 0.001$, $n=10$; group difference: $z = 2.34$, 2-tailed $P = 0.019$]. Graphs are displayed with 95% confidence bands.

Figure 2: Group Main Effect of Amygdalae-STG Connectivity.

a) Right STG Cluster with a Significant Group Difference in Connectivity with the Amygdalae. $x = -38.5$, $y = -3.2$, $z = -17.2$, volume = 381 μL . Corrected for multiple comparisons at the 0.05 level by thresholding at $F(1,22)=8.84$ and a cluster volume of 350 μL . b) Mean Amygdala Connectivity of Right STG cluster. Compared to controls, the integrity of functional amygdalae connectivity with the right STG is greater in autism [right amygdala: $t(22)=2.06$, $p=0.052$; left amygdala: $t(22)=4.43$, $p=0.002$]. Error bars are SEM. c) Left Amygdala Connectivity with the Right STG Cluster versus Eye Gaze

Behavior. This relationship is significantly different between groups [$t(19)=-2.95$, $p=0.008$]. Graphs are displayed with 95% confidence bands.

Figures:

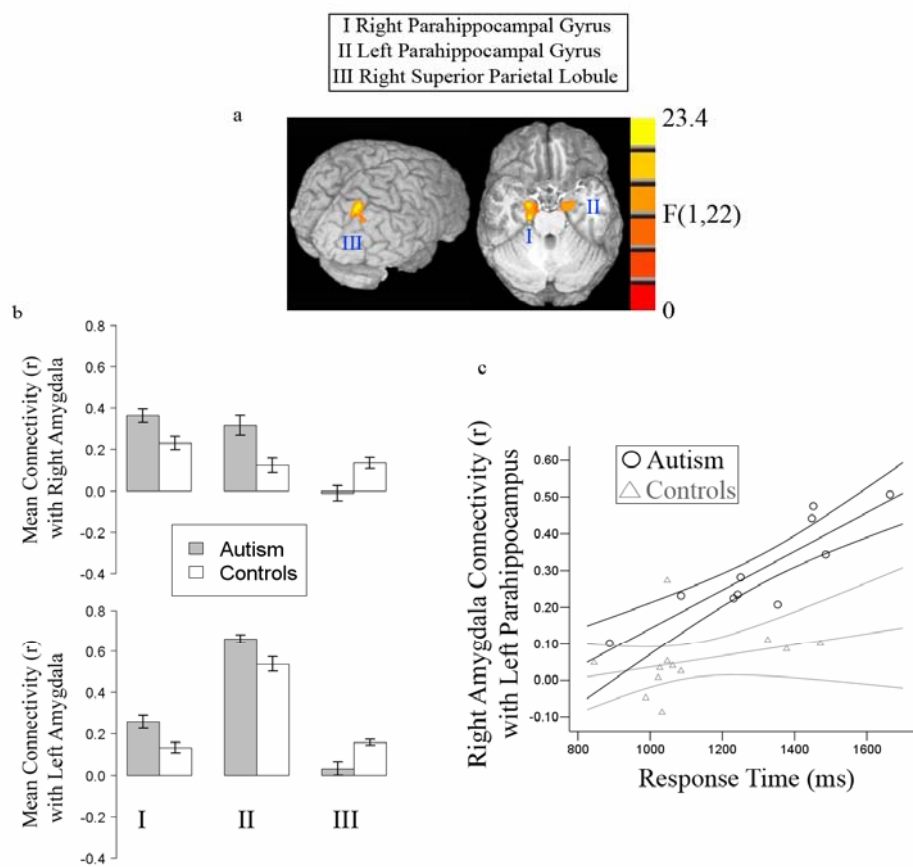


Figure 1

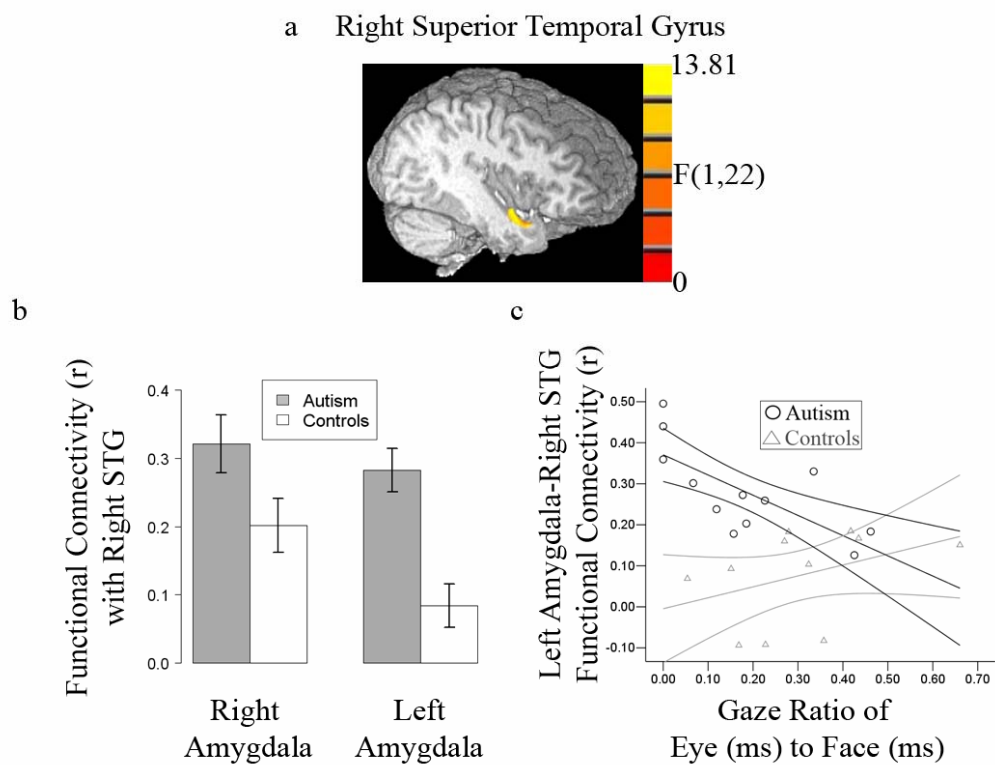


Figure 2

Supplement:

Supplementary Figure Legends

Supplementary Figure 1: a) Voxelwise Whole Brain Functional Connectivity Analysis.

Parcellation criteria and software developed in this laboratory were used to define amygdala regions of interest (ROI) on high-resolution anatomical images by an expert in the laboratory (B.N.). Amygdalae ROIs were resampled to the resolution of the echoplanar images and used to identify regions functionally connected to the amygdala. We employed a whole brain voxelwise correlative functional connectivity analysis seeded by each voxel within an amygdala ROI to map the strength and hemispheric patterns of functional connectivity with the amygdala. The timecourse of activity from a given sample voxel in the brain was correlated (Pearson's correlation) with the time course of activity (jittered ± 1 TR) contained within each voxel of an amygdala ROI. The maximum correlation was determined and stored at the spatial location of the sample voxel in a functional connectivity map. This procedure was repeated iteratively for the right and left amygdala seeds. A confirmatory analysis using the non-parametric Spearman's correlation coefficient produced clusters with a similar pattern of altered connectivity. b) Voxelwise Amygdala-STG Connectivity Analysis. A Talaraich-based bilateral STG ROI available in AFNI was used to constrain this analysis. Brain images were generated with BrainVoyager Brain Tutor (Version 1.2).

Supplementary Figure 2: Group By Hemisphere Interaction

a) Mixed Effect ANOVA Clusters of Significance. Corrected for Multiple Comparisons:

(I) Right Middle Frontal Gyrus: $x = -34.7$, $y = -58.4$, $z = 8.8$, volume = 710 μL , $t(22) = -$

5.335 (II) Left Insula: $x = 39.3$, $y = 3.5$, $z = 1.2$, volume = 638 μL , $t(22) = 4.614$ (III)

Left Parahippocampal Gyrus: $x = 24.8$, $y = 0.0$, $z = -17$, volume = 620 μL , $t(22) = 4.809$

b) Mean Connectivity versus Cluster Location. Connectivity of the Right or Left Amygdala with the (I) Right Middle Frontal Gyrus, (II) Left Insula, and (III) the Left Parahippocampal Gyrus. Error bars are SEM.

Supplementary Figure 3: Whole Brain Amygdala Connectivity versus Performance.

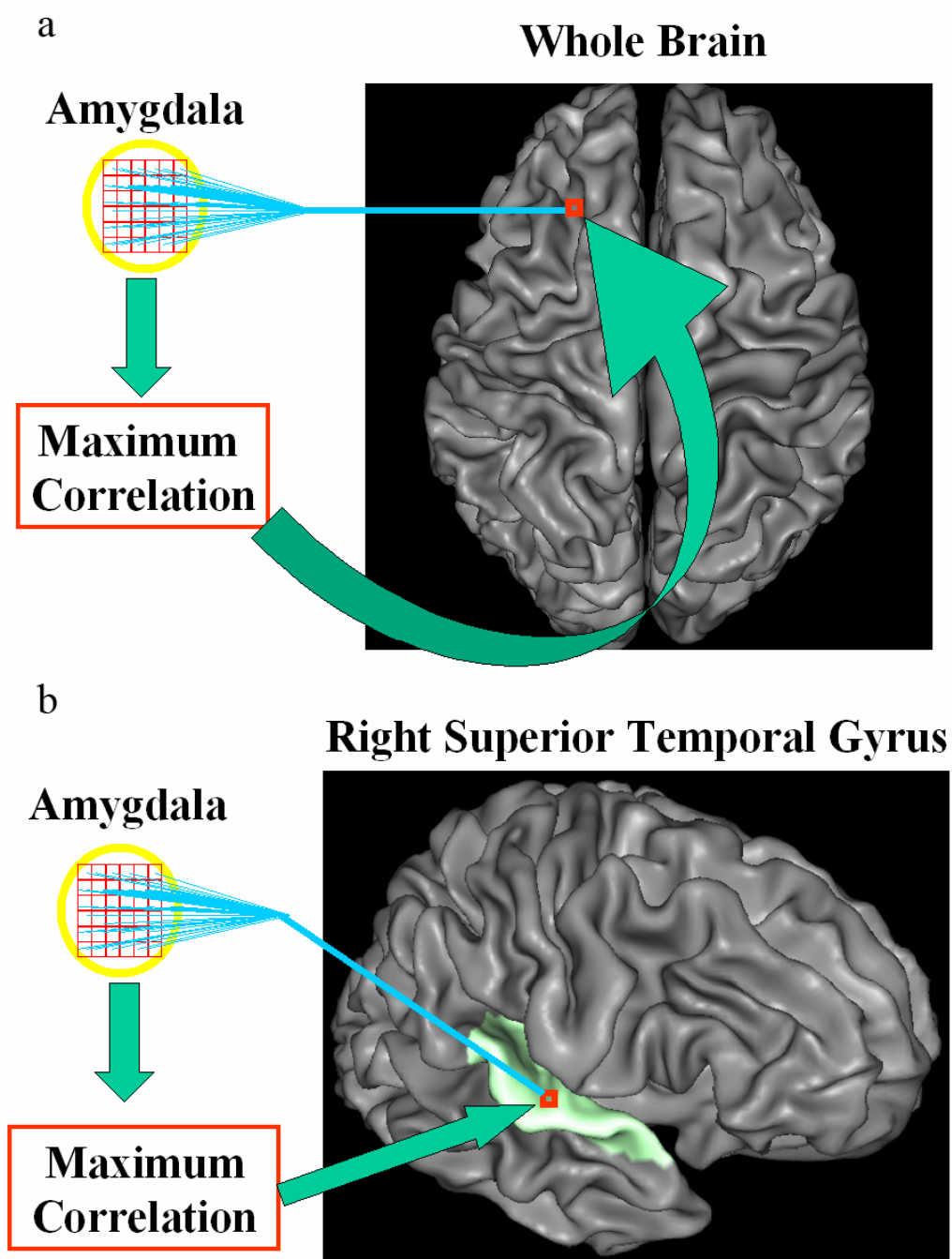
Group Main Effect Analysis: Connectivity versus Response Time to Emotional Faces

(ms) for connectivity between a) the Right Amygdala and Right Parahippocampus (control $r = 0.471$, 2-tailed $P = 0.12$, $n = 12$; autism $r = 0.658$, 2-tailed $P = 0.039$, $n = 10$), b) the Left Amygdala and Right Parahippocampus (control $r = 0.627$, 2-tailed $P = 0.029$, $n = 12$; autism $r = 0.758$, 2-tailed $P = 0.011$, $n = 10$), and c) the Right Amygdala and Left Parahippocampus (control $r = 0.191$, 2-tailed $P = 0.55$, $n = 12$; autism $r = 0.795$, 2-tailed $P = 0.006$, $n = 10$). Connectivity versus the Number of Correct Responses for connectivity between the d) Right Amygdala and Right Superior Parietal Lobule (control $r = 0.545$, 2-tailed $P = 0.067$, $n = 12$; autism $r = -0.546$, 2-tailed $P = 0.066$, $n = 12$). Group x

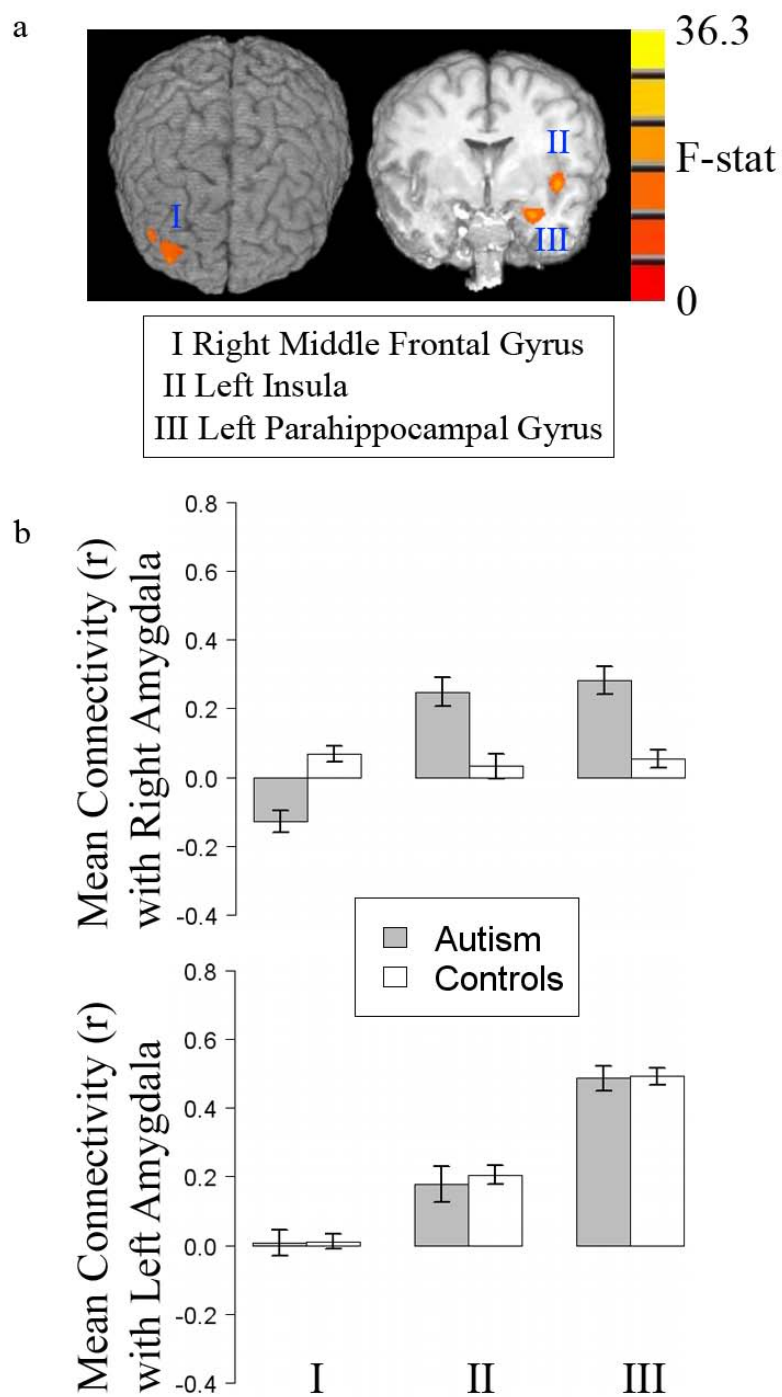
Hemisphere Interaction Analysis: Connectivity versus the Number of Correct Responses for connectivity between e) the Right Amygdala and Left Insula (control $r = 0.120$, 2-tailed $P = 0.71$, $n = 12$; autism $r = -0.598$, 2-tailed $P = 0.040$, $n = 12$) and f) the Left Amygdala and Left Insula (control $r = -0.055$, 2-tailed $P = 0.79$, $n = 12$; autism $r = -0.609$, 2-tailed $P = 0.036$, $n = 12$). Graphs are displayed with 95% confidence bands.

Supplementary Figure 4: Connectivity of STG cluster with Right Amygdala versus Eye Gaze Behavior. In autism, the connectivity integrity of the right STG cluster with the right amygdala [autism: $t(10)=-4.81$, $p=0.001$; control: $t(9)=-0.407$, $p=0.694$] predicts the percentage of time spent fixating the eyes when gazing upon emotional faces. This relationship is marginally different between groups [$t(19)=-1.828$, $p=0.083$]. 95% Confidence Bands.

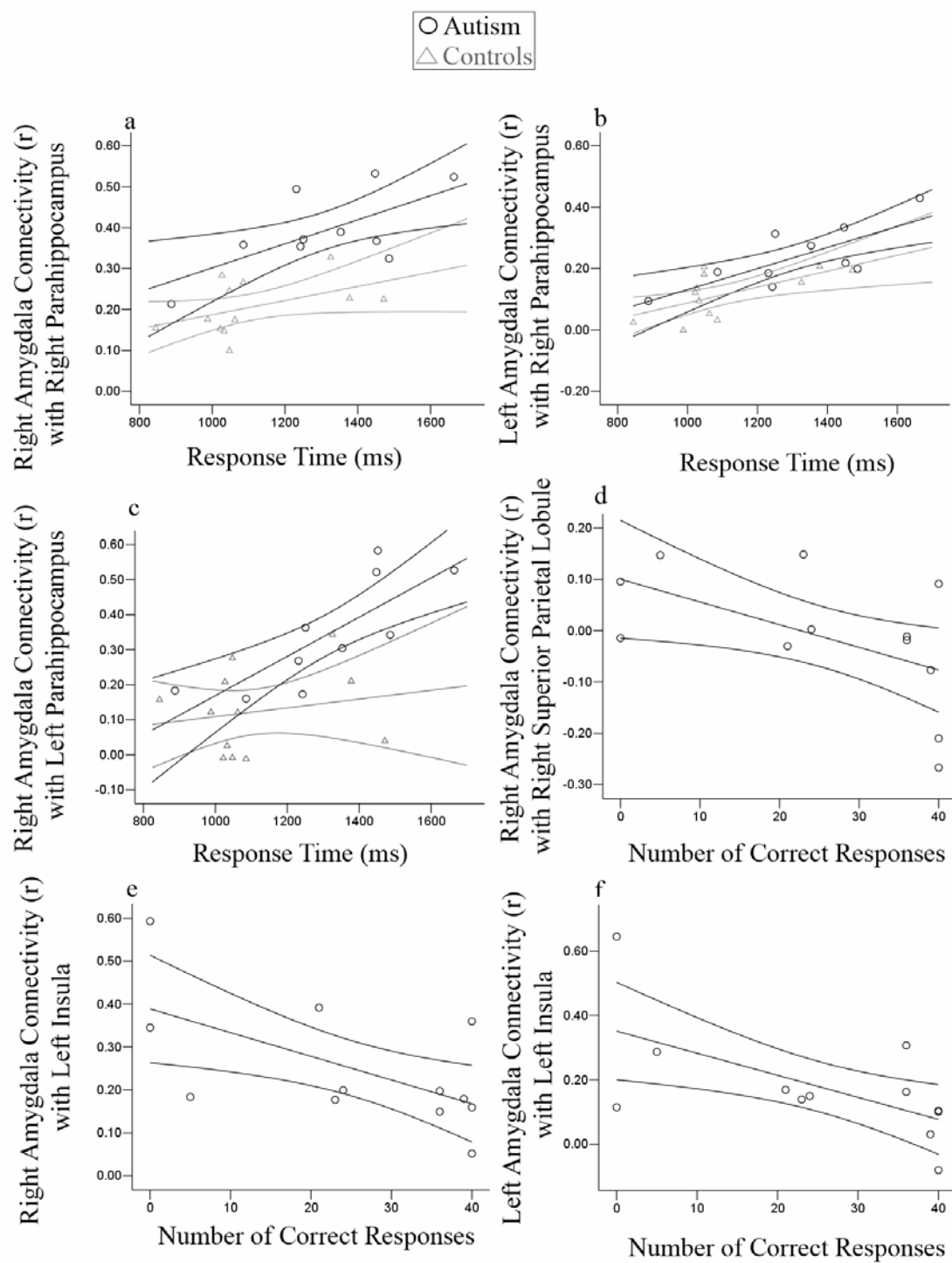
Supplementary Figures:



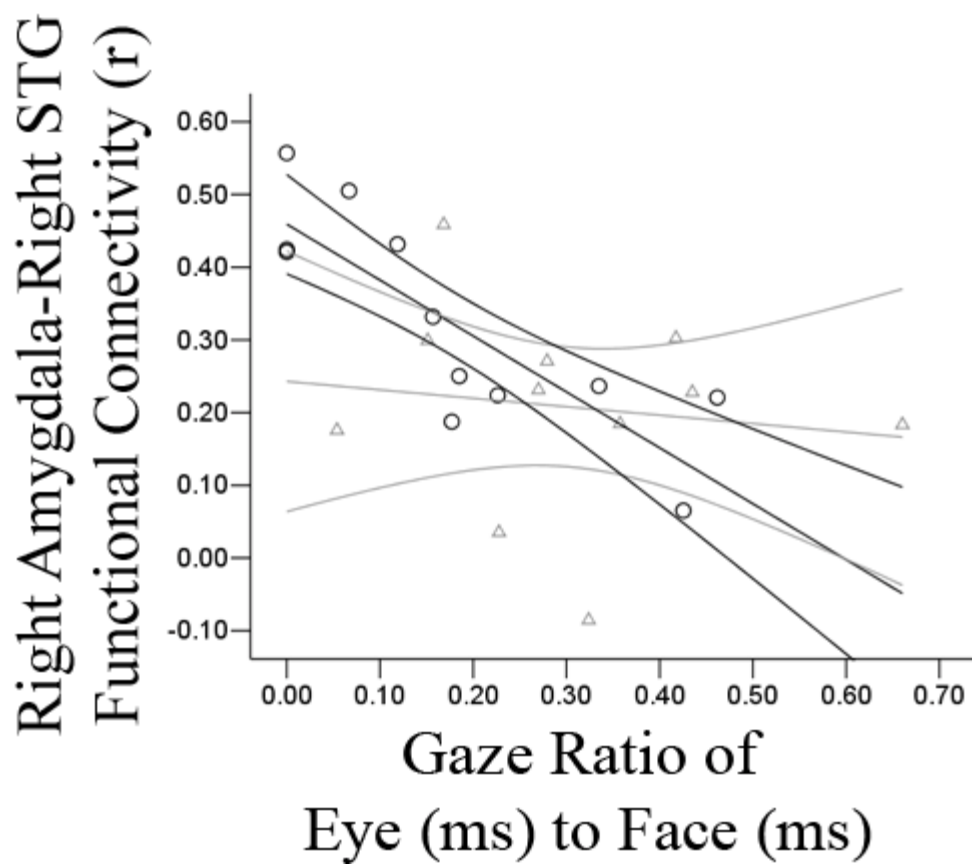
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

Chapter 3:

Amygdala Connectivity in Autism Affective Face Processing Networks

Amygdala Connectivity in Autism Affective Face Processing Networks

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Abstract

Autism is a neurodevelopmental disorder of unknown etiology with aberrant social, communicative, and behavioral domains. Dysfunctional communication of brain regions with the amygdala, a central node in affective processing, may contribute to aberrant social behavior in autism. In a functional MRI study, we examined affective face processing networks in autism and controls using a facial emotion discrimination task. Functional amygdala connectivity networks during both emotional and neutral face processing and at rest were isolated using a partial correlation analysis. We identified several visual and affective processing regions differentially connected with the amygdala in autism. The integrity of autism functional amygdala circuitry was significantly associated with autism severity scores and with behavioral task performance that differed between groups for several brain regions. Our results suggest that the amygdala is a behavioral catalyst dysfunctionally connected to visual and affective processing nodes comprising affective networks with reduced metastability and impaired multivariability.

Introduction

Autism is a neurodevelopmental disorder with a triad of deficits in the social, behavioral, and communicative domain [1]. In autism, neural alterations in function and structure produce system-level modifications in interregional brain communication with consequences for social behavior [2-5]. A connectivity framework may therefore be useful for understanding autism [6,7]. The underconnectivity hypothesis postulates that functional brain connectivity[8], the temporal association of regional brain function, is reduced in autism cortico-cortical networks based on an fMRI study of semantic processing [9]. However, the generalizability of underconnectivity to subcortical-cortical networks is unclear [10,11]. The amygdala is a subcortical region in the fronto-limbic system [12] that is a primary node of interest unifying autism neuropathology [13] and social behavior [14,15].With autism alterations in functional amygdala activation when processing facial emotions [16], amygdala communication through affective face processing networks may be disrupted and contribute to autism social behavior [17].

The amygdala is a central node in affective face processing that is part of a frontal, superior temporal, and parietal network describing autism social function and pathology [18]. Functional connectivity between the amygdala and parahippocampal gyrus among patient-volunteers with Asperger's syndrome, a high functioning form of autism on the spectrum of autistic disorders, were increased relative to controls during a fMRI block-design fearful face processing task [19]. The amygdala has a prominent role in the subcortical visual system and is positioned to influence the non-verbal communication of affect conveyed by the face and social behavior through communication with the

fusiform [20]. An fMRI study identified reduced functional connectivity between the right fusiform and the left amygdala during face processing which was negatively associated with social autism severity scores [21]. A diffusion tensor imaging study of autism revealed decreases of fractional anisotropy (FA), an index of neuronal integrity, in regions associated with affective processing including the amygdala that has direct implications for autistic deficits in socio-affective behavior [22].

In this study, we characterized affective networks through which the amygdala communicates in autism by conducting an fMRI functional amygdala connectivity analysis in which we isolated emotional and neutral networks used to process facial affect as well as resting networks. The integrity of functional amygdala circuitry in autism affective networks and their associations with behavior during task performance and with clinical behavior scores of autism severity indicate that an alternative connectivity framework to underconnectivity is necessary to explain the functional autism connectome [23].

Methods

Task design and methodology were previously reported [16]. Male control subjects ($n=12$) and twelve males with autism or Asperger's disorder participated in an event-related fMRI study using a facial emotion recognition task. Ages were not significantly different between groups [Control: 17.0 ± 2.86 ; Autism: 16.75 ± 4.52 ; $t(22) = -0.162$, $P = 0.87$]. Diagnostic criteria for autism and Asperger's Disorder were based on DSM-IV and confirmation with the Autism Diagnostic Interview Revised (ADI-R). In total 40

faces, 16 neutral and 24 emotional (8 happy, 8 fear, 8 anger), from the Karolinska Directed Emotional Faces Set [24] were presented for 3 seconds each with a jittered inter-trial interval ranging from 5 to 7 seconds. Subjects were instructed to decide if the face displayed an emotion (happy, fear, anger) or was neutral and to indicate their choice with a button box press. Both accuracy (percent correct) and reaction time were measured. Images were acquired with a quadrature head coil and consisted of 409 gradient recalled echo-planar images using BOLD contrast (TE = 30 ms; TR = 2 sec; FOV = 240x240 mm, 64x64 matrix, voxel size= 3.75 x 3.75 x 5mm) acquired sagittally (30 slices, 4mm thick with 1mm gap) with a 3.0 Tesla GE SIGNA Scanner (Waukesha, WI). Axial T1-weighted 3D SPGR images were also acquired (TE = 8 ms, TR = 35 ms, FOV = 240x240 mm, 256x192 matrix, 124 axial slices, slice thickness = 1.1-1.2 mm, NEX = 1, flip angle = 30 degrees). Analyses were carried out using AFNI [25]. Echoplanar images were Fermi filtered in the spatial frequency domain, reconstructed, motion corrected, and volume registered to the echoplanar volume collected closest in time to the T1 anatomical data prior to blurring with a Gaussian kernel (FWHM=6 mm).

Voxelwise Whole Brain Functional Connectivity Analysis

Analyses were carried out using AFNI [25], R [26] , and software developed in house. Neuroanatomical asymmetries present in the human brain[27-29] can be accounted for by conducting analyses in a symmetric Talairach template space based on voxel based morphometry analyses [27]. Manually Talairached anatomical images were averaged across all subjects in both groups. A symmetric template was generated by averaging the mean anatomical volume with the same image mirrored about the midline. Each subject's anatomical image was then automatically registered to the symmetric template using a 12

parameter affine transform. Each subject's echo-planar image underwent the same transformation as the corresponding anatomical image. We used the partial correlation between a temporal brain volume and a given amygdala's fMRI response as the connectivity statistic by partialling out motion and three physiological noise measures including global brain signal, white matter signal, and lateral ventricle signal [30]. White matter and lateral ventricle delineation was based on segmentation of T1 images in FSL [31].

Voxelwise whole brain functional connectivity maps seeded by each amygdala served as a metric for the spatial extent and degree of functional connectivity, or functional integrity, between a given region and the amygdala. Parcellation criteria and software developed in this laboratory were used to define amygdala regions of interest (ROI) on high-resolution anatomical images [32]. Amygdalae ROIs were resampled to the resolution of the echoplanar images and used to identify regions functionally connected to the amygdala. To examine emotional and neutral face networks, regressors of six TR lagged neutral or emotional impulse functions, respectively, were included as regressors of no interest [33]. The statistical resting state network was determined by simultaneously including both the emotional and neutral time lagged impulse functions as regressors of no interest in the partial correlation analysis. Activations were quantified as the area under the hemodynamic response function (HRF) curve modeled using the emotional and neutral impulse functions.

Six whole brain functional connectivity maps, three affective network maps per amygdala, were generated for each subject. Affective network maps examined functional amygdala connectivity during emotional face processing, neutral face processing, and at rest. After a Fisher transformation, whole brain z-maps were entered into a four-way, mixed effect Group x Affect x Hemisphere x Subject(Group) ANOVA in which Group and Hemisphere were fixed and Subjects were nested and randomized within Group. Clusters of significance were determined after multiple comparison correction using Monte Carlo simulations. Significant clusters were only reported for those regions with a statistical signal to noise ratio (linear baseline estimate from the GLM fit divided by the standard deviation of the residual) greater than or equal to fifty to eliminate voxels with physiological noise [34]. Masks of significant clusters were used to extract mean z-scores of connectivity and activity. Barplots show z-scores or area under the curve (AUC) measures with the standard error of the mean (SEM) calculated using the mean square error (MSE) from the denominator of the F-test [35]. Subsequent protected two-tailed t-tests were used to determine directionality of functional connectivity effects. Fisher protected t-tests were calculated using the mean square error of the F-test denominator and tested on the degrees of freedom in the denominator term of the F-test [36]. Activation maps based on AUC measures were entered into a three-way, mixed effect Group x Affect x Subject(Group) ANOVA. Significant functional connectivity clusters were used as ROIs to quantify ANOVA effects. To examine associations between connectivity and behavior, connectivity measures were corrected across subjects for activity within each group and facial affect condition according to:

$$\text{Connectivity} = b_0 + b_1 * \text{Activity} + \text{epsilon}$$

where Connectivity is a vector of subjects' interhemispheric connectivity z-scores and Activity is a vector of subjects' area under the HRF curve percent change estimates. The residual connectivity, b_0 plus epsilon, was used in subsequent behavior and functional amygdala connectivity associations. Thus, the residual connectivity measure for each group is unconfounded by variations in activation across Affect, Hemisphere, and Subjects. Group differences in connectivity correlations with performance were assessed using the Fisher z-difference. Statistical tests were conducted using R software [26].

Results

Several regions with significant group differences in functional amygdala connectivity were identified with a four-way Group x Affect x Amygdala Hemisphere x Subject(Group) (Figures 1-4). F-statistics for all clusters for each ANOVA effect were quantified (Supplementary Table 1). The Group Main effect clusters of significance (Figure 1) included the cerebellum declive [Cluster 1], left superior frontal gyrus [Cluster 2], anterior cingulate [Cluster 3], right middle temporal gyrus [Cluster 4], and (e) right postcentral gyrus [Cluster 5]. The Group x Affect interaction clusters of significance (Figure 2) included the left angular gyrus [Cluster 6], declive in cerebellum and declive of vermis [Cluster 7], left superior temporal gyrus [Cluster 8], and left middle frontal gyrus [Cluster 9]. The Group x Hemisphere clusters of significance (Figure 3) included the left insula and claustrum [Cluster 10], left middle frontal gyrus [Cluster 11], right insula [Cluster 12], left lingual gyrus and fusiform [Cluster 13], right middle temporal gyrus [Cluster 14], right fusiform gyrus [Cluster 15], right posterior cingulate [Cluster 16], and right precentral gyrus [Cluster 17]. The Group x Affect x Hemisphere clusters of

significance (Figure 4) were in the right precuneus [Cluster 18], left inferior parietal lobule [Cluster 19], left declive of cerebellum [Cluster 20], right declive of cerebellum [Cluster 21], left inferior frontal gyrus [Cluster 22], and left posterior cingulate [Cluster 23]. The volume and location of these clusters was further described in Table 1.

First we explored the pattern among clusters that had significant differences in functional amygdala connectivity (Figure 5). Unsupervised agglomerative hierarchical clustering was able to agglomerate 83% of control subjects and 75% of autism subjects.

Furthermore three primary groupings of cluster were identified. When viewing the dendrogram and z-map of functional amygdala connectivity patterns, the cluster tree brain region on the right has the highest concentration of positive connectivity z-scores and agglomerated clusters with isolateral functional connectivity to the amygdala. The middle cluster tree region agglomerated brain regions with contralateral functional connectivity to the amygdala and regions paired in their functional connectivity to the right and left amygdala. The left dendrogram cluster tree agglomerated regions with paired right and left functional amygdala connectivity (Supplementary Table 1).

Next we quantified the mean functional connectivity (Figure 6) and activity (Supplementary Figure 1 and 2) for all clusters in autism and controls. Qualitatively, autism and control subjects showed overconnectivity and underconnectivity differences. By comparing ANOVA means we identified significant group differences in connectivity (Figures 7 and 8). All Group Main effect clusters were significantly different between groups (Figure 7a). Autism was more negatively connected than controls in cerebellum

declive, left superior frontal gyrus, and right middle temporal gyrus, but autism was more positively connected than controls in the anterior cingulate and postcentral gyrus. The Group x Affect clusters were significantly different between groups for all clusters (Figure 7b). Emotional, neutral, and resting networks were more negatively connected in autism than controls in the left angular gyrus, the cerebellum declive and vermis, and the left middle frontal gyrus, but autism was more positively connected than controls for left superior temporal gyrus. These Group x Affect clusters were significantly different between affective networks only for controls and only for emotion-rest differences in the cerebellum declive and vermis, emotion-rest differences in the left superior temporal gyrus, and neutral-rest differences in the cerebellum declive and vermis (Figure 7c). The Group x Hemisphere clusters were more negatively connected in autism than controls for associations between the right amygdala and the left middle frontal gyrus, right insula, right middle temporal gyrus, right fusiform gyrus, right posterior cingulate, and the right precentral gyrus and between the left amygdala and the left insula and claustrum (Cluster 10), and the left lingual gyrus and fusiform (Cluster 13) (Figure 7d). The Group x Hemisphere clusters were significant for right - left differences (Figure 7e) in all regions for controls but only significant for autism in the left middle frontal gyrus in which the right amygdala was more negatively connected than the left amygdala. For controls the left amygdala was more positively connected than the right amygdala to the left insula and claustrum (Cluster 10) and left lingual gyrus and fusiform (Cluster 13) and the right amygdala was more positively connected than the left amygdala to the left middle frontal gyrus, right insula, right middle temporal gyrus, right fusiform gyrus, right posterior cingulate, and right precentral gyrus.

The Group x Affect x Hemisphere clusters were significant for all group difference comparisons in the left inferior parietal lobule, right cerebellum declive, and the left posterior cingulate (Figure 8a). Significant group difference comparisons were present for right amygdala connectivity with the right precuneus for emotional, neutral, and resting networks and for left amygdala connectivity with the right precuneus for emotional face processing networks. The left cerebellum declive was more positively connected in controls for left amygdala connectivity in emotional, neutral, and resting networks and for right amygdala connectivity in neutral and resting, but not emotional, networks. For the left inferior frontal gyrus, right functional amygdala connectivity was more positively connected in controls for emotional, neutral, and resting networks and the left functional amygdala connectivity was more positively connected in controls during emotional, but not neutral or resting networks (Figure 8a). Affective network differences were only significant for the control group (Figure 8b). Controls showed significant differences in affective connectivity where left amygdala was more negatively connected to right precuneus in emotional compared to neutral networks and where the left amygdala was more negatively connected to the right cerebellum declive in emotional compared to resting networks. Differences in amygdala hemisphere were significant for all amygdala hemisphere comparisons in right precuneus [Autism and Control: Right Amygdala(RA)>Left Amygdala(LA)], right cerebellum declive (Autism: RA>LA; Control: LA>RA), and left posterior cingulate (Autism and Control: LA>RA) (Figure 8c). For the left inferior parietal lobule, the right amygdala was more positively connected when compared to the left amygdala for emotional, neutral, and resting

networks in autism, but only for neutral networks in controls. The left amygdala was more connected than the right amygdala to the left cerebellum decline for emotional, neutral, and resting networks in controls, but right amygdala was more positively connected than left amygdala in autism emotional face processing networks. Compared to the left amygdala, the right amygdala was more positively connected in control neutral and resting networks (Figure 8c). We examined activity differences in the connectivity clusters and the amygdala using a Group x Affect x Subject(Group) ANOVA in these regions of interest. No differences were present for Group Main effects in the connectivity clusters (Figure 1) but were significant for a Group x Affect interaction in the right insula (Figure 2, Supplementary Table 2). Amygdala activity was not significantly different between groups based on the Group Main effect and Group x Affect interaction F-statistics (Supplementary Figure 3).

Residual connectivity associations with behavior were significantly ($p < 0.05$, uncorrected) different between groups for response times, accuracy, and eye gaze behavior (Table 2). Both ipsilateral and contralateral amygdala associations with task performance behavior were present. Emotional face response time behavior had significant group differences in its association with emotional network circuitry comprised of ipsilateral amygdala connectivity with the left lingual gyrus and fusiform, the right precuneus, and the left inferior parietal lobule. Neutral face response time behavior had significant group differences in its association with neutral network circuitry comprised of ipsilateral amygdala connectivity with the left insula and claustrum. The number of faces correctly identified had significant group differences in its association with the ipsilateral

amygdala connectivity with the right fusiform gyrus during emotional face processing. Total eye fixation time had significant group differences in its association with the ipsilateral amygdala connectivity to the left insula and claustrum during neutral face processing. The ratio of eye to face fixation time had significant group differences in its association with ipsilateral functional amygdala connectivity to both the left superior frontal gyrus and left posterior cingulate during emotional and neutral face processing.

For regions with contralateral associations between the amygdala and performance behavior (Table 2), emotional face response time behavior had group differences in its association with emotional network circuitry comprised of contralateral amygdala connectivity cerebellum declive, the left angular gyrus, left middle frontal gyrus, and the right cerebellum declive. Neutral face response time behavior had significant group differences in its association with neutral network circuitry comprised of contralateral amygdala connectivity with the left insula and claustrum and left inferior parietal lobule. The number of faces correctly identified had significant group differences in its association with the contralateral amygdala connectivity to the right postcentral gyrus during neutral face processing. Total eye fixation time had significant group differences in its association with the contralateral amygdala connectivity to the right middle temporal gyrus during emotional face processing. Both total face fixation time and the mean face fixation time per trial had significant group differences in its association with contralateral functional amygdala connectivity to the right middle temporal gyrus during emotional face processing, and the right precuneus during emotional and neutral face processing.

Having significant ($p < 0.05$, uncorrected) group differences, residual amygdala connectivity associations with task performance behavior were scatter plotted with line of best fit in 95% confidence bands for behaviors which included total eye fixation time and total face fixation time (Supplementary Figure 4), mean face fixation time/trial and the ratio of eye to face fixation time (Supplementary Figure 5), the number of facial emotions correctly identified and neutral face response time (Supplementary Figure 6), and emotional face response time (Supplementary Figure 7).

Residual connectivity associations with clinical behavior autism ratings were significant ($p < 0.05$, uncorrected) in postcentral gyrus, insula, middle temporal gyrus, fusiform, posterior cingulate, precuneus, and cerebellum declive (Table 3). Ipsilateral and contralateral associations were present for amygdala connectivity associations with clinical behavior scores of autism severity. For ipsilateral associations between the amygdala and autism severity measures, nonverbal communication scores were significant in their association with ipsilateral functional amygdala connectivity to the right postcentral gyrus during rest, the right fusiform gyrus during rest, and the left cerebellum declive in emotional neutral and resting networks. Restrictive and repetitive behavior scores were significant in their association with ipsilateral functional amygdala connectivity to the right posterior cingulate for emotional, neutral, and resting networks.

For contralateral associations between the amygdala and autism severity scores (Table 3), nonverbal communication scores were significant ($p < 0.05$, uncorrected) in their

association with contralateral functional amygdala connectivity to the right insula for emotional, neutral, and resting networks. Restrictive and repetitive behavior scores were significant in their association with contralateral functional amygdala connectivity to the right middle temporal gyrus during emotional face processing. Verbal communication scores were significant in their association with contralateral functional amygdala connectivity to both the right precuneus and left posterior cingulate for emotional, neutral, and resting networks.

Significant ($p < 0.05$, uncorrected) residual connectivity associations with clinical behavior autism ratings associations were scatter plotted with line of best fit in 95% confidence bands for autism ratings which included nonverbal communication (Supplementary Figure 8), verbal communication (Supplementary Figure 9), and restricted and repetitive behavior (Supplementary Figure 10). Autism amygdala connectivity associations ($p < 0.1$, uncorrected) with ADI-R social scores were also reported (Supplementary Figure 11).

Discussion

In this paper, we examined the functional connectivity architecture of autism affective face processing networks and identified significant group differences in the magnitude of functional connectivity with the left and right amygdala and the association of functional connections with task related behavior and autism scores. Through partial correlation analysis, we identified several regions differentially connected with the amygdala (Table 1, Figures 1-4). Unsupervised agglomerative hierarchical clustering analysis distinguished 75% of autism subjects and 83% of control subjects on the basis of patterns in the underlying structure of functional connectivity. The dendrograms indicated more

positive connectivity was associated with intrahemispheric amygdala associations and less positive connectivity was associated with regions comparably paired with the right and left amygdala (Figure 5).

The amygdala overconnectivity and underconnectivity present in autism affective networks suggests that metastability [37,38], the tendency of brain regions to be both segregated and integrated, is functionally disrupted in autism. For the functional amygdala connectivity of GM clusters, the comparison of ANOVA means (Figure 7a) indicated that autism was more negatively connected than controls in the cerebellum declive, left superior frontal gyrus, and right middle temporal gyrus, but autism was more positively connected than controls in the anterior cingulate and postcentral gyrus. For the functional amygdala connectivity with GA clusters, the comparison of ANOVA means (Figure 7b,c) indicated that, compared to autism, the cerebellum declive and vermis were significantly more positively connected in control emotional, neutral, and resting networks and that controls, but not autism, were more positively connected in emotional networks compared to rest and neutral networks compared to rest. In addition, the left superior temporal gyrus was significantly more negatively connected in controls' emotional, neutral, and resting networks compared to autism and that controls, but not autism, were more positively connected in emotional networks compared to rest. For the functional amygdala connectivity with GH clusters, the comparison of ANOVA means (Figure 7 d,e) indicated that the autism group was significantly more negatively connected with the amygdala than controls. For the functional amygdala connectivity with GAH clusters, the comparison of ANOVA means indicated that only the control

group showed significant affective network differences and that the right precuneus and the right cerebellum declive showed significant differences in amygdala connectivity by group, affect, and hemisphere (Figure 8 a,b,c). Autism was more positively connected than controls for left amygdala connectivity with the right precuneus for emotional networks, but functional connectivity in this circuit was comparable in autism and controls for neutral networks (Figure 8a). In controls, the left amygdala was more negatively connected to right precuneus in emotional compared to neutral networks (Figure 8b). The right amygdala was more positively connected than the left amygdala to the right precuneus in both autism and controls for emotional and neutral networks (Figure 8c). Autism was more negatively connected than controls for left amygdala connectivity with the right cerebellum declive for both emotional and resting networks (Figure 8a). In controls, the left amygdala was more positively connected to the right cerebellum declive for emotional compared to resting networks (Figure 8b). The left amygdala was more negatively connected than the right amygdala to the right cerebellum declive for autism emotional and resting networks, but in controls, the left amygdala was more positively connected than the right amygdala for emotional and resting networks (Figure 8c). The pattern of differences for affective network comparisons (Figure 8b) in autism further suggests that metainstability is a phenotypic feature of amygdala affective networks. In union, affective amygdala networks might be described as having functional metainstability in autism.

In addition to metainstability, several other aspects of the autism functional connectome are altered. The changes between emotional, neutral, and resting functional amygdalae

(left and right) connectivity magnitudes for GA clusters in cerebellum and left superior temporal gyrus (Figure 7b,c) suggest that affective connectivity dynamics is reduced in autism and that the amygdalae are behavioral catalysts [39], nodes involved in transitioning between brain states, that have impaired functional catalysis of affective network behaviors in autism. Also, autism affective networks have reduced multivariability [37], the ability to form multiple brain states. For example, the comparison of ANOVA means for the functional amygdala connectivity with GH clusters (Figure 7 d,e) indicated that controls demonstrated significant differential amygdala connectivity that was either altered or lacking in autism. The GH clusters were significantly different between groups (Figure 7d) for connectivity with the right amygdala (left middle frontal gyrus, right insula, right middle temporal gyrus, right fusiform gyrus, right posterior cingulate, right precentral gyrus) and left amygdala (left insula and claustrum, left lingual gyrus and fusiform). The GH clusters were significant for differential right - left amygdala connectivity differences in controls for all clusters (Figure 7e). However, the left middle frontal gyrus was the only GH cluster region in autism with significant differential amygdala connectivity. The left middle frontal gyrus was more negatively connected to the right amygdala in autism than controls and was significantly more positively connected to the left than the right amygdala in autism; in contrast, this region was significantly more negatively connected to the left than the right amygdala in controls. Unlike autism, multiple differentially connected affective circuits dependent on amygdala hemisphere in controls, may reflect distinct temporal dynamics with or in left and right amygdala when processing emotions [40].

To address the behavioral consequence of impaired amygdala functional catalysis, metainstability, and reduced multivariability in autism affective networks, we examined whether the integrity of ipsilateral and contralateral functional amygdala connections (residual connectivities) in affective networks had significant group differences in its association with performance behavior (Table 2) and had significant associations with clinical scores of severity in autism (Table 3). Group differences in ipsilateral associations between the amygdala and performance behavior were present for the left superior frontal gyrus, left insula and claustrum, left lingual gyrus and fusiform, right fusiform gyrus, right precuneus, left inferior parietal lobule, and left posterior cingulate (Table 2). Ipsilateral associations between the amygdala and autism severity scores were significant in the right postcentral gyrus, right fusiform gyrus, right posterior cingulate, and the left cerebellum declive (Table 3). Group differences in contralateral associations between the amygdala and performance behavior were present for the cerebellum declive, right postcentral gyrus, left angular gyrus, left middle frontal gyrus, left insula and claustrum, right middle temporal gyrus, right precuneus, left inferior parietal lobule and the right cerebellum declive (Table 2). Contralateral associations between the amygdala and autism severity scores were significant in the right insula, right middle temporal gyrus, right precuneus, and left posterior cingulate (Table 3). Several of these regions are part of the social brain network and mirror neuron system involved in affective face processing [41]. In union, these results support a role for the amygdala in affective networks which functionally connect visual and social processing regions [42].

In summary, metastability, multivariability, and behavioral catalysts provide a useful and generalizable framework for interpreting autism functional connectivity architecture. Underconnectivity was previously hypothesized to be the leading connectivity framework in understanding autism [9]. However functional connectivity is task specific and the features of affective face processing networks were not previously considered in the nosology of autism connectivity. In the present study, we identified features of overconnectivity and underconnectivity with the amygdala as evidence of affective network metainstability in cerebrum and cerebellum, a region involved in affective face processing [43-46] and previously hypothesized to contribute to behavioral, cognitive, and emotional deficits in autism [47]. A potential biological mechanism is that an increased ratio of excitation to inhibition through glutamatergic or GABAergic signaling within autism affective networks could increase or decrease functional connectivity by reducing the signal to noise ratio of information transfer through increased non-affective noise or decreased affective signal, respectively [48,49]. In autism, the lack of magnitude changes in amygdala connectivity integrity with the cerebellum declive and vermis and the left superior temporal gyrus (Figure 7c) between emotional, neutral, and resting states suggests that the amygdala was a behavioral catalyst in affective processing networks with disrupted catalysis in autism. In addition, the metainstability of autism affective networks and significant group differences in connectivity associations with behavior (Table 2) indicate that autism affective networks have reduced multivariability. Furthermore, the extent of significant differences in autism functional amygdala circuitry associations with behavior and functional amygdala connectivity associations with autism severity scores indicate that behavioral performance and clinical severity of autism

behavior are associated with the integrity of ipsilateral and contralateral functional amygdala connections in affective networks that have reduced metastability and reduced multivariability in autism. Further studies using combined fMRI and DTI will be necessary to examine the autism functional connectome and the contribution of the amygdala as a behavioral catalyst to reduced metastability and multivariability in autism networks and to phenotypic autism behavior.

Conclusion

The amygdala is a central node in autism affective face processing networks. The overconnectivity and underconnectivity with the amygdala we identified in the functional emotional, neutral, and resting network circuitry indicates that the amygdala is a behavioral catalyst dysfunctionally connected to autism visual and affective subcortical processing regions with behavioral consequences. The integrity of amygdala circuitry in affective networks is disrupted in autism and suggests that the reduced metastability and multivariability of affective networks are phenotypic features of the functional autism connectome.

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Table:

Table 1: Summary of Cluster Index, Region, Broadman Area (BA), Volume, and Center of Volume Coordinates

ANOVA Effects	Cluster	Region	BA	Volume (uL)	RL x-axis	AP y-axis	IS z-axis
Group Main	1	Cerebellum, Declive	*	2376	-4.1	72.7	-22.4
	2	Left Superior Frontal Gyrus	8	1998	15.1	-36.3	44.5
	3	Anterior Cingulate	24	1944	-0.8	-27.8	22.2
	4	Right Middle Temporal Gyrus	21	1350	-59.7	30.5	-7.9
	5	Right Postcentral Gyrus	3	1269	-13.4	36.9	69.6
Group x Affect	6	Left Angular Gyrus	39	5049	41.7	59.3	35.7
	7	Cerebellum, Declive and Declive of Vermis	*	2322	0.8	70.7	-11.9
	8	Left Superior Temporal Gyrus	22	2052	48.9	-12.1	-6.2
	9	Left Middle Frontal Gyrus	10	1350	29.1	-49.8	17.2
Group x Hemisphere	10	Left Insula and Claustrum	*	11259	37.1	4.7	3.7
	11	Left Middle Frontal Gyrus	8	6723	31.9	-20.9	44.5
	12	Right Insula	13	2700	-42.5	16.7	11.1
	13	Left Lingual Gyrus and Fusiform	19	1701	14.0	49.7	-1.5
	14	Right Middle Temporal Gyrus	39	1674	-39.2	57.4	25.1
	15	Right Fusiform Gyrus	37	1134	-29.7	43.9	-11.1
	16	Right Posterior Cingulate	30	1107	-5.3	50.8	17.4
	17	Right Precentral Gyrus	9	783	-41.6	-14.7	38.5

Group x Affect x Hemisphere	18	Right Precuneus	31	2700	-12.4	57.4	23.3
	19	Left Inferior Parietal Lobule	40	2700	56.8	34.5	35.2
	20	Left Cerebellum, Declive	*	2106	19.4	80.1	-19.8
	21	Right Cerebellum, Declive	*	1998	-24.9	79.9	-20.6
	22	Left Inferior Frontal Gyrus	45	1215	52.8	-19.2	14.4
	23	Left Posterior Cingulate	30	1161	12.7	54.7	11.8

Table 2: Brain Regions with Significant Group Differences (z-difference $p \leq 0.05$, uncorrected) in Residual Connectivity Associations with Performance Behavior.

Brain regions with connectivity to the isolateral amygdala are italicized. Association plots are available in the supplement.

Brain Region	Cluster	Affect Group Main	Amygdala	Behavior
Cerebellum, Declive	1	E	R	Emotional Face Response Time
Cerebellum, Declive	1	E	L	Emotional Face Response Time
<i>Left Superior Frontal Gyrus</i>	2	<i>E</i>	<i>L</i>	<i>Ratio of Eye to Face Fixation Time</i>
<i>Left Superior Frontal Gyrus</i>	2	<i>N</i>	<i>L</i>	<i>Ratio of Eye to Face Fixation Time</i>
Right Postcentral Gyrus	5	N	L	Number Correct
Group x Affect				
Left Angular Gyrus	6	E	R	Emotional Face Response Time
Left Middle Frontal Gyrus	9	E	R	Emotional Face Response Time
Group x Hemisphere				
Left Insula and Claustrium	10	N	R	Neutral Face Response Time
<i>Left Insula and Claustrium</i>	10	<i>N</i>	<i>L</i>	<i>Total Eye Fixation Time</i>
<i>Left Lingual Gyrus and Fusiform</i>	13	<i>E</i>	<i>L</i>	<i>Neutral Face Response Time</i>
				<i>Emotional Face Response Time</i>

Right Middle Temporal Gyrus	14	E	L	Total Eye Fixation Time Total Face-Fixation Time Mean Face-Fixation Time per Trial
<i>Right Fusiform Gyrus</i>	15	<i>E</i>	<i>R</i>	<i>Number Correct</i>
Group x Affect x Hemisphere				
<i>Right Precuneus</i>	18	<i>E</i>	<i>R</i>	<i>Emotional Face Response Time</i>
Right Precuneus	18	<i>E</i>	L	Total Face-Fixation Time Mean Face-Fixation Time per Trial
Right Precuneus	18	<i>N</i>	L	Total Face-Fixation Time Mean Face-Fixation Time per Trial
<i>Left Inferior Parietal Lobule</i>	19	<i>E</i>	<i>L</i>	<i>Emotional Face Response Time</i>
Left Inferior Parietal Lobule	19	<i>N</i>	R	Neutral Face Response Time
Right Cerebellum, Declive	21	<i>E</i>	L	Emotional Face Response Time
<i>Left Posterior Cingulate</i>	23	<i>E</i>	<i>L</i>	<i>Ratio of Eye to Face Fixation Time</i>
<i>Left Posterior Cingulate</i>	23	<i>N</i>	<i>L</i>	<i>Ratio of Eye to Face Fixation Time</i>

Table 3: Brain Regions with Significant ($p \leq 0.05$, uncorrected) Autism Residual Connectivity Associations with Clinical Behavior Scores of Autism Severity. Brain regions with connectivity to the isolateral amygdala are italicized. Association plots are available in the supplement.

Brain Region	Cluster	Affect Group Main	Amygdala	Autism Behavior
<i>Right Postcentral Gyrus</i>	5	<i>R</i>	<i>R</i>	<i>Nonverbal Communication</i>
Group x Hemisphere				
Right Insula	12	E	L	Nonverbal Communication
Right Insula	12	N	L	Nonverbal Communication
Right Insula	12	R	L	Nonverbal Communication
Right Middle Temporal	14	E	L	Restricted and Repetitive

Gyrus				Behavior
<i>Right Fusiform Gyrus</i>	15	<i>R</i>	<i>R</i>	<i>Nonverbal Communication</i>
<i>Right Posterior Cingulate</i>	16	<i>E</i>	<i>R</i>	<i>Restricted and Repetitive Behavior</i>
<i>Right Posterior Cingulate</i>	16	<i>N</i>	<i>R</i>	<i>Restricted and Repetitive Behavior</i>
<i>Right Posterior Cingulate</i>	16	<i>R</i>	<i>R</i>	<i>Restricted and Repetitive Behavior</i>
Group x Affect x Hemisphere				
Right Precuneus	18	<i>E</i>	<i>L</i>	Verbal Communication
Right Precuneus	18	<i>N</i>	<i>L</i>	Verbal Communication
Right Precuneus	18	<i>R</i>	<i>L</i>	Verbal Communication
<i>Left Cerebellum, Declive</i>	20	<i>E</i>	<i>L</i>	<i>Nonverbal Communication</i>
<i>Left Cerebellum, Declive</i>	20	<i>N</i>	<i>L</i>	<i>Nonverbal Communication</i>
<i>Left Cerebellum, Declive</i>	20	<i>R</i>	<i>L</i>	<i>Nonverbal Communication</i>
Left Posterior Cingulate	23	<i>E</i>	<i>R</i>	Verbal Communication
Left Posterior Cingulate	23	<i>N</i>	<i>R</i>	Verbal Communication
Left Posterior Cingulate	23	<i>R</i>	<i>R</i>	Verbal Communication

Figures

Figure 1: Group Main Effect Clusters of Significance.

A 4-way ANOVA identified five clusters with a significant main effect of Group. Shown are regions of the (a) Declive in Cerebellum [Cluster 1] (b) Left Superior Frontal Gyrus [Cluster 2] (c) Anterior Cingulate [Cluster 3] (d) Right Middle Temporal Gyrus [Cluster 4] (e) Right Postcentral Gyrus [Cluster 5].

Figure 2: Group x Affect Interaction Clusters of Significance.

A 4-way ANOVA identified four clusters with a significant Group x Affect interaction in the (a) Left Angular Gyrus [Cluster 6] (b) Declive in Cerebellum and Declive of Vermis [Cluster 7] (c) Left Superior Temporal Gyrus [Cluster 8] (d) Left Middle Frontal Gyrus [Cluster 9].

Figure 3: Group x Hemisphere Clusters of Significance.

A 4-way ANOVA identified eight clusters with a significant Group x Hemisphere interaction in the (a) Left Insula and Claustrum [Cluster 10] (b) Left Middle Frontal Gyrus [Cluster 11] (c) Right Insula [Cluster 12] (d) Left Lingual Gyrus and Fusiform [Cluster 13] (e) Right Middle Temporal Gyrus [Cluster 14] (f) Right Fusiform Gyrus [Cluster 15] (g) Right Posterior Cingulate [Cluster 16] (h) Right Precentral Gyrus [Cluster 17].

Figure 4: Group x Affect x Hemisphere Clusters of Significance.

A 4-way ANOVA identified six clusters with a significant Group x Affect x Hemisphere interaction in the (a) Right Precuneus [Cluster 18] (b) Left Inferior Parietal Lobule [Cluster 19] (c) Left Declive of Cerebellum [Cluster 20] (d) Right Declive of Cerebellum [Cluster 21] (e) Left Inferior Frontal Gyrus [Cluster 22] (f) Left Posterior Cingulate [Cluster 23].

Figure 5: Heatmap and Unsupervised Clustering of Functional Amygdala Connectivity.

Dendrograms indicate agglomerations of functional amygdala connectivity clusters. The matrix of z-scores are displayed as a heatmap and sorted based on dendrogram agglomeration and mean column z-scores. Autism subjects are indicated in red and controls in black. Ten of twelve control subjects (83%) and nine of twelve autism subjects (75%) were correctly agglomerated based on the pattern of functional amygdala connectivity of clusters with significant ANOVA effects.

Figure 6: Mean Connectivity Values for Significant Clusters.

Mean cluster z-scores were plotted for the (a) Group Main effect and the (b) Group x Affect, (c) Group x Hemisphere, and (d) Group x Hemisphere x Affect interactions in the four-way Group x Affect x Hemisphere x Subject(Group) ANOVA. Labels indicate Group (A=autism, C=control), Affect (E=emotional face, N=neutral face, R=rest), Hemisphere (R=right, L=left), and Cluster number as indexed in Table 1.

Figure 7: Comparison of ANOVA Cluster Means.

Protected t-tests decompose the (a) Group Main Effect, (b,c) Group x Affect, and (d,e) Group x Hemisphere clusters according to differences in Group (a,b,d), Affect (c), and Hemisphere (e). Horizontal lines indicate the critical threshold of significance (Bonferroni corrected within clusters, $p=0.05$, two-tailed). Cluster labels refer to Group (A=autism, C=control), Affect (E= emotion, N=neutral, R = rest), Hemisphere (R=right amygdala connectivity, L=left amygdala connectivity) and the Cluster number based on Table 1. If two levels of one label factor are listed in succession before a period, this denotes the second level was subtracted from the first level.

Figure 8: Comparison of Group x Affect x Hemisphere Cluster Means.

Protected t-tests decompose the Group x Affect x Hemisphere clusters according to differences in Group (a), Affect (b), and Hemisphere (c). Horizontal lines indicate the critical threshold of significance (Bonferroni corrected within clusters, $p=0.05$, two-tailed). Cluster labels refer to Group (A=autism, C=control), Affect (E= emotion, N=neutral, R = rest), Hemisphere (R=right amygdala connectivity, L=left amygdala connectivity) and the Cluster number based on Table 1. If two levels of one label factor are listed in succession before a period, this denotes the second level was subtracted from the first level.

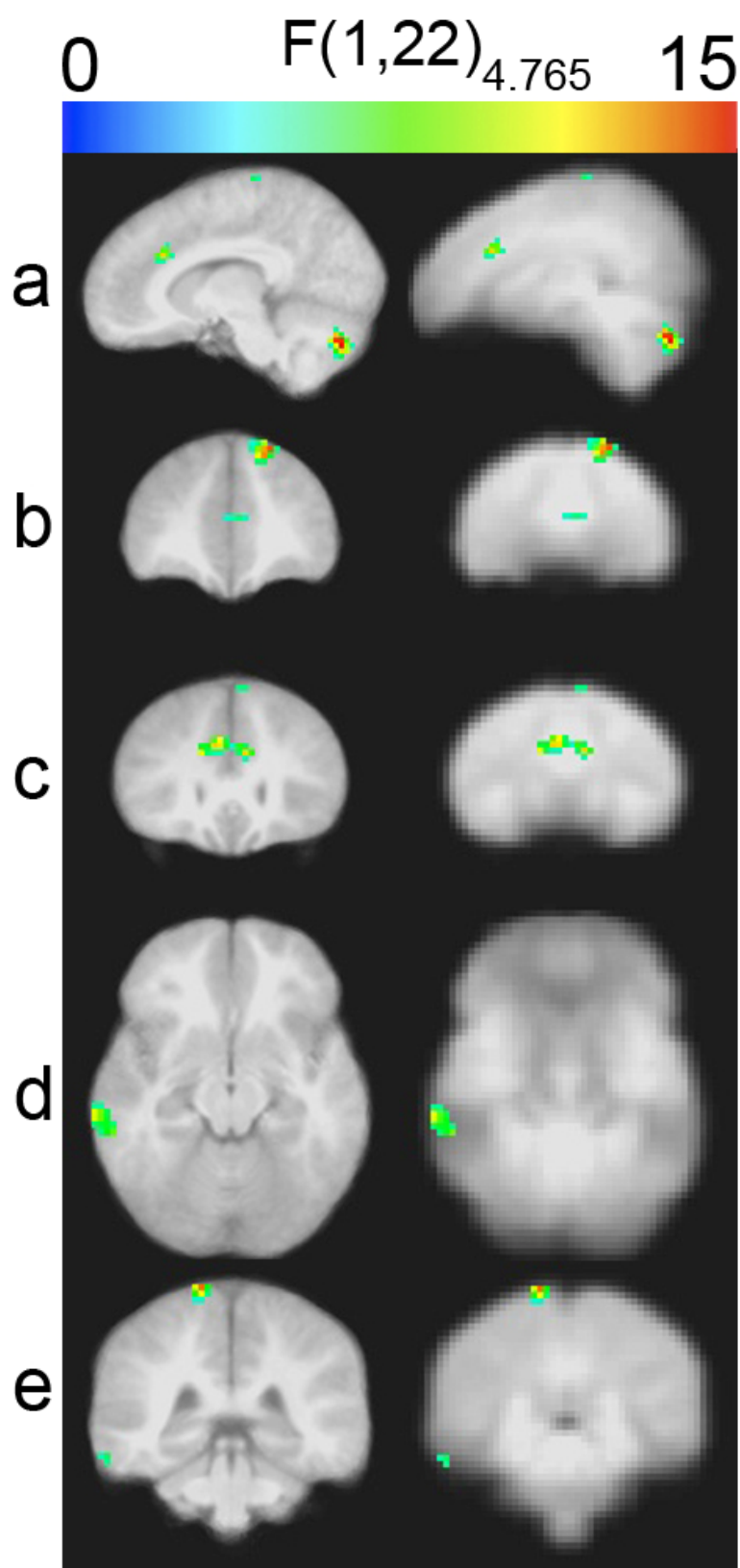


Figure 1

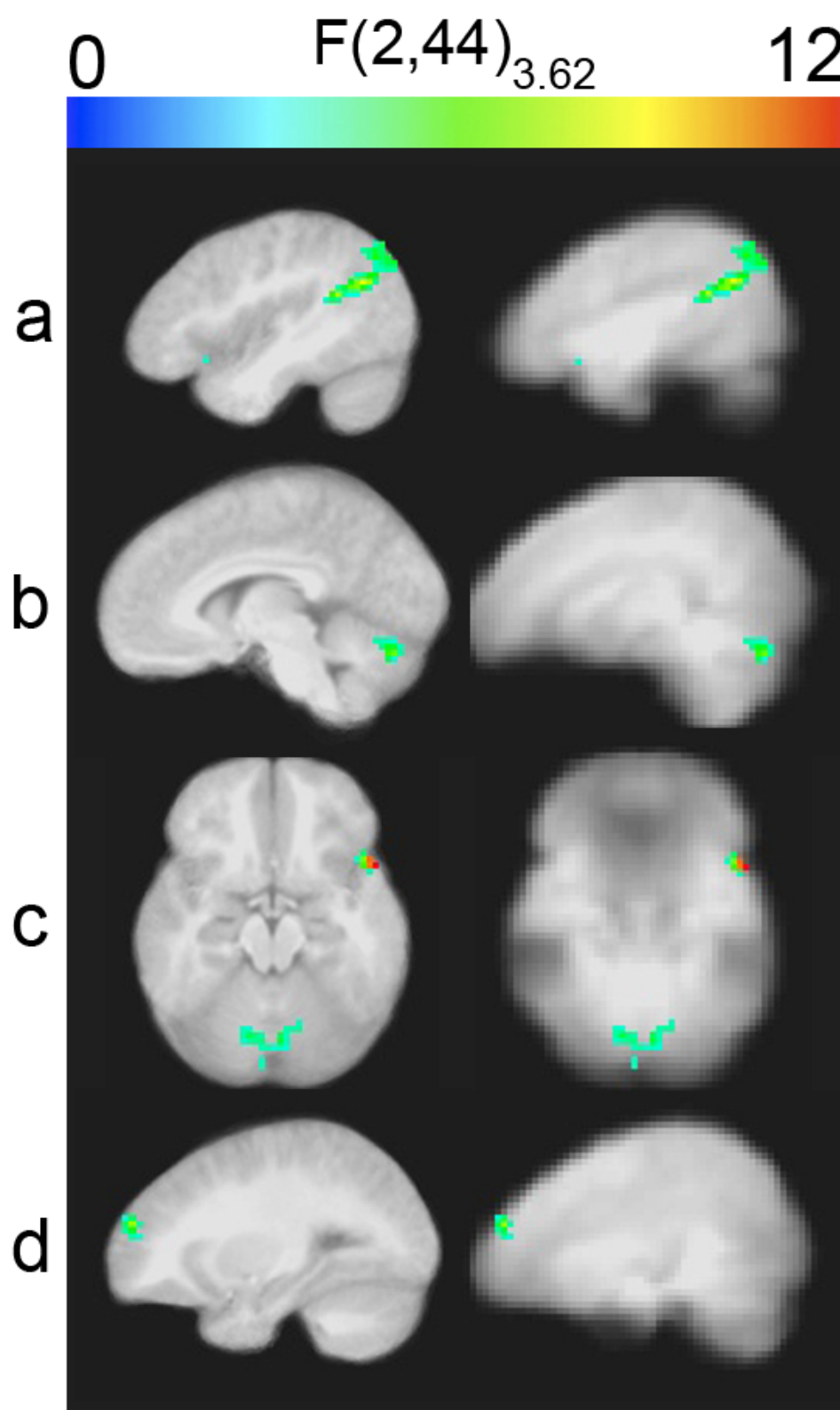


Figure 2

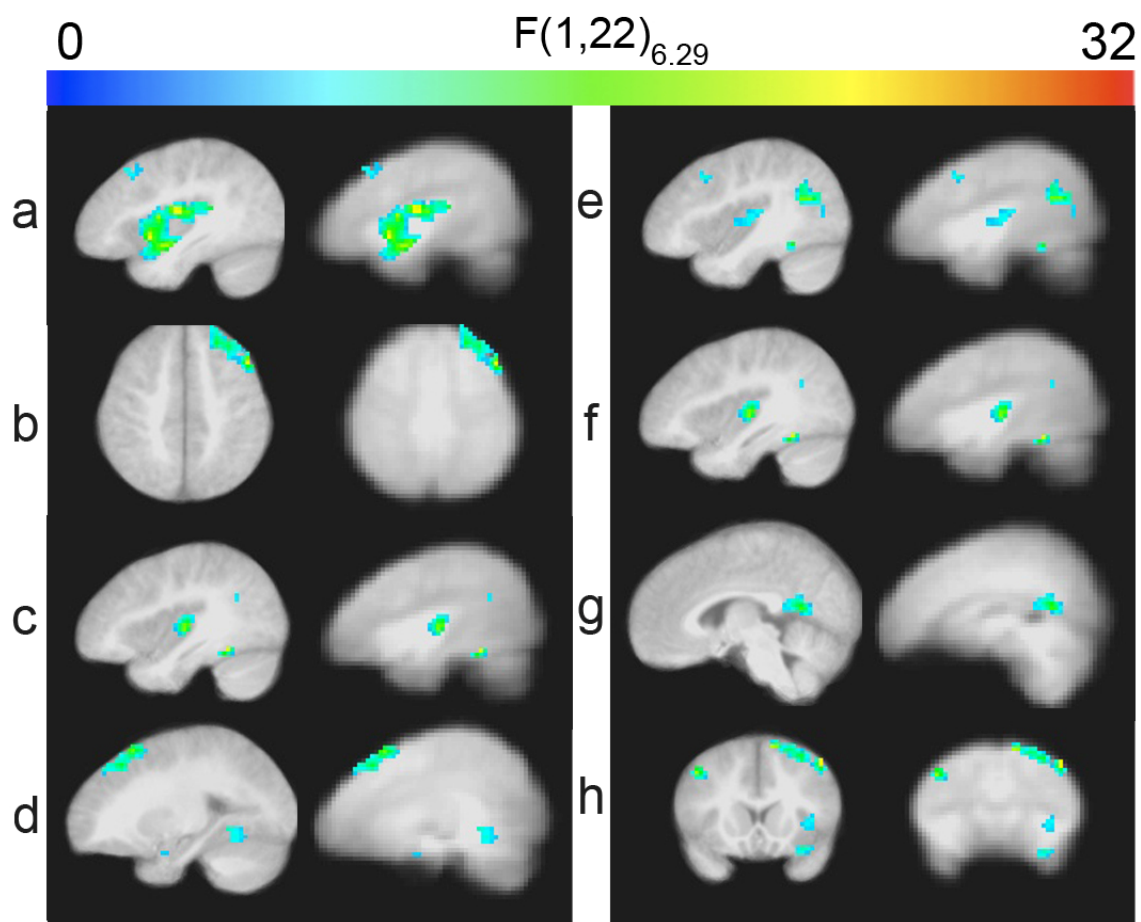


Figure 3

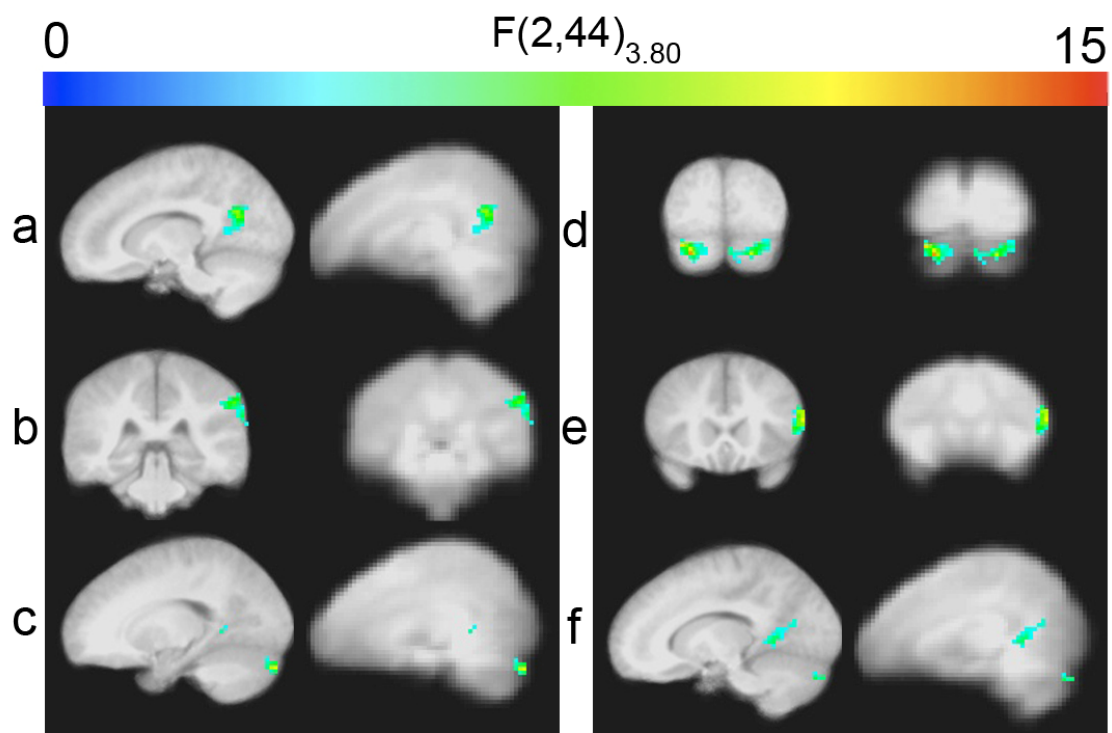


Figure 4

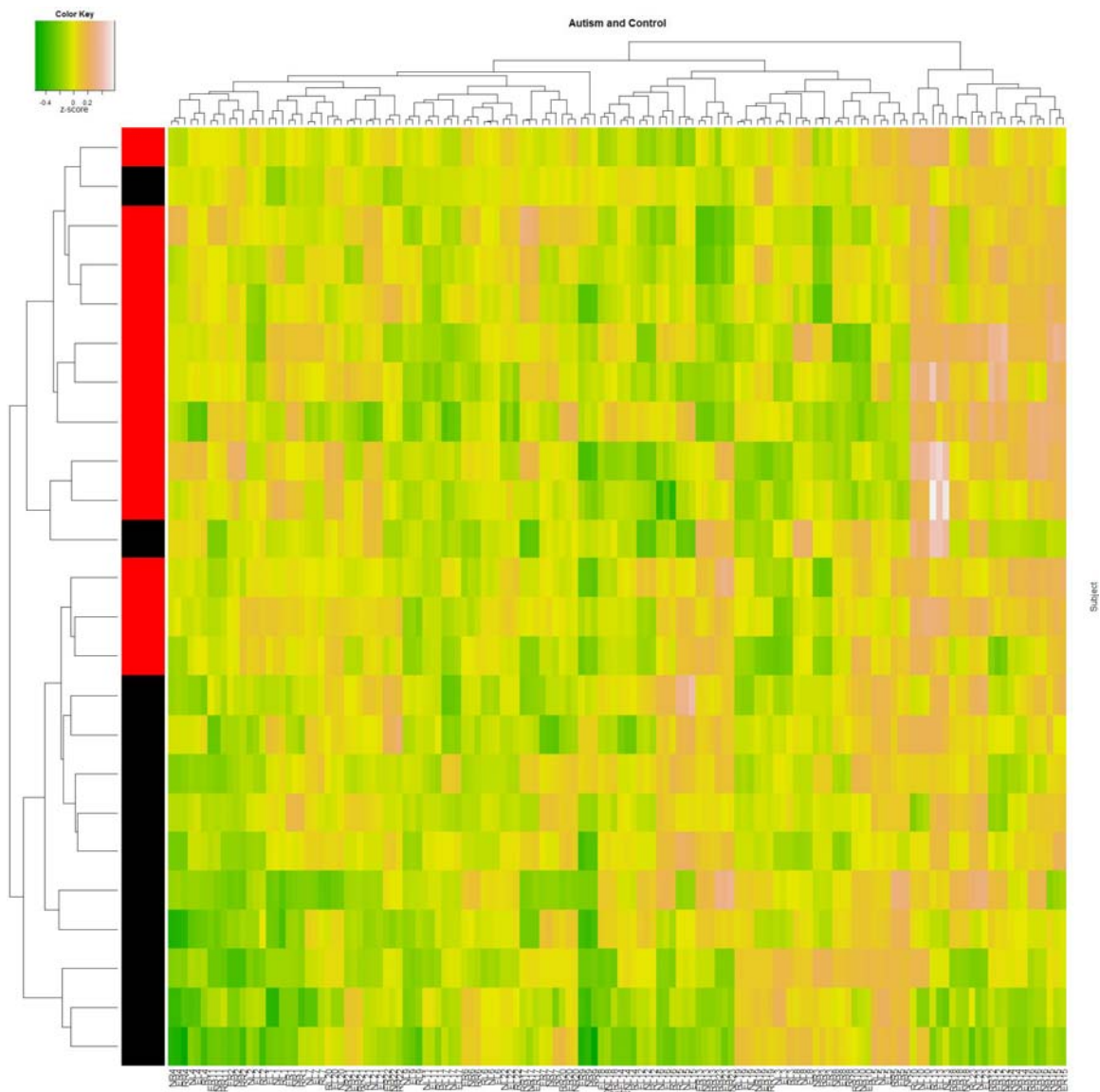


Figure 5

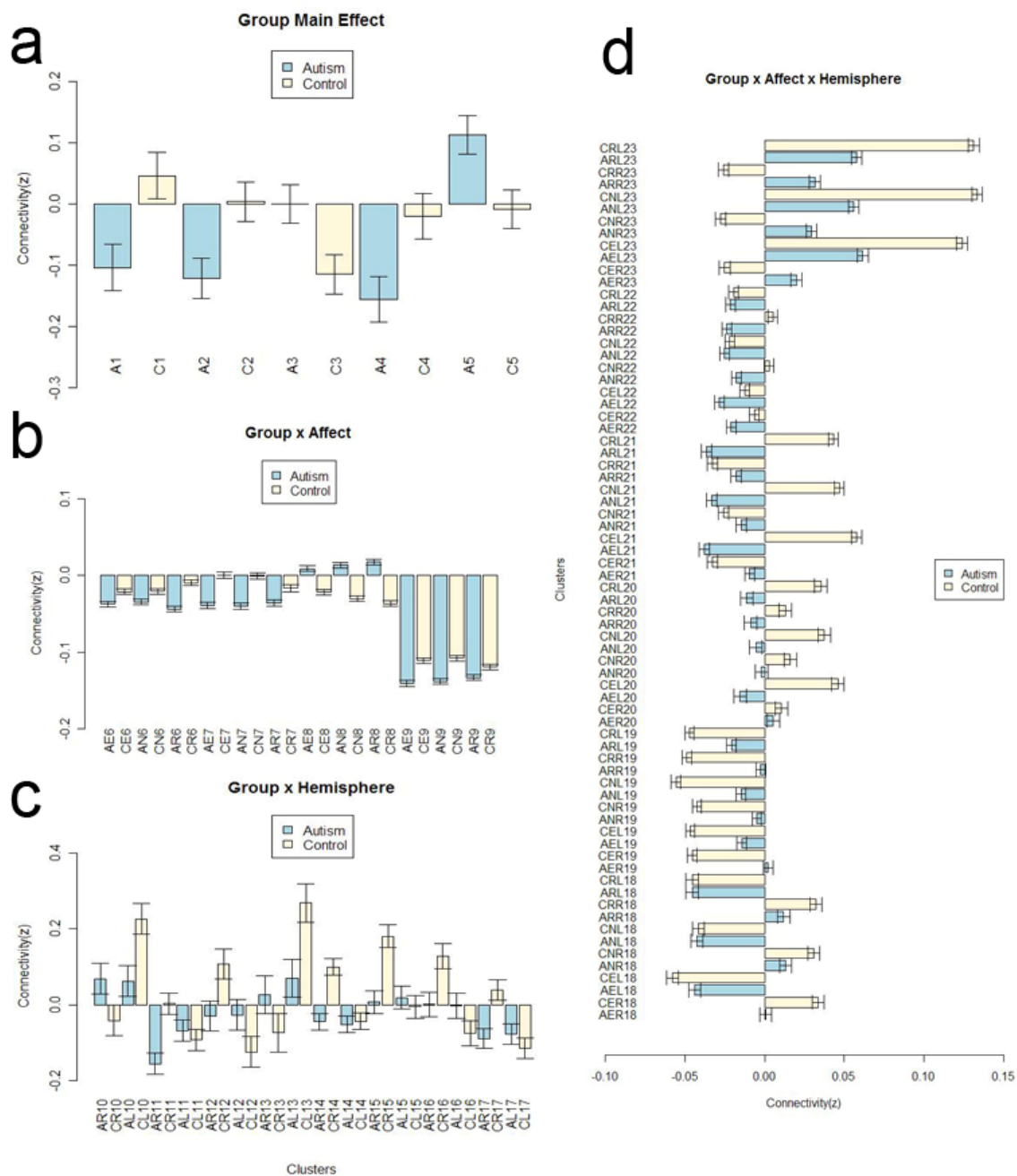


Figure 6

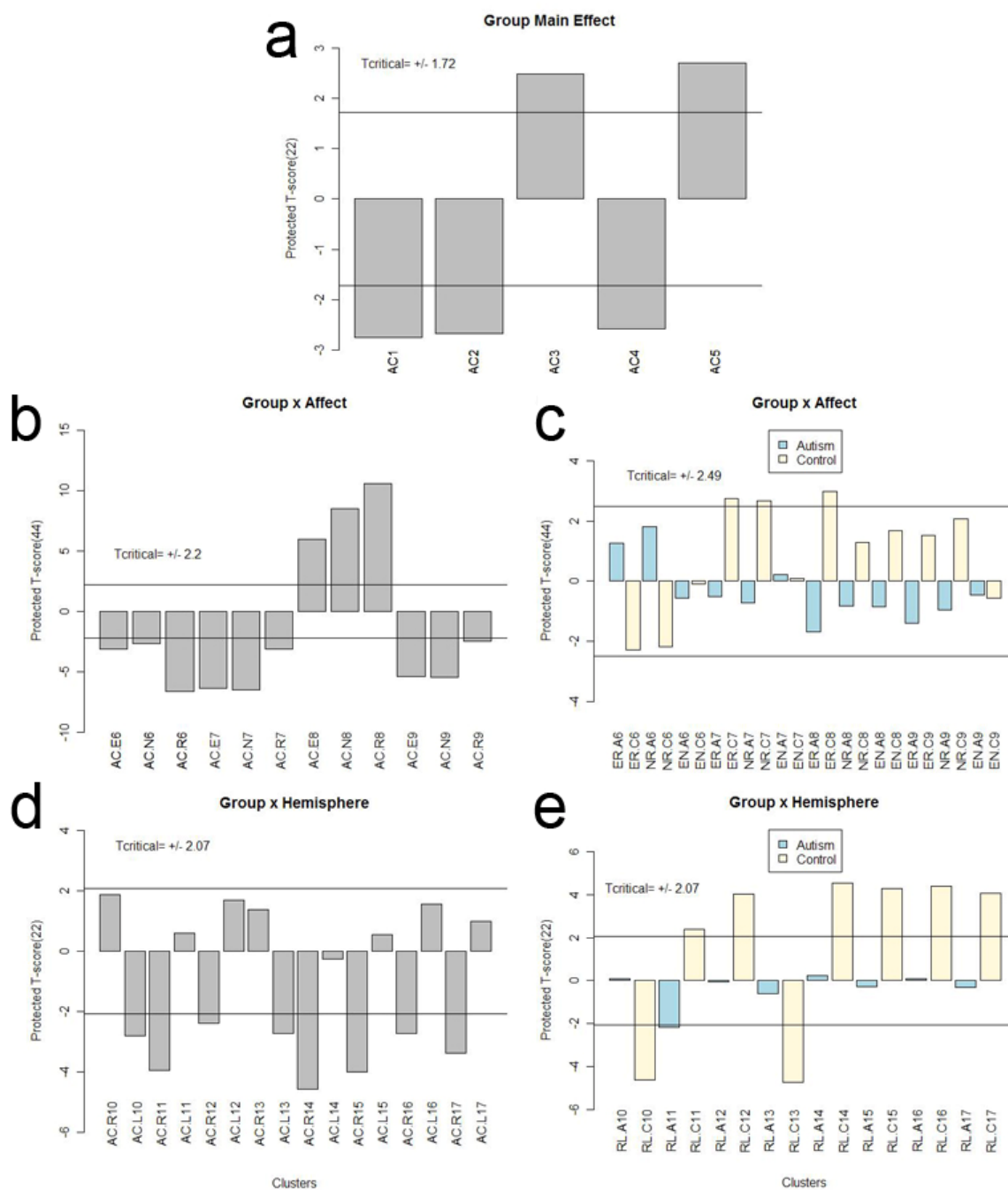


Figure 7

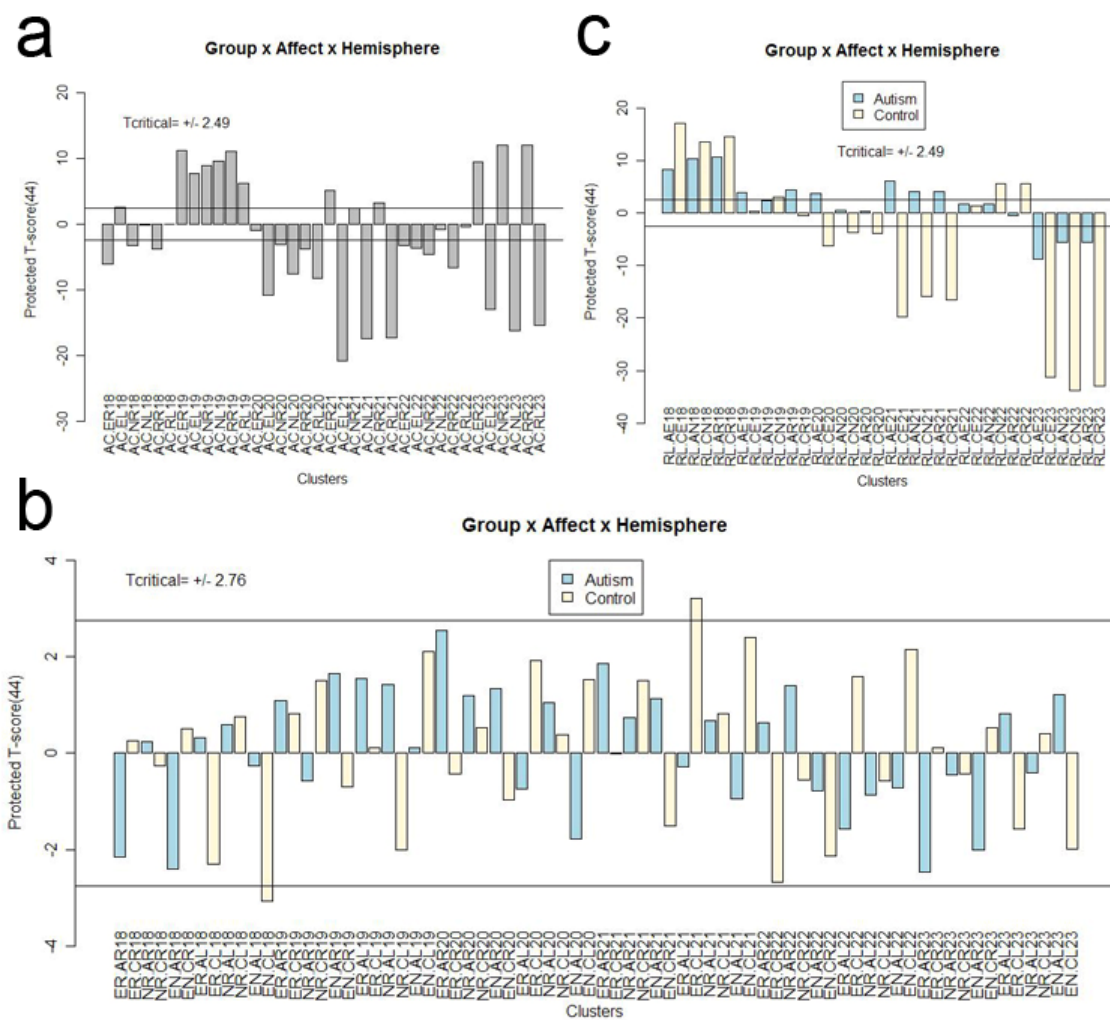


Figure 8

Supplementary Tables

Supplementary Table 1: Dendrogram Results from Unsupervised Clustering of Functional Amygdala Connectivity Z-scores.

Amygdala	Cluster	Brain Region
Right Cluster Tree		
L	10	Left insula and claustrum
L	13	Left lingual gyrus and fusiform
R	18	Right precuneus
L	23	Left posterior cingulate
R	12	Right insula
R	14	Right middle temporal gyrus
R	16	Right posterior cingulate
R	15	Right fusiform gyrus
Middle Cluster Tree		
L	18	Right precuneus
L	14	Right middle temporal gyrus
L	12	Right insula
L	16	Right posterior cingulate
L	15	Right fusiform gyrus
R	13	Left lingual gyrus and fusiform
R	23	Left posterior cingulate
L	19	Left inferior parietal lobule
R	19	Left inferior parietal lobule
L	3	Anterior cingulate
L	8	Left superior temporal gyrus
R	3	Anterior cingulate
R	8	Left superior temporal gyrus
R	10	Left insula and claustrum
L	5	Postcentral gyrus
R	5	Postcentral gyrus
Left Cluster Tree		
R	4	Right middle temporal gyrus
L	4	Right middle temporal gyrus
R	11	Left middle frontal gyrus
R	2	Left superior frontal gyrus
L	2	Left superior frontal gyrus
L	1	Cerebellum declive
R	1	Cerebellum declive
L	7	Cerebellum declive and declive of vermis
L	20	Left cerebellum declive
R	21	Right cerebellum declive

L	21	Right cerebellum declive
R	22	Left inferior frontal gyrus
L	9	Left middle frontal gyrus
L	11	Left middle frontal gyrus
L	17	Right precentral gyrus
R	6	Left angular gyrus
L	6	Left angular gyrus
L	22	Left inferior frontal gyrus
R	17	Right precentral gyrus
R	7	Cerebellum declive and declive of vermis
R	20	Left cerebellum declive
R	9	Left middle frontal gyrus

Supplementary Table 2: Summary of F-statistics for Cluster Connectivity and Activity. The connectivity F-statistics for cluster-specific ANOVA effects itemized in Table 1. Activity F-statistics for the Group main effect and the Group x Affect interaction were reported for all regional clusters of interests with significant connectivity differences. Significant F-statistics were denoted with a “ * ”. Activity F-statistics were not volume thresholded since the ROI of clusters with significant connectivity differences was used to quantify the F-statistic.

Cluster	Connectivity F-statistic	Activity F-statistic (Group Main)	Activity F-statistic (Group x Affect)
1	8.05*	0.35	0.48
2	7.45*	0.80	0.84
3	6.70*	1.34	1.06
4	6.82*	0.49	0.47
5	7.65*	0.71	0.66
F(1,22)=4.765			
Threshold	Volume(uL)= 1054	F(1,22)= 4.30	F(2,44)=3.21
	Voxelwise p = 0.04 (p=0.05 corrected)	Voxelwise p = 0.05	Voxelwise p = 0.05
6	5.26*	0.56	0.70
7	4.54*	1.69	1.52
8	5.76*	2.40	2.15
9	4.46*	0.92	0.83
F(2,44)=3.62			
Threshold	Volume(uL)=984	F(1,22)= 4.30	F(2,44)=3.21
	Voxelwise p = 0.035 (p=0.05 corrected)	Voxelwise p = 0.05	Voxelwise p = 0.05
10	11.71*	2.20	2.23
11	11.08*	0.68	0.77

12	9.25*	4.12	3.31*
13	8.97*	2.86	2.28
14	9.93*	1.70	1.39
15	11.05*	1.52	1.63
16	9.49*	1.01	0.88
17	10.14*	0.15	0.27
F(1,22)=6.29			
Threshold	Volume(uL)=773	F(1,22)= 4.30	F(2,44)=3.21
	Voxelwise p = 0.02 (p=0.05 corrected)	Voxelwise p = 0.05	Voxelwise p = 0.05
18	5.41*	0.71	0.88
19	5.64*	1.33	1.24
20	5.54*	0.48	0.49
21	5.79*	2.05	1.82
22	6.48*	1.84	1.41
23	5.00*	2.05	1.66
F(2,44)=3.80			
Threshold	Volume(uL)=984	F(1,22)= 4.30	F(2,44)=3.21
	Voxelwise p = 0.03 (p=0.05 corrected)	Voxelwise p = 0.05	Voxelwise p = 0.05

Supplementary Figures

Supplementary Figure 1: Group Mean Activity Values for Significant Connectivity

Clusters.

Mean activity values for each group were plotted for each group. Error bars are SEM

based on the Group Main Effect for the three-way GroupxAffectxSubject(Group)

ANOVA of area under the curve activity estimates.

Supplementary Figure 2: Group x Affect Mean Activity for Significant Connectivity

Clusters

Mean activity values for each Group and Affect were plotted for each group. Error bars are SEM based on the GroupxAffect interaction for the three-way GroupxAffectxSubject(Group) ANOVA of area under the curve activity estimates.

Supplementary Figure 3: Amygdala Activity ANOVA Effects.

The F-statistics for the three-way Group x Affect x Subject(Group) ANOVA of amygdala activity (area under the curve) estimates were plotted. The autism and control groups were not significantly different ($p=0.05$, uncorrected, two-tailed) in amygdala for the Group Main effect or Group x Affect interaction. Error bars are SEM based on the Group Main effect [$F(1,22)$] and Group x Affect interaction [$F(2,44)$].

Supplementary Figure 4: Autism and Control Residual Connectivity Associations with Total Eye Fixation Time and Total Face-Fixation Time.

Autism and control association between total eye fixation time and functional connectivity of the (a) left insula and claustrum and left amygdala during neutral face processing [Autism: $r(10)=-0.362$, $p=0.248$; Control: $r(9)=0.699$, $p=0.0166$; z-difference= -3.53 , $p=0.000$], (b) right middle temporal gyrus and left amygdala during emotional face processing [Autism: $r(10)=0.206$, $p=0.520$; Control: $r(9)=-0.643$, $p=0.033$; z-difference= 2.50 , $p=0.012$]. Autism and control association between total face-fixation time and functional connectivity of (c) right middle temporal gyrus and left amygdala during emotional face processing [Autism: $r(10)=0.239$, $p=0.455$; Control: $r(9)=-0.619$, $p=0.0425$; z-difference= 2.39 , $p=0.017$], (d) right precuneus and left amygdala during emotional face processing [Autism: $r(10)=0.419$, $p=0.175$; Control: $r(9)=-0.448$, $p=0.167$; z-difference=

2.07, $p=0.038$], (e) right precuneus and left amygdala during neutral face processing [Autism: $r(10)=0.411$, $p=0.184$; Control: $r(9)=-0.552$, $p=0.0784$; z-difference=2.46, $p=0.0139$].

Supplementary Figure 5: Autism and Control Residual Connectivity Associations with Mean Face-Fixation Time/Trial and Ratio Eye to Face Fixation Time.

Autism and control association between mean face-fixation time/trial and functional connectivity of the (a) right middle temporal gyrus and left amygdala during emotional face processing [Autism: $r(10)=0.239$, $p=0.455$; Control: $r(9)=-0.619$, $p=0.0425$; z-difference=2.39, $p=0.167$], (b) right precuneus and left amygdala during emotional face processing [Autism: $r(10)=0.419$, $p=0.175$; Control: $r(9)=-0.448$, $p=0.167$; z-difference=2.07, $p=0.0384$], (c) right precuneus and left amygdala during neutral face processing [Autism: $r(10)=0.411$, $p=0.184$; Control: $r(9)=-0.552$, $p=0.0784$; z-difference=2.46, $p=0.0139$]. Autism and control association between ratio eye to face fixation time and functional connectivity of (d) left superior frontal gyrus and left amygdala during emotional face processing [Autism: $r(10)=0.372$, $p=0.233$; Control: $r(9)=-0.522$, $p=0.0997$; z-difference=2.21, $p=0.0271$], (e) left superior frontal gyrus and left amygdala during neutral face processing [Autism: $r(10)=0.505$, $p=0.0942$; Control: $r(9)=-0.592$, $p=0.055$; z-difference=3.00, $p=0.00270$], (f) left posterior cingulate and left amygdala during emotional face processing [Autism: $r(10)=0.560$, $p=0.0584$; Control: $r(9)=-0.313$, $p=0.348$; z-difference=2.23, $p=0.0260$], (g) left posterior cingulate and left amygdala during neutral face processing [Autism: $r(10)=0.648$, $p=0.0226$; Control: $r(9)=-0.182$, $p=0.592$; z-difference=2.49, $p=0.0127$].

Supplementary Figure 6: Autism and Control Residual Connectivity Associations with Number of Facial Expressions Judged Correctly and Response Time to Neutral Faces.

Autism and control association between number of facial expressions judged correctly and functional connectivity of the (a) right postcentral gyrus and left amygdala during neutral face processing [Autism: $r(10)=-0.169$, $p=0.600$; Control: $r(10)=-0.863$, $p=0.000302$; $z\text{-difference}=2.41$, $p=0.0161$], (b) right fusiform gyrus and right amygdala during emotional face processing [Autism: $r(10)=-0.214$, $p=0.504$; Control: $r(10)=0.622$, $p=0.0308$; $z\text{-difference}=-2.43$, $p=0.0151$]. Autism and control association between response time(ms) to neutral faces and functional connectivity of the (c) left insula and claustrum and right amygdala during neutral face processing [Autism: $r(8)=0.656$, $p=0.0396$; Control: $r(10)=-0.227$, $p=0.478$; $z\text{-difference}=2.57$, $p=0.0101$], (d) left insula and claustrum and left amygdala during neutral face processing [Autism: $r(8)=0.740$, $p=0.0145$; Control: $r(10)=-0.0464$, $p=0.886$; $z\text{-difference}=3.74$, $p=-0.000186$], (e) left inferior parietal lobule and right amygdala during neutral face processing [Autism: $r(8)=-0.509$, $p=0.133$; Control: $r(10)=0.697$, $p=0.0117$; $z\text{-difference}=-3.84$, $p=0.000124$].

Supplementary Figure 7: Autism and Control Residual Connectivity Associations with Response Time to Emotional Faces.

Autism and control association between the response time (ms) to emotional faces and functional connectivity of the (a) cerebellum declive and right amygdala during emotional face processing [Autism: $r(8)=-0.747$, $p=0.013$; Control: $r(10)=0.0295$, $p=0.928$;

z-difference=-3.97, $p=0.0000716$], (b) cerebellum declive and left amygdala during emotional face processing [Autism: $r(8)=-0.690$, $p=0.0373$; Control: $r(10)=0.475$, $p=0.119$; z-difference=-3.61, $p=0.000304$], (c) left angular gyrus and right amygdala during emotional face processing [Autism: $r(8)=0.488$, $p=0.152$; Control: $r(10)=-0.436$, $p=0.156$; z-difference=2.19, $p=0.0288$], (d) left middle frontal gyrus and right amygdala during emotional face processing [Autism: $r(8)=-0.656$, $p=0.0394$; Control: $r(10)=0.376$, $p=0.228$; z-difference=-2.93, $p=0.00335$], (e) left lingual gyrus and fusiform and left amygdala during emotional face processing [Autism: $r(8)=-0.685$, $p=0.0288$; Control: $r(10)=0.354$, $p=0.259$; z-difference=-3.18, $p=0.00145$], (f) right precuneus and right amygdala during emotional face processing [Autism: $r(8)=-0.668$, $p=0.0346$; Control: $r(10)=0.146$, $p=0.651$; z-difference=-2.51, $p=0.0119$], (g) left inferior parietal lobule and left amygdala during emotional face processing [Autism: $r(8)=0.760$, $p=0.0108$; Control: $r(10)=-0.285$, $p=0.37$; z-difference=6.82, $p=0.000$], (h) right cerebellum declive and left amygdala during emotional face processing [Autism: $r(8)=-0.665$, $p=0.0358$; Control: $r(10)=0.379$, $p=0.224$; z-difference=-3.03, $p=0.00247$].

Supplementary Figure 8: Autism Residual Connectivity Associations with Non-Verbal Communication.

Autism association between non-verbal communication and functional connectivity of the (a) right postcentral gyrus and right amygdala during rest [Autism: $r(6)=-0.763$, $p=0.0277$], (b) right insula and left amygdala during emotional face processing [Autism: $r(6)=-0.757$, $p=0.0296$], (c) right insula and left amygdala during neutral face processing [Autism: $r(6)=-0.741$, $p=0.0354$], (d) right insula and left amygdala during rest

[Autism: $r(6)=-0.743$, $p=0.0346$], (e) right fusiform gyrus and right amygdala during rest [Autism: $r(6)=0.743$, $p=0.0345$], (f) left cerebellum declive and left amygdala during emotional face processing [Autism: $r(6)=0.715$, $p=0.0463$], (g) left cerebellum declive and left amygdala during neutral face processing [Autism: $r(6)=0.712$, $p=0.0474$], (h) left cerebellum declive and left amygdala during rest [Autism: $r(6)=0.744$, $p=0.0345$].

Supplementary Figure 9: Autism Residual Connectivity Associations with Verbal Communication.

Autism association between verbal communication and functional connectivity between (a) right precuneus and left amygdala during emotional face processing [Autism: $r(5)=-0.768$, $p=0.0438$], (b) right precuneus and left amygdala during neutral face processing [Autism: $r(5)=-0.799$, $p=0.0313$], (c) right precuneus and left amygdala during rest [Autism: $r(5)=-0.785$, $p=0.0366$], (d) left posterior cingulate and right amygdala during emotional face processing [Autism: $r(5)=-0.840$, $p=0.018$], (e) left posterior cingulate and right amygdala during neutral face processing [Autism: $r(5)=-0.862$, $p=0.0125$], (f) left posterior cingulate and right amygdala during rest [Autism: $r(5)=-0.862$, $p=0.0127$].

Supplementary Figure 10: Autism Residual Connectivity Associations with Restricted and Repetitive Behavior.

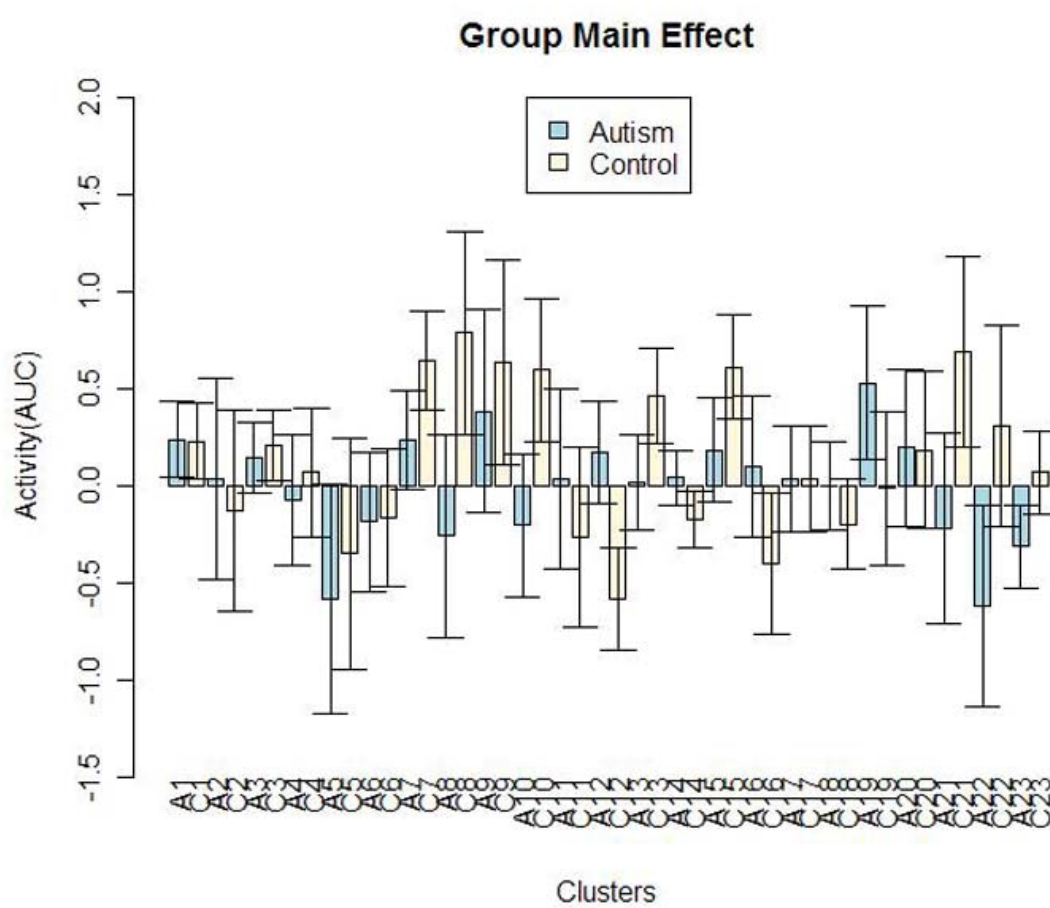
Autism association between restricted and repetitive behavior and functional connectivity between (a) right middle temporal gyrus and left amygdala during emotional face processing [Autism: $r(6)=0.751$, $p=0.0319$], (b) right posterior cingulate and right amygdala during emotional face processing [Autism: $r(6)=0.737$, $p=0.0371$], (c) right

posterior cingulate and right amygdala during neutral face processing

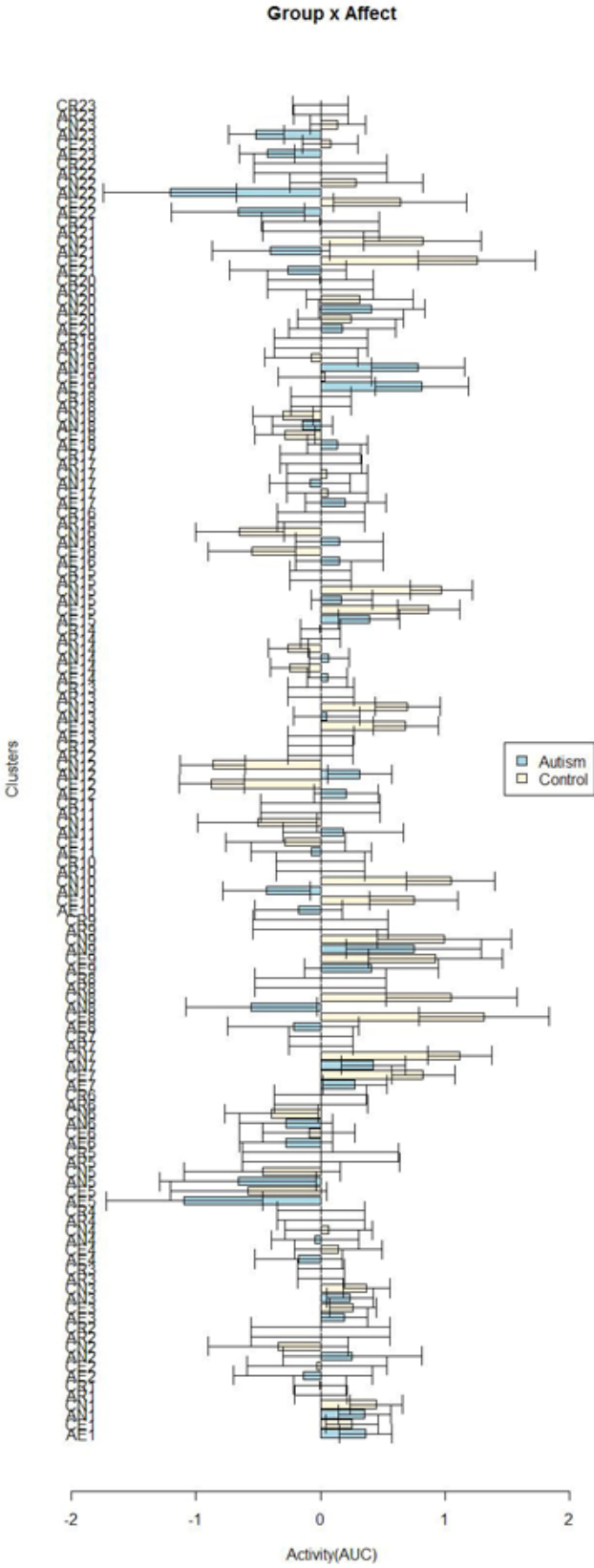
[Autism: $r(6)=0.773$, $p=0.0244$], (d) right posterior cingulate and right amygdala during rest [Autism: $r(6)=0.726$, $p=0.0414$].

Supplementary Figure 11: Autism Residual Connectivity Associations with ADI-R Social Scores.

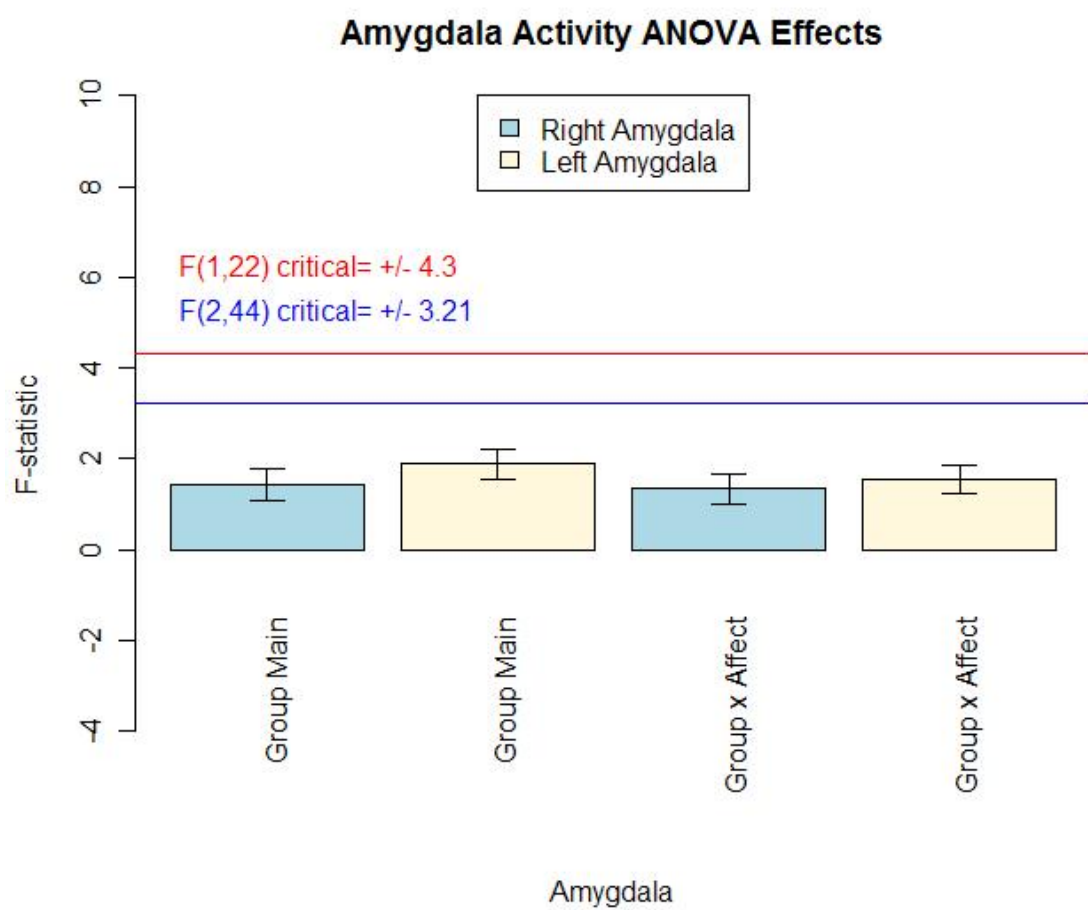
Autism association ($p < 0.1$, uncorrected) between restricted and repetitive behavior and functional connectivity between (a) cerebellum declive and right amygdala at rest [Autism: $r(6)=-0.691$, $p=0.0577$], (b) left insula/claustrum and left amygdala at rest [Autism: $r(6)=0.662$, $p=0.0737$], (c) right posterior cingulate and left amygdala during neutral face processing [Autism: $r(6)=-0.687$, $p=0.0598$], (d) right posterior cingulate and left amygdala during rest [Autism: $r(6)=-0.658$, $p=0.0763$], (e) right precuneus and right amygdala during emotional face processing [Autism: $r(6)=-0.648$, $p=0.082$], (f) right precuneus and left amygdala during emotional face processing [Autism: $r(6)=-0.643$, $p=0.0856$], (g) right precuneus and right amygdala during neutral face processing [Autism: $r(6)=-0.654$, $p=0.0786$], (h) right precuneus and left amygdala during neutral face processing [Autism: $r(6)=-0.667$, $p=0.0705$], (i) right precuneus and right amygdala during rest [Autism: $r(6)=-0.636$, $p=0.0903$], (j) left posterior cingulate and left amygdala during emotional face processing [Autism: $r(6)=-0.627$, $p=0.0961$], (k) left posterior cingulate and left amygdala during neutral face processing [Autism: $r(6)=-0.694$, $p=0.0563$].



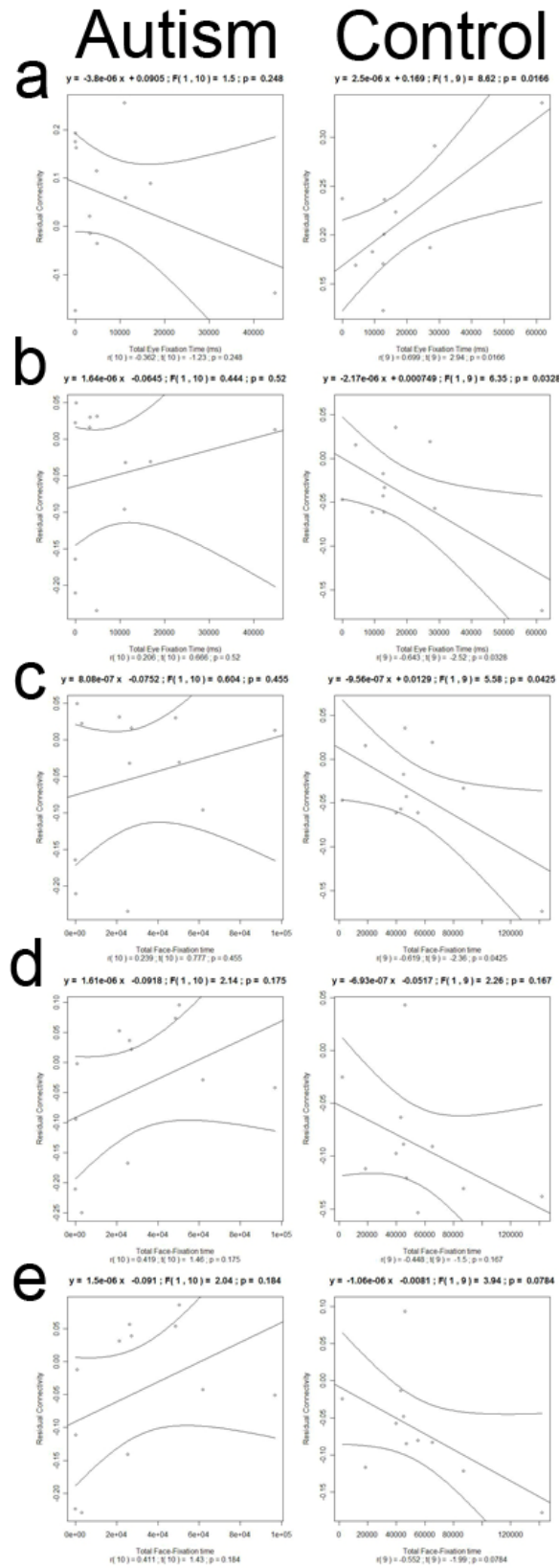
Supplementary Figure 1



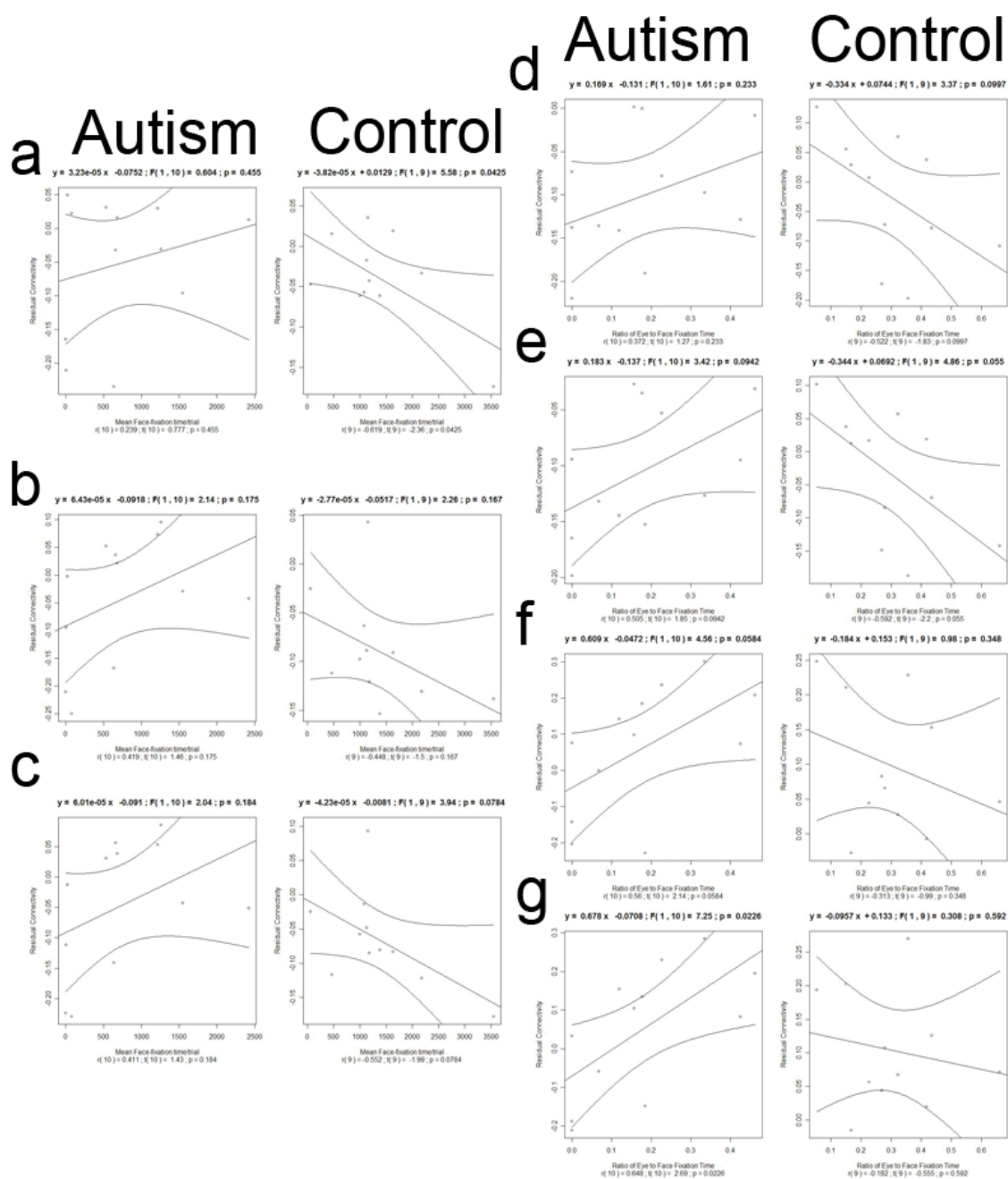
Supplementary Figure 2



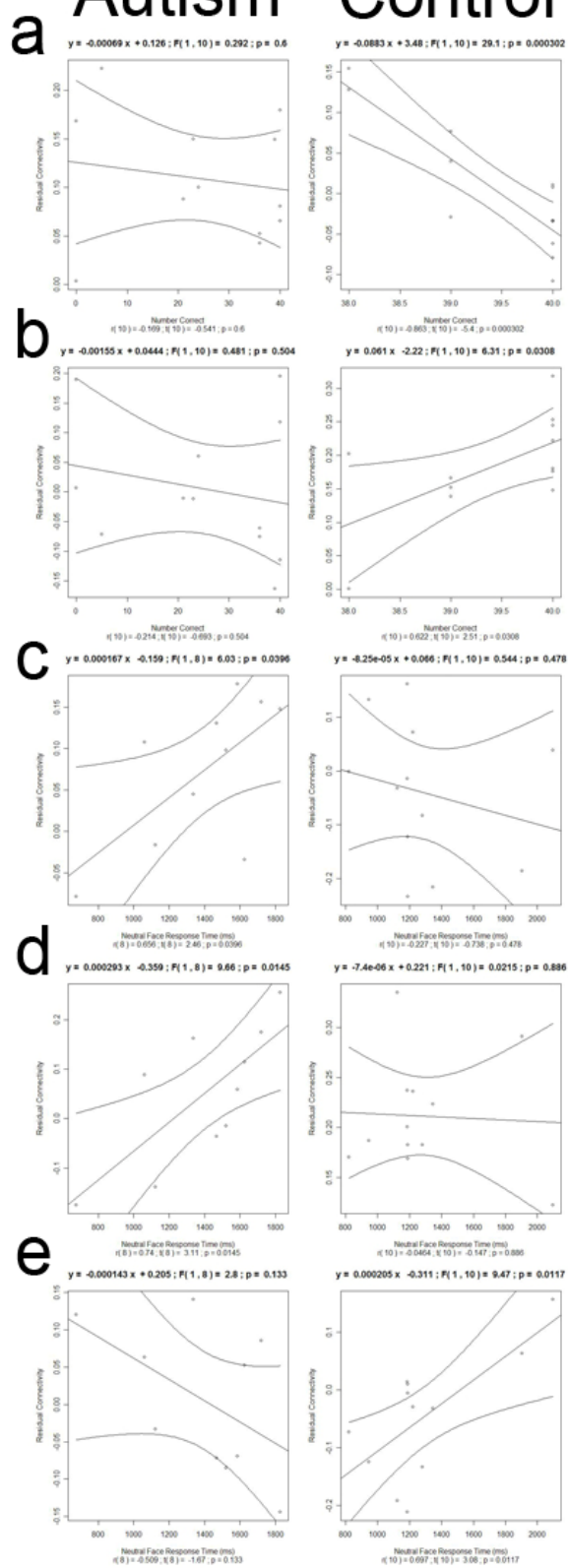
Supplementary Figure 3

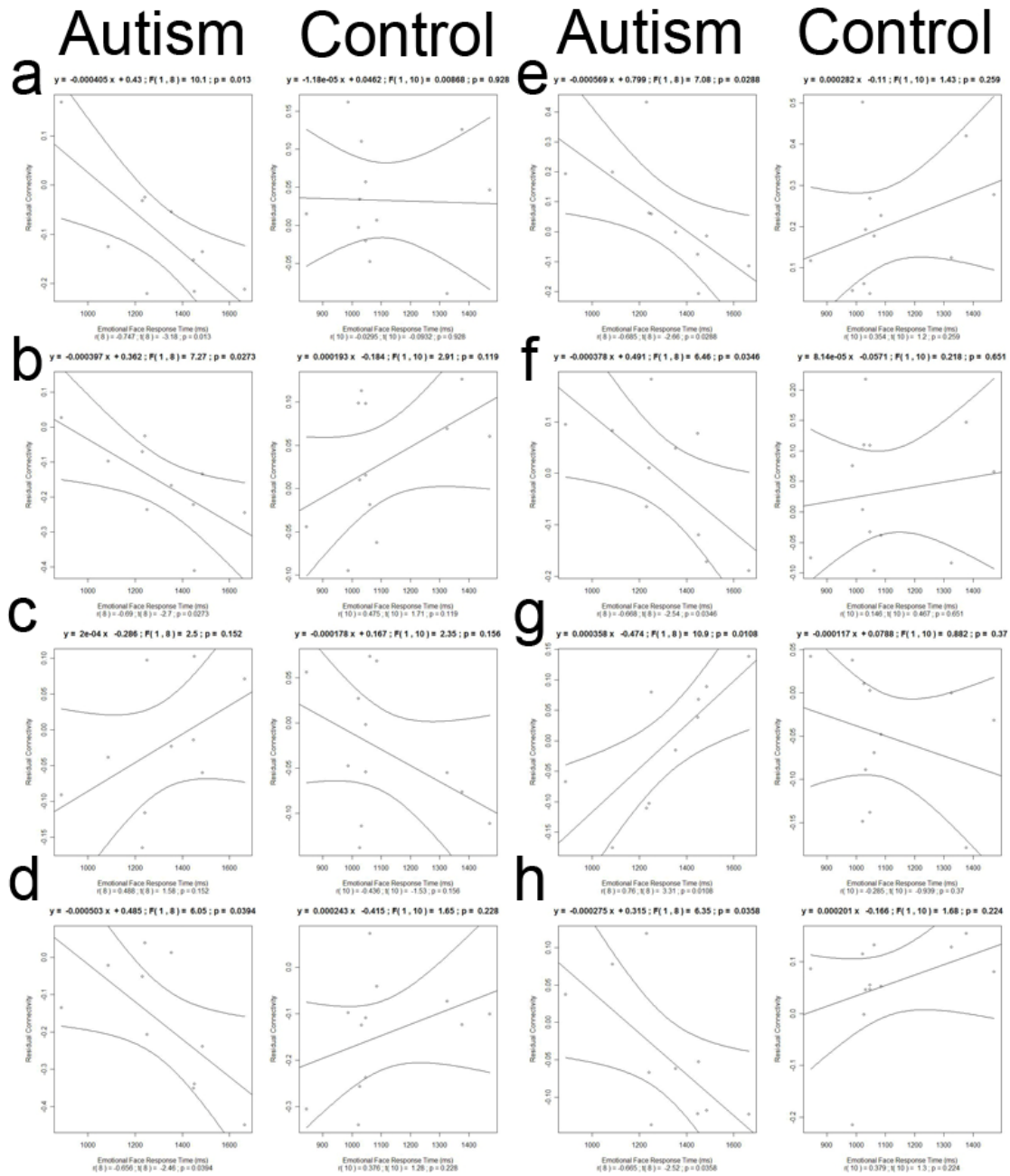


Supplementary Figure 4

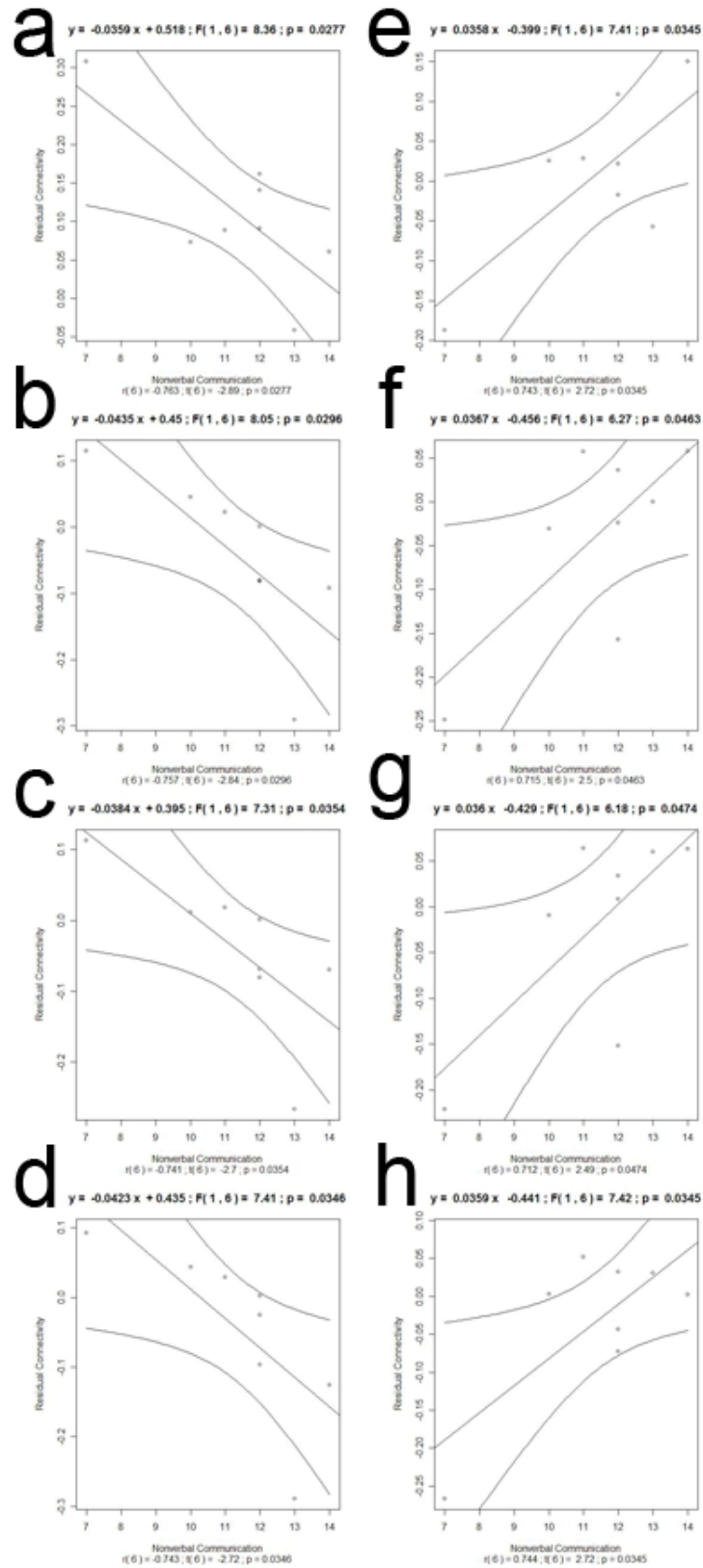


Supplementary Figure 5

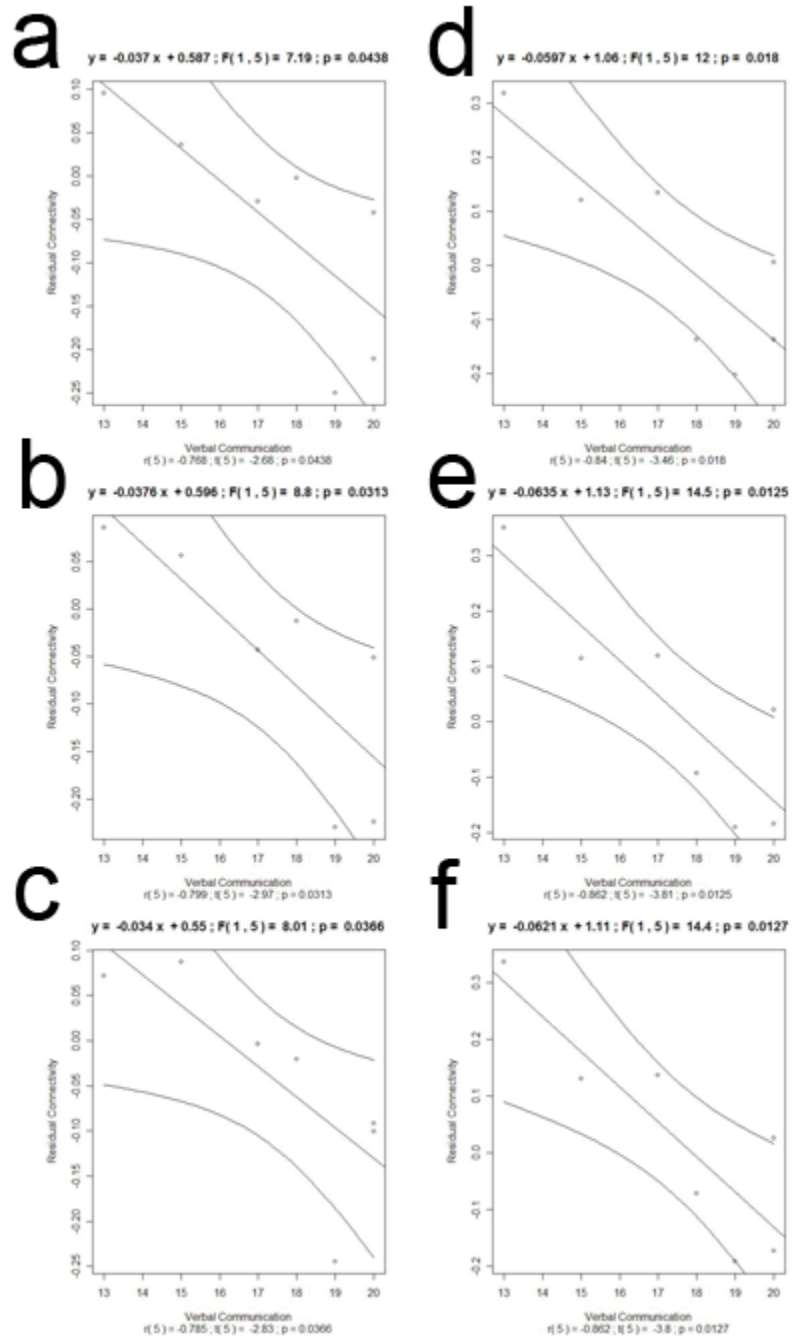




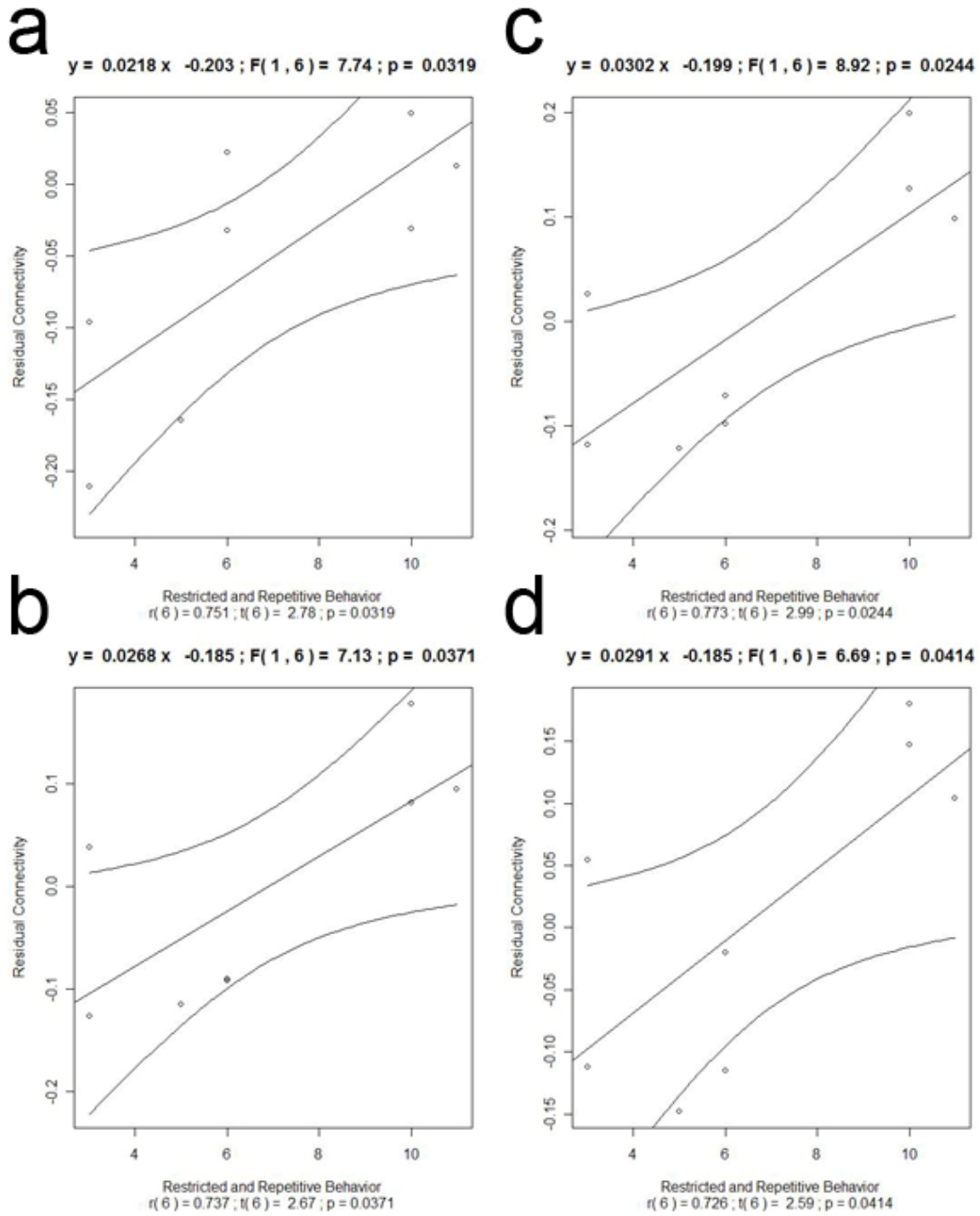
Supplementary Figure 7



Supplementary Figure 8



Supplementary Figure 9



Supplementary Figure 10

Supplementary Figure 11

Chapter 4:

Interhemispheric Connectivity Dynamics of Affective Face Integration in Autism

Interhemispheric Connectivity Dynamics of Affective Face Integration in Autism

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Running Title: Interhemispheric Autism Connectivity

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Abstract

Altered connectivity of brain function is implicated in the autism phenotype. We used functional MRI, a facial emotion discrimination task, and an interhemispheric partial correlation analysis to assess the functional networks of affective social processing in autism and controls. We identified interhemispheric patterns of overconnectivity and underconnectivity that differed between groups and across facial affect. In addition, the reduced magnitude of connectivity differences between autism affective processing networks indicated that dynamic connectivity capacity was limited. Furthermore, the association of dynamic connectivity capacity with corpus callosum density showed group differences across network transitions. Based on the functional dysmetria of face processing networks in autism, we propose that deficits in affective social behavior are related to an imbalance in affective network dynamics.

Introduction

Autism is a neurodevelopmental disorder with a classic triad of deficits in the social, behavioral, and communicative domains. Autism deficits in these domains can be attributed to alterations in both neurotransmission and synaptic structure with consequences for cognition and behavior (Wickelgren 2005; Uhlhaas and Singer 2006; Courchesne, Pierce et al. 2007; Garber 2007). System level investigations of autism functional brain connectivity, the temporal association of regional brain function, have largely focused on intrahemispheric associations (Minshew and Williams 2007) but not on integration between the hemispheres. Among those studies examining the interhemispheric connectivity of a predefined set of brain regions, autism presents with functional underconnectivity both within and across the hemispheres during specific tasks (Just, Cherkassky et al. 2004; Kana, Keller et al. 2006; Just, Cherkassky et al. 2007) and during rest (Cherkassky, Kana et al. 2006). These intrahemispheric and interhemispheric studies have led to formulation of an “underconnectivity hypothesis” in which the absolute magnitude of functional connectivity is lower in autism compared to controls. A voxelwise, whole brain interhemispheric functional connectivity analysis between homotopic brain regions at rest and during task could provide insight into the functional consequences of callosum alterations in autism.

Functional interhemispheric brain networks can influence behavior through the corpus callosum (Clarke and Zaidel 1994), a key anatomical structure connecting the hemispheres. Interhemispheric callosal projections connect homotopic and heterotopic brain regions (de Lacoste, Kirkpatrick et al. 1985) and are topographically organized

along the anterior-posterior dimension of the brain as a whole and within brain regions (Paul, Brown et al. 2007). In monkeys, for example, interhemispheric fibers of the insula cross in the ventral portion of the corpus callosum and are topographically organized with rostral insula fibers crossing in the rostral corpus callosum body and caudal insula fibers traveling through caudal portions of the corpus callosum body (Reeves 1985). The corpus callosum has several integrative roles including asymmetric interhemispheric inhibition, information transfer, and hemispheric recruitment (Stephan, Marshall et al. 2007). Interhemispheric information transfer requires an intact corpus callosum based on studies of split brain and corpus callosum agenesis patients (Paul, Brown et al. 2007). A functional connectivity study of corpus callosum agenesis identified the presence of intrahemispheric connectivity, but largely absent interhemispheric connectivity measures, during auditory and motor tasks (Quigley, Cordes et al. 2003). Children with agenesis of the corpus callosum present with autism-like social interaction and communication behavior (Badaruddin, Andrews et al. 2007; Paul, Brown et al. 2007). The relationship between corpus callosum integrity and network integration varies in its extent among individuals (Belger and Banich 1998) and can influence behaviors such as reaction time (Baird, Colvin et al. 2005). Individuals with autism demonstrate impaired interhemispheric transfer based on performance during motor, visual, and auditory neuropsychological tasks (Nyden, Carlsson et al. 2004). With implications for aberrant emotional face processing in autism, behavioral studies in controls indicate that interhemispheric transfer of emotional information is a function of emotional significance (Compton, Wilson et al. 2004), that interhemispheric processing increases the accuracy of face and facial expression matching (Compton 2002), and that emotional faces have an

interhemispheric processing advantage over neutral faces (Compton, Feigenson et al. 2005). Production of functional interhemispheric networks for affective face processing requires an intact callosum.

Corpus callosum alterations in autism may contribute to unique interhemispheric connectivity patterns. Reduced fractional anisotropy measures using diffusion tensor imaging are noted in the corpus callosum and indicate that interhemispheric processing is altered (Alexander, Lee et al. 2007; Keller, Kana et al. 2007). The volume of the callosum body and splenium are reduced in autism after controlling for brain volume, gender, and performance IQ (Piven, Bailey et al. 1997). A 2D voxel-based morphometric analysis of midsagittal corpus callosum identified reductions in autism genu and splenium white matter density after controlling for age (Chung, Dalton et al. 2004). Furthermore, volumetric measures of corpus callosum are significantly and positively associated with functional interhemispheric connectivity differences in autism during task (Kana, Keller et al. 2006; Just, Cherkassky et al. 2007) and at rest (Cherkassky, Kana et al. 2006). In union, the functional and anatomical studies suggest that an altered relationship exists in autism between corpus callosum measures and functional transcallosal integration.

Here we describe a functional connectivity approach that uses partial correlation analysis to examine interhemispheric integration during rest and both emotional and neutral face processing, a task central to aberrant social behavior in autism. The comparison of whole brain functional connectivity measures for both the rest and task state within the same

study could provide insight into the connectivity dynamics of autism by respectively discriminating trait-based from state-based connectivity networks and by clarifying the relationship between homotopic connectivity and both callosum and behavioral measures. In addition, we report autism alterations in the relationship between homotopic connectivity dynamics and callosum density.

Methods

Study design and task methodology have been reported elsewhere (Dalton, Nacewicz et al. 2005). Briefly, twelve male control subjects ($n=12$) with a mean age ($\pm$ SD) of 17.0 ($\pm$ 2.86) and twelve males with autism ($n=12$) or Asperger's disorder aged 16.75 ($\pm$ 4.52) years were recruited from a list of available autism volunteers maintained at the University of Wisconsin-Madison Waisman Center. Ages were not significantly different [$t(22) = -0.162$, $P = 0.87$] between groups. Criteria for diagnosing autism and Asperger's Disorder included DSM-IV and confirmation with the Autism Diagnostic Interview Revised (ADI-R). Subjects participated in an event-related fMRI study using a facial emotion recognition task. In total 40 faces, 16 neutral and 24 emotional (8 happy, 8 fear, 8 anger), from the Karolinska Directed Emotional Faces Set (Lundqvist) were presented with a frontal or side orientation for 3 seconds each with a jittered inter-trial interval ranging from 5 to 7 seconds. Subjects were instructed to decide if the face displayed an emotion (happy, fear, anger) or was neutral and to indicate their choice with a button box press. Both accuracy (percent correct) and reaction times were measured. Images were acquired with a quadrature head coil and consisted of 409 gradient recalled echo-planar images using BOLD contrast ($TE = 30$ ms; $TR = 2$ sec; $FOV = 240 \times 240$ mm,

64x64 matrix, voxel size= 3.75 x 3.75 x 5mm) acquired sagittally (30 slices. 4mm thick with 1mm gap) with a 3.0 Tesla GE SIGNA Scanner (Waukesha, WI). Axial T1-weighted 3D SPGR images were also acquired (TE = 8 ms, TR = 35 ms, FOV = 240x240 mm, 256x192 matrix, 124 axial slices, slice thickness = 1.1-1.2 mm, NEX = 1, flip angle = 30 degrees). Analyses were carried out using AFNI (Cox 1996). Echoplanar images were Fermi filtered in the spatial frequency domain, reconstructed, motion corrected, and volume registered to the echoplanar volume collected closest in time to the T1 anatomical images prior to blurring with a Gaussian kernel (FWHM=6 mm).

Interhemispheric Connectivity Analysis

Neuroanatomical asymmetries present in the human brain (Watkins, Paus et al. 2001; Toga and Thompson 2003; Luders, Gaser et al. 2004) can be accounted for by conducting analyses in a symmetric Talairach template space based on voxel based morphometry analyses (VBM) (Watkins, Paus et al. 2001). Manually Talairached anatomical images were averaged across all subjects in both groups. A symmetric template was generated by averaging the mean anatomical volume with the same image mirrored about the midline. Each subject's anatomical image was then automatically registered to the symmetric template using a 12 parameter affine transform. Each subject's echoplanar image underwent the same transformation as the corresponding anatomical image. We used the partial correlation between a temporal brain volume and its right-left flipped mirrored volume as the connectivity statistic by partialling out motion and three physiological noise measures including global brain signal, white matter signal, and lateral ventricle signal (Birn, Diamond et al. 2006). Given that the spatial heterogeneity of HRFs complicates estimation of functional connectivity between heterotopic brain regions

(Bandettini, Birn et al. 2002; Birn and Bandettini 2005), our partial correlation approach using a symmetric template has the advantage of comparing regions with similar hemodynamic transfer functions based on the greater similarity of homologous neurovascular architectures. White matter and lateral ventricle delineation was based on segmentation of T1 images in FSL (Smith, Jenkinson et al. 2004). To examine emotional and neutral face networks, regressors of six TR lagged neutral or emotional impulse functions, respectively, were included as regressors of no interest (Fair, Schlaggar et al. 2007). The statistical resting state network was determined by simultaneously including both the emotional and neutral time lagged impulse functions as regressors of no interest in the partial correlation analysis. Activations were quantified as the homotopic sum of hemodynamic response function (HRF) area under the curve (AUC) estimates that were modeled using the emotional and neutral impulse functions. A representative design matrix is shown in Supplementary Figure 1.

The partial correlation statistic for each affective network (emotional, neutral, and rest) was converted to a z-statistic using the Fisher transform. We tested the null hypothesis that mean interhemispheric connectivity was comparable between autism and controls using a type five, three-way mixed effects Group x Affect x Subject(Group) ANOVA with Subject nested and randomized within Group. Clusters of significance were determined after multiple comparison correction using Monte Carlo simulations. Significant clusters were only reported for those regions with a statistical signal to noise ratio (linear baseline estimate from the GLM fit divided by the standard deviation of the residual) greater than or equal to fifty to eliminate voxels with physiological noise

(Murphy, Bodurka et al. 2007). Masks of significant clusters were used to extract mean z-scores of connectivity and activity.

Connectivity Associations with Behavior and Callosum Density

Connectivity measures were corrected across subjects for activity within each group and facial affect condition according to:

$$\text{Connectivity} = b_0 + b_1 * \text{Activation} + \text{epsilon}$$

where Connectivity is a vector of subjects' interhemispheric connectivity z-scores and Activation is a vector of subjects' area under the HRF curve percent change estimates. The residual connectivity, epsilon, was used in subsequent behavior and callosum density associations. Thus, the connectivity measure used here is unconfounded by variations in activation across Affect conditions and Subjects. Callosum density estimates for the autism and control subjects were based on previously reported (Chung, Dalton et al. 2004) 2D voxel-based morphometry measures of mid-sagittal corpus callosum white matter probability maps. Residual interhemispheric connectivity was correlated with callosum density across subjects for individual differences in connectivity dynamics (emotion - rest, neutral – rest, and emotion – neutral) of the 8 brain regions (insula, cuneus, lingual, etc.) previously identified in the connectivity ANOVA. Statistical maps were Fisher transformed and entered into a three-way ANOVA (Group[autism, control] x Affect[emotion – rest, neutral – rest, emotion - neutral] x Cluster[insula, lingual, cuneus, etc.]) with Group and Affect fixed and Cluster randomized.

Results

We used partial correlation analysis to examine the interhemispheric connectivity of affective facial networks in autism and controls. A three-way ANOVA of connectivity scores identified significant (corrected, $p \leq 0.05$) Group Main (GM) effect and Group x Affect (GA) clusters. The group differences were homotopically detected and identified in the insula, inferior cuneus, inferior parietal, lateral lingual, and superior cuneus (Figure 1). Mean connectivity z-scores and activity in these regions are shown in Figure 2. Group x Affect interactions were detected bilaterally in fusiform, medial lingual, and cingulate regions (Figure 3). Mean connectivity z-scores and activity in these regions are shown in Figure 4. Protected t-scores, Bonferroni corrected for within cluster comparisons, were used to compare the GM and GA cluster means according to differences in group and affect (Figure 5). Among the GM clusters, insula, inferior cuneus, lateral lingual, and superior cuneus showed more positive interhemispheric connectivity in autism, but interhemispheric inferior parietal connectivity was more positively connected in controls than autism (Figure 5 a). There was no Group x Affect interaction in the GM clusters (Figure 5 b).

Next we compared GA cluster means (Figure 5 c). Compared to controls, the autism fusiform was more positively connected at rest, the medial lingual was more positively connected in all affective networks (emotional, neutral, and rest); and the cingulate was less positively connected during emotional face processing, but more positively connected during neutral face processing. In controls, fusiform showed marginally greater interhemispheric connectivity during emotional face processing than autism.

Interhemispheric connectivity of the fusiform, when processing neutral faces, and the resting cingulate were comparable in autism and controls. When examining the GA clusters for within group differences in connectivity between affective face networks (Figure 5 d), the autism group did not significantly change its connectivity from one affective network to another. This indicates a reduced dynamic interhemispheric connectivity capacity in the autism group. Control interhemispheric connectivity was greater during emotional than neutral face viewing in fusiform and cingulate, but not in medial lingual regions. Controls showed greater interhemispheric connectivity during emotional face viewing compared to rest in fusiform, medial lingual, and cingulate. Controls showed greater connectivity during neutral face processing compared to rest in fusiform and medial lingual regions.

Relationship between Activation and Connectivity

Interhemispheric connectivity z-scores were pooled across GM clusters for autism and controls. GM connectivity associations with activation were not significant in either group. When the interhemispheric connectivity z-scores were pooled across GA clusters for autism and controls and plotted against activation, the connectivity associations with activation were significant in both groups [Autism: $r(106) = 0.205$; $t(106) = 2.15$; $p = 0.0335$; Control: $r(106) = 0.492$; $t(106) = 5.82$; $p = 0.000$] and significantly different between groups [$z\text{-difference}(\text{Autism-Control}) = -2.40$; $p = 0.0166$]. Clusterwise associations between connectivity (z-scores) and activation (area under the curve) for the GM and GA cluster were not significant for either group after adjusting p-values for multiple comparisons ($FDR \leq 0.05$). No group differences were significant after FDR

adjusting p-values for multiple comparisons. Nevertheless, the connectivity and activation associations between homologous affect and cluster regions indicated that removing the activation effect from connectivity would improve the specificity of connectivity measures. For this reason, residual connectivity was used in subsequent analyses. For further details see Supplementary Note 1.

Residual Interhemispheric Connectivity Associations with Behavior and ADI-R

Next we examined the relationship between residual connectivity and behavioral measures focusing on gaze behavior, response time, and autism ADI-R measures. Residual interhemispheric connectivity associations with the ratio of time spent looking at the eyes compared to the face were significantly associated in controls, but not autism, in the insula during neutral face viewing [Autism: $r(10)=-0.0376$, $p=0.91$; Control: $r(9)=0.634$, $p=0.036$] and in the fusiform during emotion [Autism: $r(10)=-0.0331$, $p=0.92$; Control: $r(9)=-0.651$, $p=0.030$] and neutral [Autism: $r(10)=-0.0706$, $p=0.827$; Control: $r(9)=-0.626$, $p=0.0393$] face processing (Figure 6). Residual interhemispheric connectivity associations with response time to emotional faces were significant for controls, but not autism, in the inferior cuneus [Autism: $r(8)=0.568$, $p=0.0867$; Control: $r(10)=0.612$, $p=0.0344$] and significant for autism but not controls in the lateral lingual region [Autism: $r(8)=0.693$, $p=0.0263$; Control: $r(10)=0.206$, $p=0.521$] (Figure 7). We then examined residual interhemispheric connectivity measures with ADI-R scores. Autism residual interhemispheric connectivity associations with restricted and repetitive behavior (Figure 8) were significant in the cingulate at rest [Autism: $r(6)=-0.844$;

$p=0.00834$] and during emotional [Autism: $r(6)=-0.842$; $p=0.00867$] and neutral face processing [Autism: $r(6)=-0.874$; $p=0.00452$]. Additional associations of residual interhemispheric connectivity with behavior and ADI-R are reported in Supplementary Note 2. To directly examine the consequences of altered affective connectivity dynamics, behavioral and ADI-R associations of individual differences in residual interhemispheric connectivity are reported in Supplementary Note 3.

Corpus Callosum Density Associations with Individual Differences in Residual Interhemispheric Connectivity Dynamics

To determine whether corpus callosum (CC) contributed to dynamic functional interhemispheric connectivity alterations in autism, we examined the association between corpus callosum white matter probability density and individual differences in residual connectivity among interhemispheric affective face networks (Figure 9). After a Fisher transform, z-maps from the corpus callosum individual difference connectivity associations were entered into a Group x Affect x Cluster ANOVA with Group and Affect fixed and the Cluster factor randomized. The Affect factor in the individual difference analysis included emotion - rest, neutral - rest, and emotion - neutral connectivity differences (dynamics). Both GM and GA clusters from Table 1 were included in the Cluster factor. For the association of individual residual connectivity differences with CC density, the Group Main effect analysis indicated no group differences were present; however, one Group x Affect cluster survived multiple comparison correction in the CC body. This cluster of significance (Fig. 9 a) was used to extract z-scores of association for averaging and mean comparisons (Figure 9 b and c).

The callosum GA clusters indicated that CC density associations with individual residual interhemispheric connectivity differences were more negative in autism than controls for all affective network dynamics in insula, lateral lingual gyrus, and superior cuneus. In the callosum body, the dynamic neutral - rest and emotion - neutral connectivity capacity of middle cingulate was more negatively associated with callosum density in autism compared to controls. Autism associations were more positive than controls for emotion - neutral dynamics in the inferior cuneus and emotion - rest dynamics in the fusiform.

Conclusion

Given that functional connectivity patterns are task and seed region dependent, we tested whether the underconnectivity hypothesis (Just, Cherkassky et al. 2004), based in part on results from an fMRI sentence processing task, was generalizable and comprehensively described autism connectivity dynamics during facial emotion discrimination. In this fMRI study of affective face processing, we used a voxelwise whole brain interhemispheric partial correlation analysis that enabled us to quantify homotopic emotional and neutral affective face processing networks, homotopic resting networks, and their relationship with behavior and callosum density.

The hypothesis that functional networks are underconnected in autism (Just, Cherkassky et al. 2004) is controversial (Muller 2007). Both subcortico-cortical and cortico-cortical functional connectivity studies have reported overconnectivity in the autism connectivity phenotype (For review, see Muller 2007). In a complex information model of autism, functional overconnectivity is aligned with normal or enhanced autism performance during complex tasks (Williams, Goldstein et al. 2006). Overconnectivity may explain

autism abnormalities in sensory arousal and selective attention (Baron-Cohen and Belmonte 2005). A potential biological mechanism in autism is that an increased ratio of excitation to inhibition through respective glutamatergic and GABAergic signaling in sensory, social, and emotional systems could increase functional connectivity by reducing the signal to noise (SNR) ratio of information transfer (Rubenstein and Merzenich 2003). However, underconnectivity or overconnectivity may be associated with the reduced information content in low SNR neural circuitry either through reductions in signal or through increases in noise (Belmonte, Cook et al. 2004). With implications for both functional intrahemispheric and interhemispheric processing in autism (Muller 2007), a model of intrahemispheric connections based on enhanced white matter volume in autism (Herbert, Ziegler et al. 2004) concluded that anatomical overconnectivity of cortico-cortical connections due to increased numbers of smaller, less densely populated cortical minicolumns in autism (Casanova, Buxhoeveden et al. 2002) is associated with increased autism brain volume (Manuel F. Casanova 2004). Alternative network descriptions than overconnectivity and underconnectivity may be useful in describing the autism connectome.

The underconnectivity, overconnectivity, and reduced dynamics of affective face processing networks in autism we describe (Figure 5) negates underconnectivity as a generalizable framework and suggests that a more general “dynamic network framework” beyond overconnectivity or underconnectivity may be beneficial in understanding autism affective networks. Metastability (Bressler 2003; Fingelkurts and Fingelkurts 2004), the tendency of brain regions to be both segregated and integrated, is altered in autism based

on the patterns of both overconnectivity and underconnectivity in homotopic affective face networks (Figure 5). In autism, the asymmetry of homotopic temporal responses is reduced (more positively connected compared to controls) in nodes involved in visual and emotional face processing (insula, inferior cuneus, lateral lingual, and superior cuneus) and enhanced in a dorsal stream attentional circuit (inferior parietal). Homotopic connectivity at rest and during task in these regions was comparable in both autism and controls (Figure 5 b) and suggests that the metainstability produced by the overconnectivity and underconnectivity is an autism trait comparable across affective face networks. Autism metainstability may explain the variation in association magnitude between the response time to emotional faces and functional homotopic inferior cuneus and lateral lingual circuits processing emotional faces (Figure 7).

We identified nodes involved in the transition between brain states, behavioral catalysts (McIntosh 2004), that were impaired in autism based on the reduced interhemispheric connectivity capacity dynamics of autism. The interhemispheric functional connectivity analysis of homotopic brain regions that vary their connectivity with facial affect provided insight into the state of autism affective face networks and into the functional contributions of callosum density. In autism, fusiform, medial lingual, and cingulate regions did not significantly change the extent of their homotopic connectivity between affective networks but these behavioral catalysts had a greater dynamic capacity in the control group (Figure 5 d). The autism inability for connectivity to change according to facial affect in these regions may require a more generalized network framework to

characterize these patterns and suggests that autism has reduced network multivariability (Fingelkurts and Fingelkurts 2004), the ability to form multiple brain states.

Reduced network multivariability may explain group variations in the extent to which functional homotopic circuits are associated with behavior and callosum density (Figure 6, Figure 8, Figure 9). The reduced integrity of callosum structure in autism (Chung, Dalton et al. 2004) may require greater activity to produce meaningful dynamic connectivity across the corpus callosum (Chawla, Lumer et al. 1999; Chawla, Lumer et al. 2000). Across the GA connectivity regions, both the autism and control groups showed a significant positive association between activity and connectivity, but this association was weaker in autism [Autism: $r(106) = 0.205$; $t(106) = 2.15$; $p = 0.0335$; Control: $r(106) = 0.492$; $t(106) = 5.82$; $p = 0.000$] and significantly different between groups [$z\text{-difference}(\text{Autism-Control}) = -2.40$; $p = 0.0166$]. Positive and negative callosum body associations with functional connectivity dynamics indicate that homotopic circuits in affective networks may draw upon differential functions of the callosum body which include asymmetric interhemispheric inhibition, information transfer, and hemispheric recruitment (Stephan, Marshall et al. 2007). The white matter structure of the midsagittal callosum may contribute to functional alterations in homotopic connectivity, reduced multivariability in affective networks, and ultimately to phenotypic autism social behavior.

In summary, the interhemispheric functional connectivity patterns we describe indicate that affective face processing produces over and underconnected homotopic networks in

autism with altered metastable dynamics, reduced multivariability, and reduced dynamic connectivity capacity. Combined DTI-fMRI studies in autism will be necessary to further examine anatomical associations with interhemispheric functional connectivity, functional network dynamics, and behavior.

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Figures

Figure 1: Group Main Effect.

Group differences were identified in the insula and lateral lingual gyrus (top row), inferior parietal gyrus (middle), and the superior and inferior cuneus (bottom). Insula also appears in the middle row. Significant clusters were corrected for multiple comparisons ($p=0.05$). Statistical maps were overlaid on the mean T1 anatomical and echoplanar images.

Figure 2: Bar Plots of Group Main Effect Clusters.

(a) Cluster averages ($n=12$) for connectivity (z-scores) and (b) activation (area under the curve, AUC). Cluster labels refer to Group (A=autism, C=control), Affect (E= emotion, N=neutral, R = rest), and the Cluster number based on Table 1. For example, AE1 denotes autism during emotional face processing in the insula. Error bars are SEM.

Figure 3: Group x Affect Interaction Clusters.

Group x Affect interactions were identified in fusiform (top), medial lingual (middle), and middle cingulate (bottom). Significant clusters were corrected for multiple comparisons ($p=0.05$). Statistical maps were overlaid on the mean T1 anatomical and echoplanar images.

Figure 4: Group x Affect Cluster Bar Plots.

Cluster averages ($n=12$) for (a) connectivity (z-scores) and (b) activation (area under the curve). Cluster labels refer to Group (A=autism, C=control), Affect (E= emotion,

N=neutral, R = rest), and the Cluster number based on Table 1. For example, AE6 denotes autism during emotional face processing in the fusiform. Error bars are SEM.

Figure 5: Comparison of ANOVA Cluster Means.

Protected t-test (Bonferroni corrected, $p=0.05$) decompose the Group Main Effect (a and b) and Group x Affect clusters(c and d) according to differences in Group (a,c) and Affect (b,d). Horizontal lines indicate the critical threshold of significance ($p=0.05$, two-tailed). Cluster labels refer to Group (A=autism, C=control), Affect (E= emotion, N=neutral, R = rest), and the Cluster number based on Table 1. If two levels of one label factor are listed in succession, this denotes the second level was subtracted from the first level. For example, ACE1 denotes the contrast of autism - controls for insula during emotional face processing and AEN1 denotes the contrast of emotion - neutral face processing for the insula in autism.

Figure 6: Residual Connectivity Associations with Gaze Behavior in Autism and Controls.

Residual interhemispheric connectivity associations with the ratio of eye to face gaze were significantly associated in controls, but lacking in autism, in the insula during neutral face viewing [Autism: $r(10)=-0.0376$; $p=0.91$; Control: $r(9)=0.634$; $p=0.036$]; and in the fusiform during emotion [Autism: $r(10)=-0.0331$; $p=0.92$; Control: $r(9)=-0.651$; $p=0.030$] and neutral [Autism: $r(10)=-0.0706$; $p=0.827$; Control: $r(9)=-0.626$; $p=0.0393$] face processing. Regression line and 95% confidence bands are shown.

Figure 7: Residual Connectivity Associations with Response Time to Emotional Faces in Autism and Controls.

Residual interhemispheric connectivity associations with response time to emotional faces was significant for controls, but not autism, in the inferior cuneus [Autism: $r(8)=0.568$; $p=0.0867$; Control: $r(10)=0.612$; $p=0.0344$] and significant for autism but not controls in the lateral lingual region [Autism: $r(8)=0.693$; $p=0.0263$; Control: $r(10)=0.206$; $p=0.521$]. Regression line and 95% confidence bands are shown.

Figure 8: Autism Residual Connectivity Associations with Restricted and Repetitive Behavior.

Autism residual interhemispheric connectivity associations with restricted and repetitive behavior were significant in the cingulate during emotional [Autism: $r(6)=-0.842$; $p=0.00867$] and neutral face processing [Autism: $r(6)=-0.874$; $p=0.00452$] and at rest [Autism: $r(6)=-0.844$; $p=0.00834$]. Regression line and 95% confidence bands are shown.

Figure 9: Corpus Callosum Density Associations with Individual Connectivity Differences.

(a) Individual differences in connectivity dynamics (Affect factor: Emotion – Rest, Neutral – Rest, Emotion – Neutral) were associated with CC density and entered into a three-way Group x Affect x Cluster ANOVAs with Cluster randomized. Individual connectivity differences showed no group differences in associations with CC density. Significant Group x Affect clusters were identified in the callosum body for individual connectivity difference associations with CC density [$F(1,7)=11.25$]. Clusters were

corrected for multiple comparisons ($p=0.05$, corrected) and thresholded at $F(1,7)=5.59$ and $F(2,14)=3.74$. (b and c) Bar plots of significant callosum clusters. Group x Affect individual connectivity difference associations with CC density are (b) averaged and (c) tested for group differences using Fisher's protected t-test. Cluster labels refer to factors Group (A=autism, C=control), Affect (E= emotion, N=neutral, R = rest), and the Cluster number based on Table 1. If two levels of one label factor are listed in succession, this denotes the second level was subtracted from the first level. For example, AC.ER1 denotes the contrast of autism and controls in insula for the difference between emotion and rest connectivity.

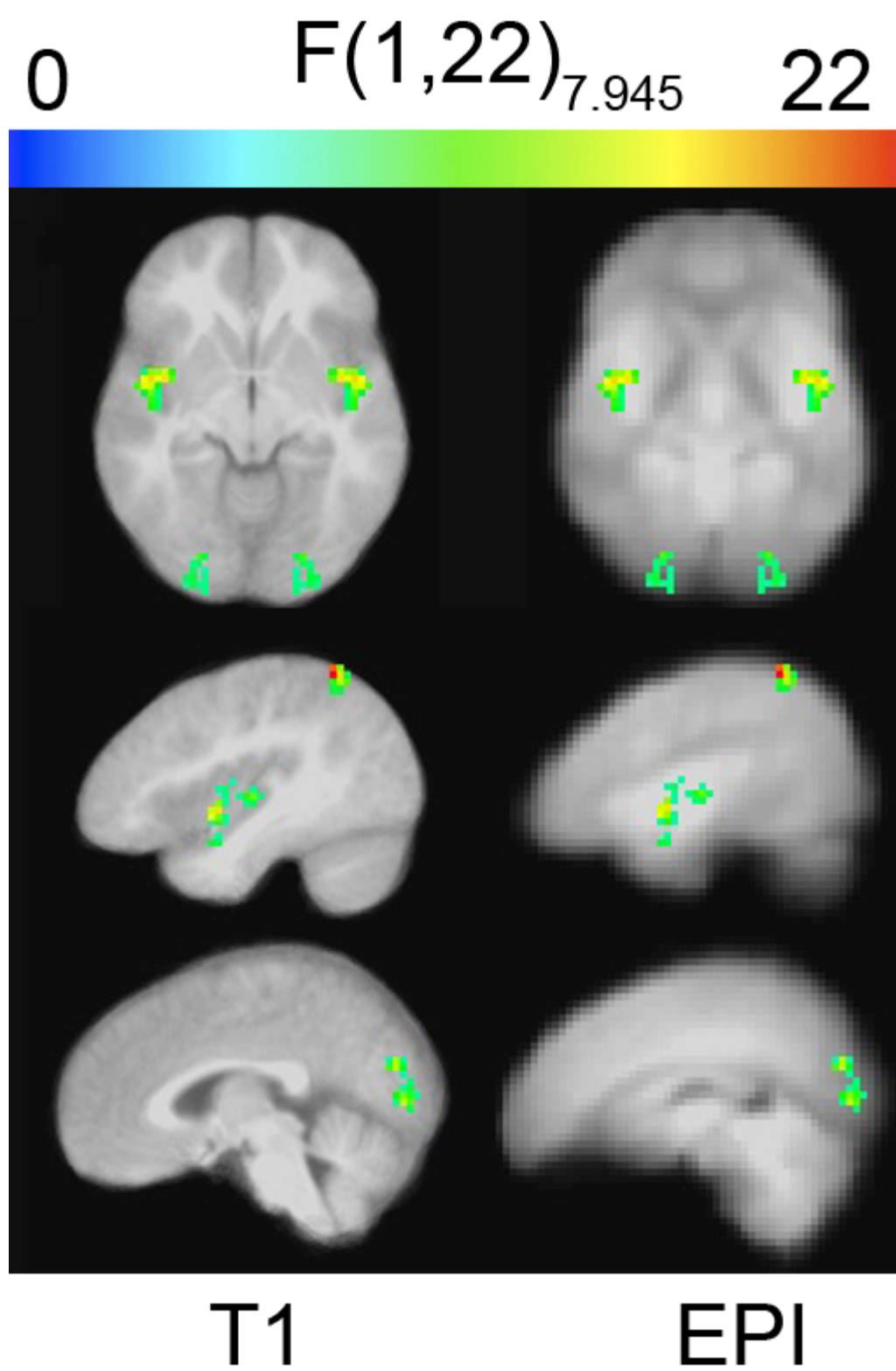


Figure 1

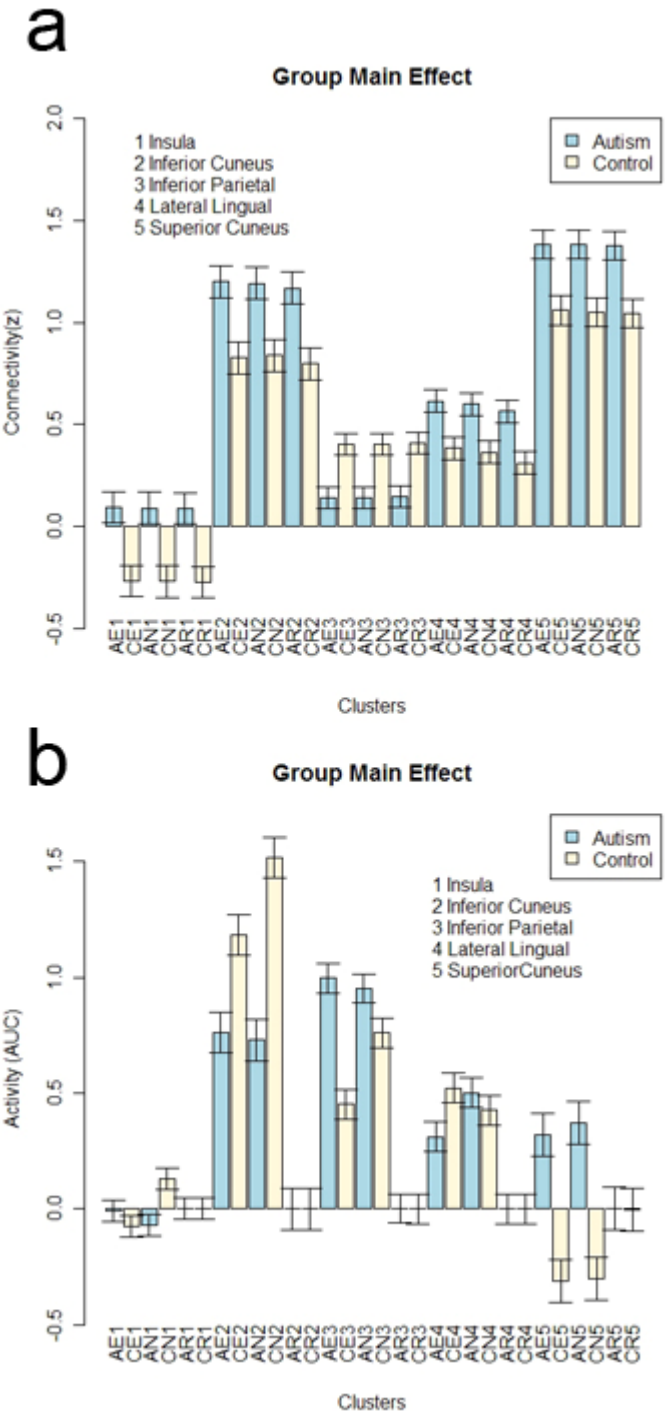


Figure 2

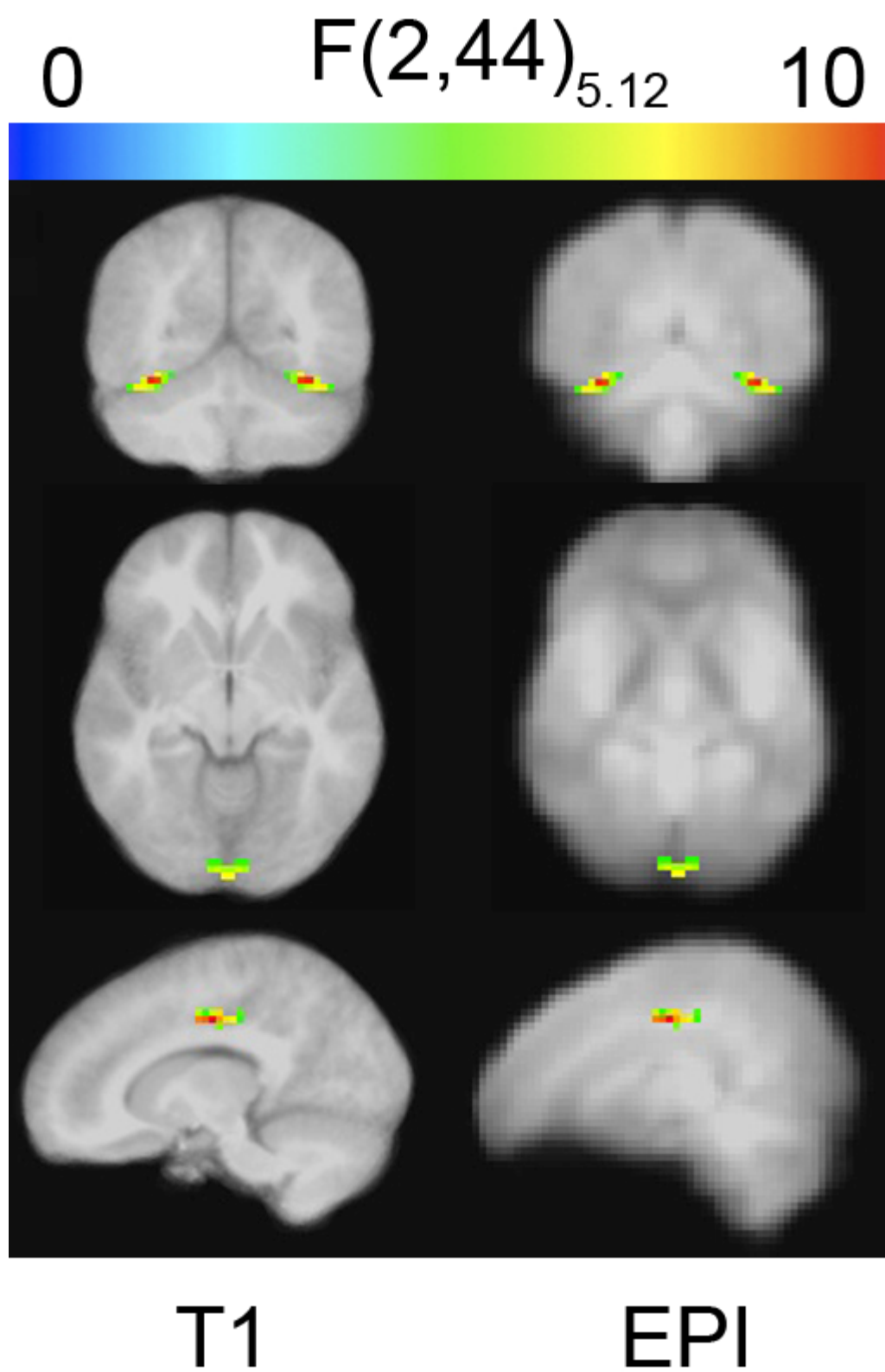


Figure 3

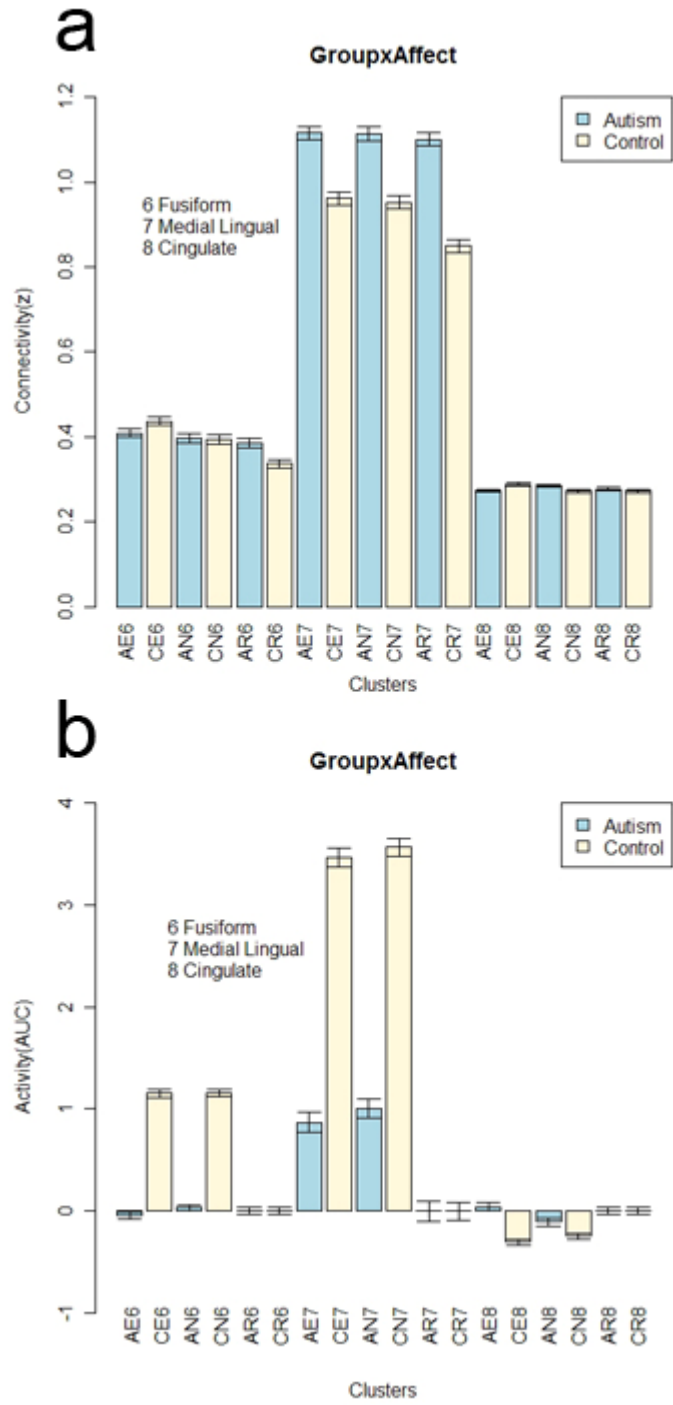


Figure 4

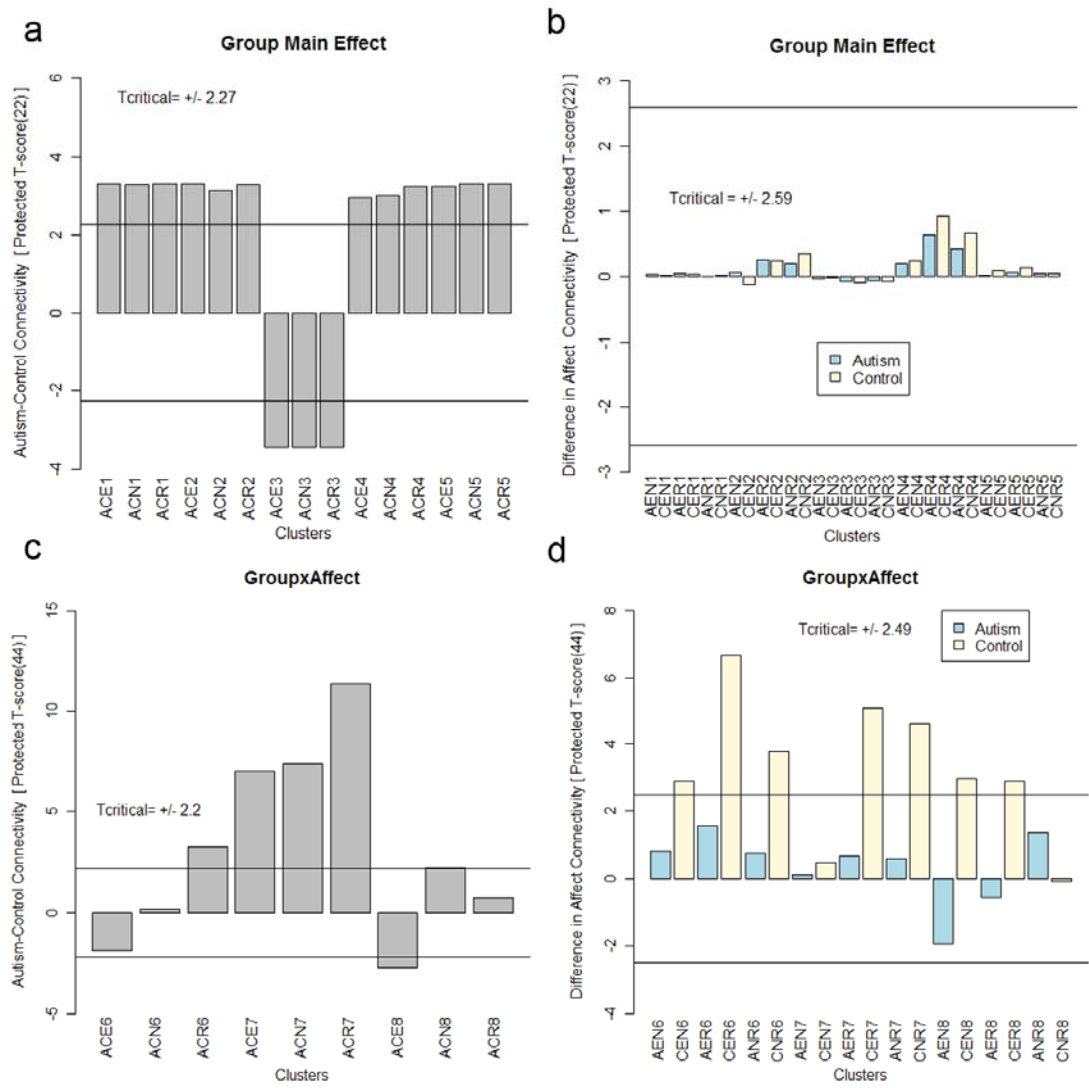


Figure 5

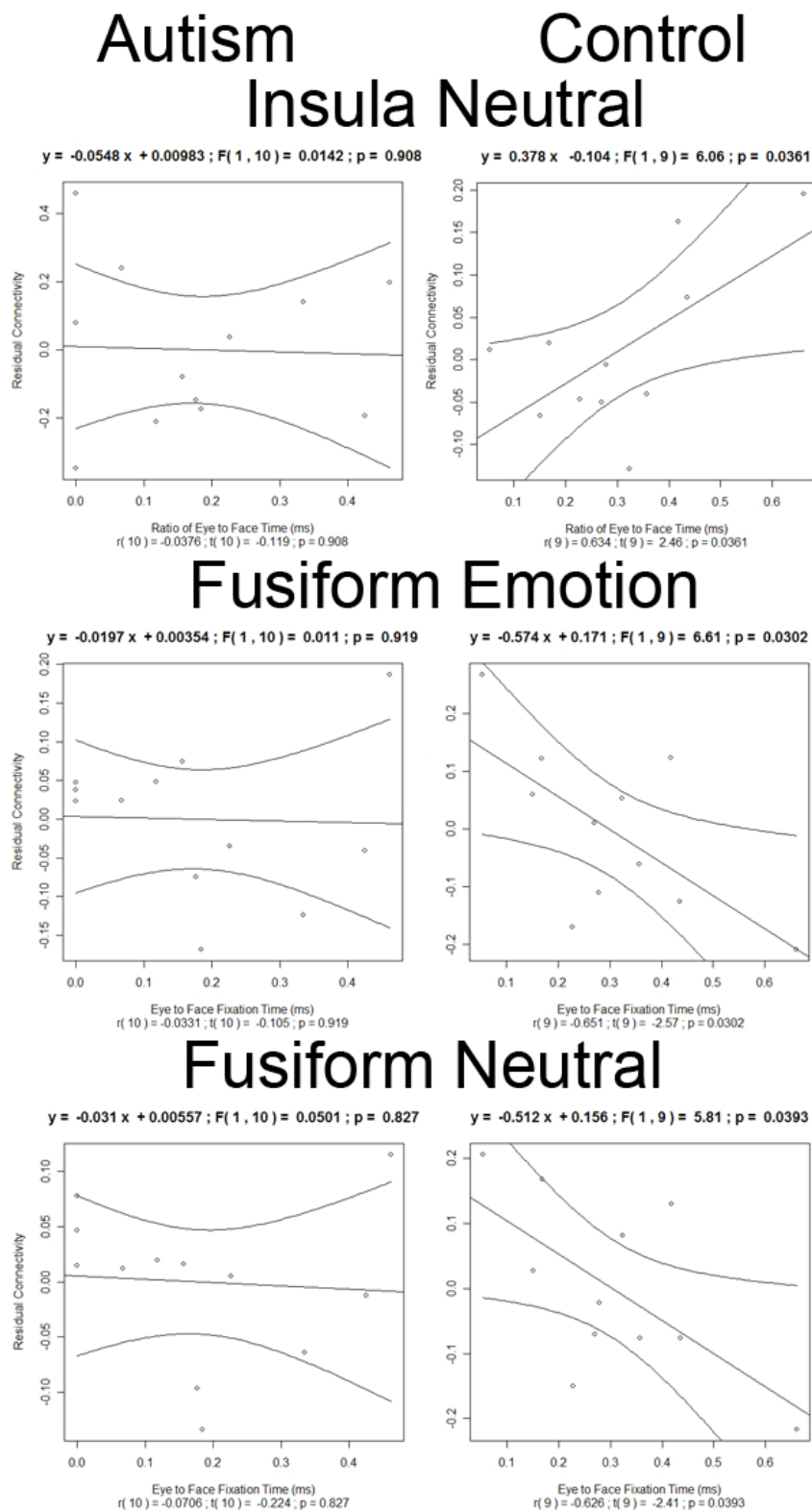
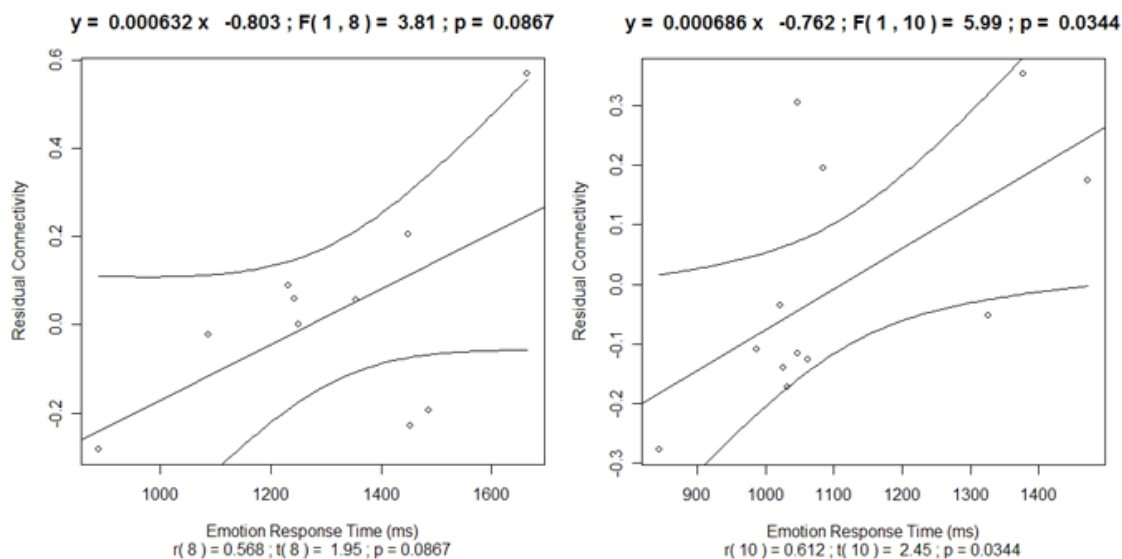


Figure 6

Autism Control

Inferior Cuneus Emotion



Lingual (Group Main) Emotion

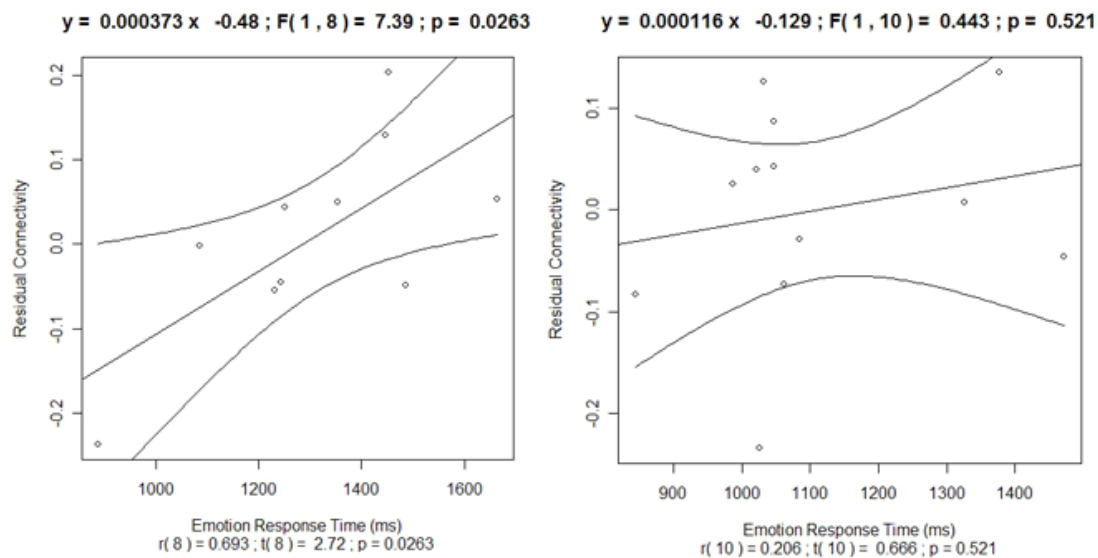
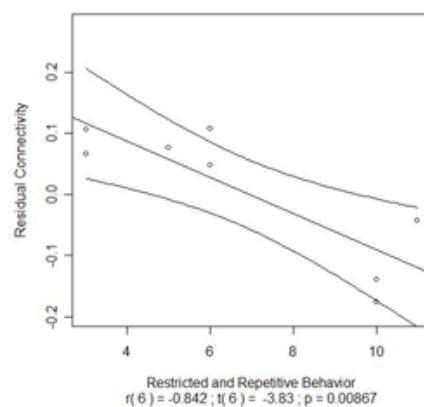


Figure 7

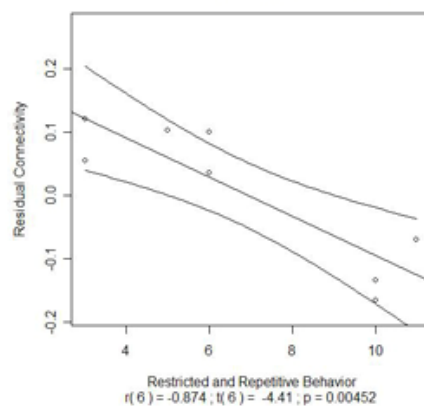
Cingulate Emotion

$$y = -0.0294x + 0.204; F(1, 6) = 14.7; p = 0.00867$$



Cingulate Neutral

$$y = -0.031x + 0.215; F(1, 6) = 19.5; p = 0.00452$$



Cingulate Rest

$$y = -0.0294x + 0.202; F(1, 6) = 14.9; p = 0.00834$$

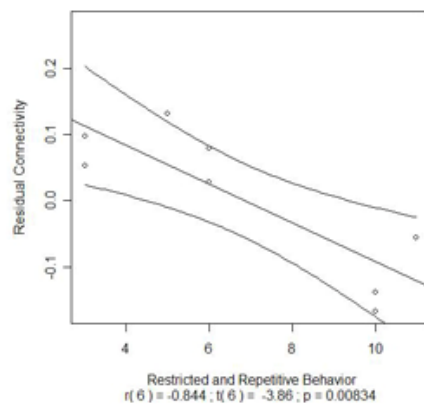


Figure 8

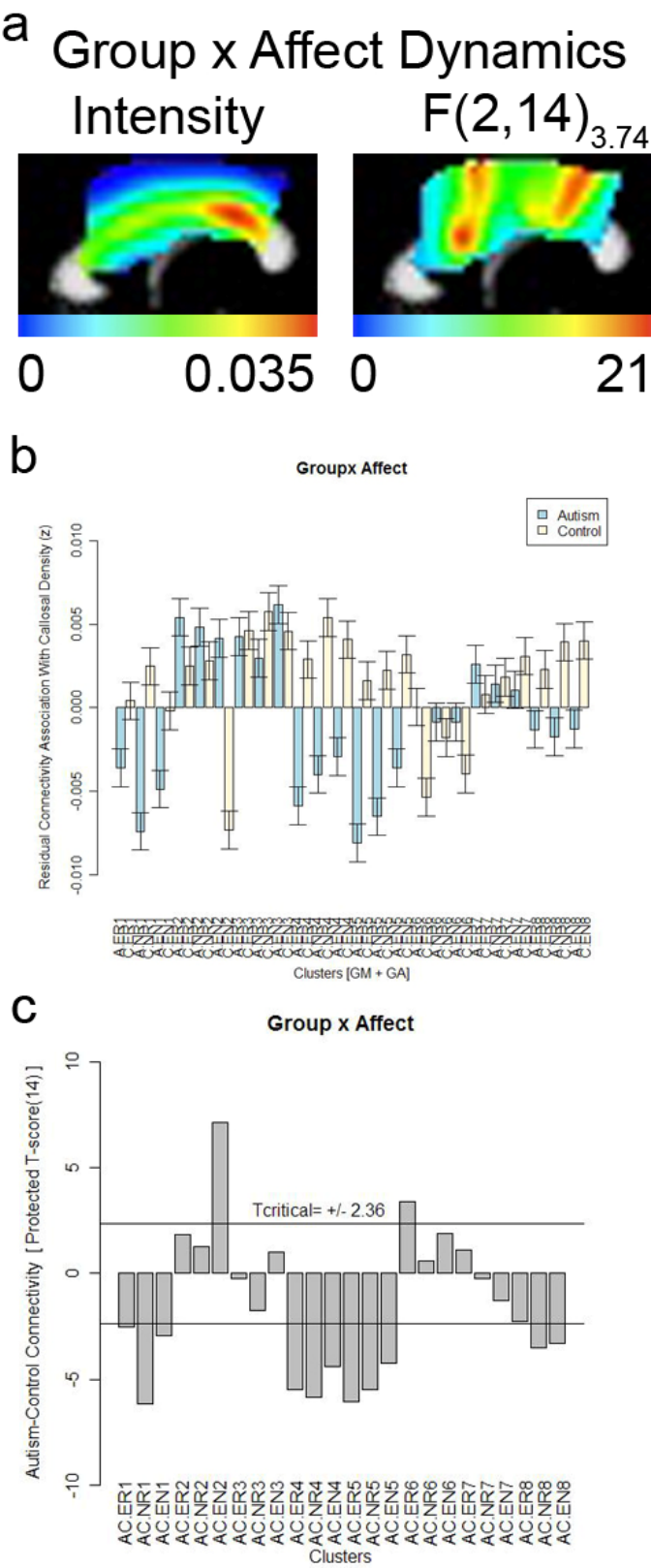


Figure 9

Tables

Table 1: Significance of Cluster Connectivity and Activity

Cluster	Group Main Effect	Volume	RPI Tailarach Coordinate	Connectivity	Activity
		(uL)	[x,y,z]		
				F(1,22)	F(1,22)
1	Insula	3726	-41.9, 7.4, 2.5	11.03*	0.71
	Inferior				
2	Cuneus	1404	0, 81.2, 12.1	10.60*	0.90
	Inferior				
3	Parietal	1134	-36.5, 50.9, 57.3	12.22*	0.62
	Lateral				
4	Lingual	675	-21.3, 84.7, 0.9	9.43*	0.35
	Superior				
5	Cuneus	594	0, 77.6, 23.2	11.18*	1.50
	Threshold				
	(p=0.05				
	corrected)			7.95	7.95
	GroupxAffect			F(2,44)	F(2,44)
6	Fusiform	810	-33.8, 56.8, -8.8	6.63*	3.98
	Medial				
7	Lingual	648	0, 83.8, -1.5	6.22*	5.16*
8	Cingulate	648	-9.1, 17.4, 39.4	7.14*	1.09
	Threshold				
	(p=0.05				
	corrected)			5.12	5.12

Supplementary Note 1

Relationship between Activation and Connectivity

Interhemispheric connectivity z scores were pooled across GM clusters for autism and controls and plotted against activity (Supplementary Figure 2). GM connectivity associations with activity were not significant in either group. When the interhemispheric connectivity z-scores were pooled across GA clusters for autism and controls and plotted against activity, the connectivity associations with activity were significant in both groups [Autism: $t(106)=2.15$; $p=0.0335$; Control: $t(106)=5.82$; $p=0.000$] (Supplementary Figure 2, bottom row). To examine clusterwise associations between connectivity and activity, GM and GA cluster z-scores and area under the curve measures were entered into a correlation matrix (Supplementary Figure 3). Correlations were converted into z scores for the autism and control group and subsequently tested for group differences. No associations were significant ($FDR \leq 0.05$) in either group for GM or GA clusters after FDR adjusting p values; and, no group differences were significant ($FDR \leq 0.05$) after FDR adjusting p values for multiple comparisons. Although no connectivity associations with activation were significant, the nondiagonal elements suggested that the association between interhemispheric connectivity and activation from nonhomologous elements was more varied in the GM matrix compared to the GA matrix. In addition, the diagonal of the correlation matrices, in which connectivity and activation associations were between homologous affect and cluster regions, indicated that removing the activation effect from connectivity would improve the specificity of connectivity measures. For this reason, residual connectivity was used in subsequent analyses.

Supplementary Note 2

Residual Interhemispheric Connectivity Associations with Behavior and ADI-R

Residual interhemispheric connectivity associations with response time to affect by face orientation were significant in the autism group but not the control group (Supplementary Figure 4). For autism, residual interhemispheric connectivity of the superior cuneus during emotional face processing was significantly associated with the response time to emotional straight faces [Autism: $r(8)=0.668$; $p=0.0346$; Control: $r(10)=0.39$; $p=0.21$]; inferior cuneus during neutral face processing was significantly associated with the response time to neutral side faces [Autism: $r(8)=0.636$; $p=0.048$; Control: $r(10)=0.123$; $p=0.702$] and response time to side faces [Autism: $r(8)=0.763$; $p=0.0102$; Control: $r(10)=0.246$; $p=0.44$]; the lateral lingual gyrus (GM cluster) during emotion face processing was significantly associated with the response time to emotional straight faces [Autism: $r(8)=0.787$; $p=0.00688$; Control: $r(10)=0.289$; $p=0.363$]; and the fusiform during neutral face processing was significantly associated with response time to side faces [Autism: $r(8)=-0.643$; $p=0.0451$; Control: $r(10)=-0.281$; $p=0.377$].

We then examined residual interhemispheric connectivity measures with ADI-R scores. Autism residual interhemispheric connectivity associations with nonverbal IQ (Supplementary Figure 5) were significant in the insula during emotional face processing [Autism: $r(5)=-0.805$; $p=0.029$] and at rest [Autism: $r(5)=-0.802$; $p=0.0301$] and in the cingulate during emotional face processing [Autism: $r(5)=-0.774$; $p=0.0412$] and at rest

[Autism: $r(5)=-0.784$; $p=0.0368$]. Autism residual interhemispheric connectivity associations with verbal behavior and full scale IQ (FSIQ) were examined (Supplementary Figure 6). In autism, during emotional face processing residual interhemispheric fusiform connectivity was significantly associated with verbal communication scores [Autism: $r(5)=0.814$; $p=0.0258$]; during neutral face processing insula was significantly associated with the number of verbal items correct [Autism: $r(6)=0.74$; $p=0.0358$]; and, at rest, cingulate was significantly associated with FSIQ [Autism: $r(5)=-0.793$; $p=0.0335$].

Supplementary Note 3

Individual Residual Interhemispheric Connectivity Difference Associations with Behavior and ADI-R

Based on the reduced dynamic interhemispheric connectivity capacity of autism in GA clusters (Figure 5d), we examined the association of individual connectivity differences of GA clusters with behavior (Supplementary Figure 7). Significant autism associations were detected between fusiform neutral-rest dynamics and mean face fixation time per trial [Autism: $r(10)=-0.697$; $p=0.0118$; Control: $r(9)=0.0931$; $p=0.785$]; cingulate emotion-rest dynamics and the response time to emotional straight faces [Autism: $r(8)=-0.723$; $p=0.0182$; Control: $r(10)=0.0511$; $p=0.875$]; cingulate emotion-neutral dynamics and the difference in response time to emotional and neutral faces [Autism: $r(8)=-0.71$; $p=0.0215$; Control: $r(10)=0.258$; $p=0.419$] and fusiform emotion-neutral dynamics and age [Autism: $r(10)=0.584$; $p=0.046$; Control: $r(10)=0.104$; $p=0.748$]. Significant control

associations were detected between medial lingual emotion–rest dynamics and the number of facial affects correctly identified [Autism: $r(10)=0.424$; $p=0.17$; Control: $r(10)=-0.628$; $p=0.0287$]. Individual connectivity differences were examined for their association with verbal and nonverbal ADI-R measures and were significant in autism between the fusiform emotion-rest dynamics and nonverbal IQ [Autism: $r(5)=0.781$; $p=0.0382$] and between cingulate neutral-rest dynamics and the number of verbal items correct [Autism: $r(6)=-0.731$; $p=0.0395$] (Supplementary Figure 8).

Supplementary Figures

Supplementary Figure 1: Design Matrix.

From left to right: Lane 0-3 Zero through third order polynomials, Lane 4-10 Time lagged emotional face events, Lane 5-11 Lagged neutral face event, Lane 6 Whole brain average time series, Lane 7 White matter average time series, Lane 8 Ventricle average time series, Lane 9 Rotation about the I-S axis (roll), Lane 10 Rotation about the R-L axis (pitch), Lane 11 Rotation about the A-P axis (yaw), Lane 12 Displacement in the superior direction, Lane 13 Displacement in the left direction, Lane 14 Displacement in the posterior direction.

Supplementary Figure 2: Connectivity Associations with Activity Pooled across Clusters.

Connectivity associations with activity pooled across Group Main Effect (GM) or Group x Affect (GA) clusters indicate a lack of association in GM clusters (top) and a positive association across GA clusters (bottom) in both the autism and control groups.

Supplementary Figure 3: Correlation Matrix of Connectivity Associations with Activity.

Group Main Effect (a-d) and Group x Affect (e-h) clusters of connectivity were not significantly associated with either group at $FDR < 0.05$ (a,b,e,f) and were not significantly different between groups (c,g,d,h) at $FDR < 0.05$. However, differences in patterns of these associations across clusters warranted removal of activity from connectivity measures.

Supplementary Figure 4: Autism and Control Residual Connectivity Associations with Response Time to Affect by Face Orientation.

For autism, residual interhemispheric connectivity of the superior cuneus during emotional face processing was significantly associated with the response time to emotional straight faces [Autism: $r(8)=0.668$; $p=0.0346$; Control: $r(10)=0.39$; $p=0.21$]; inferior cuneus during neutral face processing was significantly associated with the response time to neutral side faces [Autism: $r(8)=0.636$; $p=0.048$; Control: $r(10)=0.123$; $p=0.702$] and response time to side faces [Autism: $r(8)=0.763$; $p=0.0102$; Control: $r(10)=0.246$; $p=0.44$]; the lateral lingual gyrus (GM cluster) during emotion face processing was significantly associated with the response time to emotional straight faces [Autism: $r(8)=0.787$; $p=0.00688$; Control: $r(10)=0.289$; $p=0.363$]; and the fusiform during neutral face processing was significantly associated with response time to side faces [Autism: $r(8)=-0.643$; $p=0.0451$; Control: $r(10)=-0.281$; $p=0.377$].

Supplementary Figure 5: Autism Residual Connectivity Associations with Non-verbal IQ.

Autism residual interhemispheric connectivity associations with nonverbal IQ were significant in the insula during emotional face processing [Autism: $r(5)=-0.805$; $p=0.029$] and at rest [Autism: $r(5)=-0.802$; $p=0.0301$] and in the cingulate during emotional face processing [Autism: $r(5)=-0.774$; $p=0.0412$] and at rest [Autism: $r(5)=-0.784$; $p=0.0368$].

Supplementary Figure 6: Autism Residual Connectivity Associations with Verbal Behavior and Full Scale IQ (FSIQ).

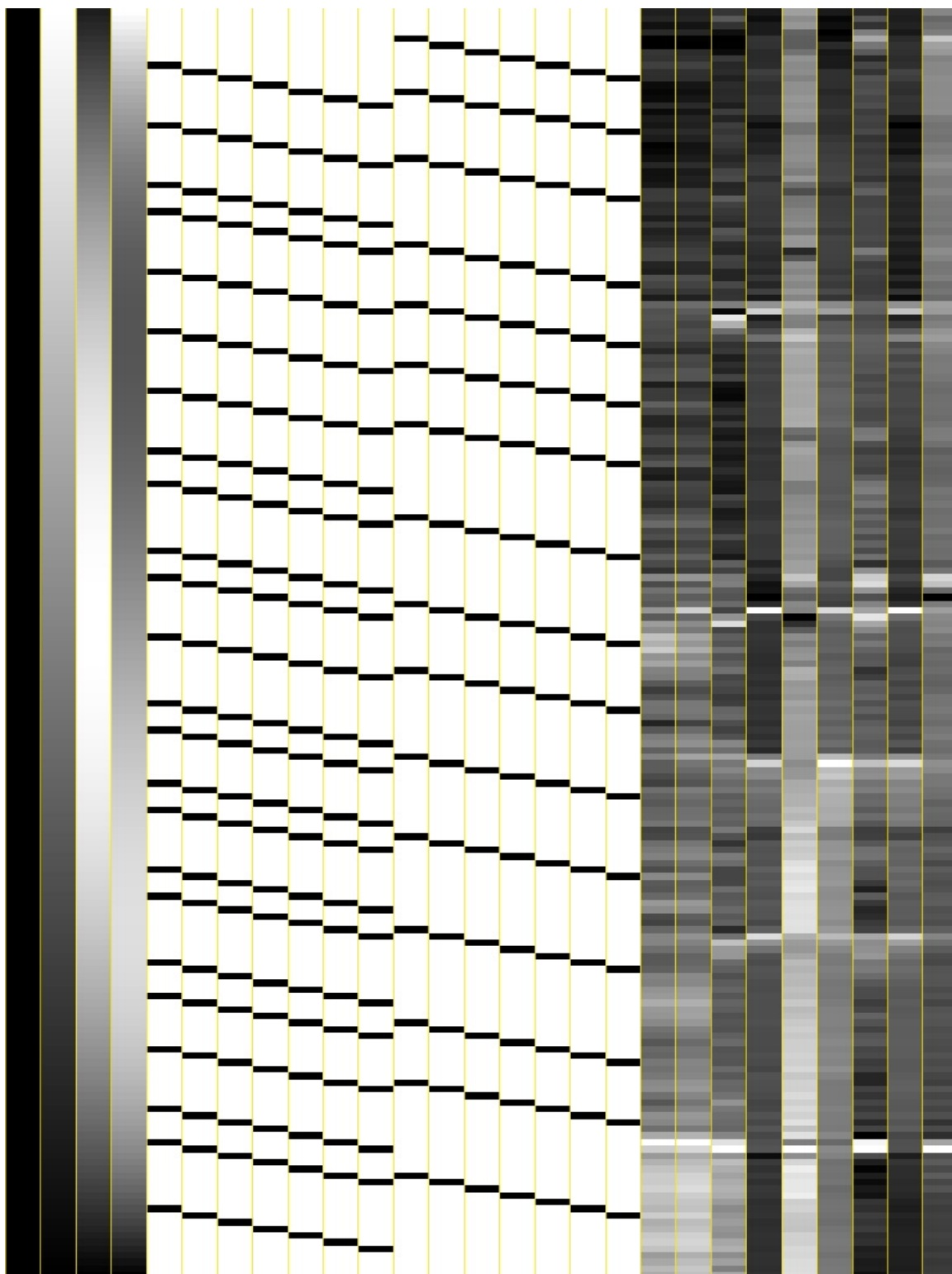
In autism, during emotional face processing residual interhemispheric fusiform connectivity was significantly associated with verbal communication scores [Autism: $r(5)=0.814$; $p=0.0258$]; during neutral face processing insula was significantly associated with the number of verbal items correct [Autism: $r(6)=0.74$; $p=0.0358$]; and, at rest, cingulate was significantly associated with FSIQ [Autism: $r(5)=-0.793$; $p=0.0335$].

Supplementary Figure 7: Association of Autism and Control Behavior with Individual Connectivity Differences for Group x Affect Clusters.

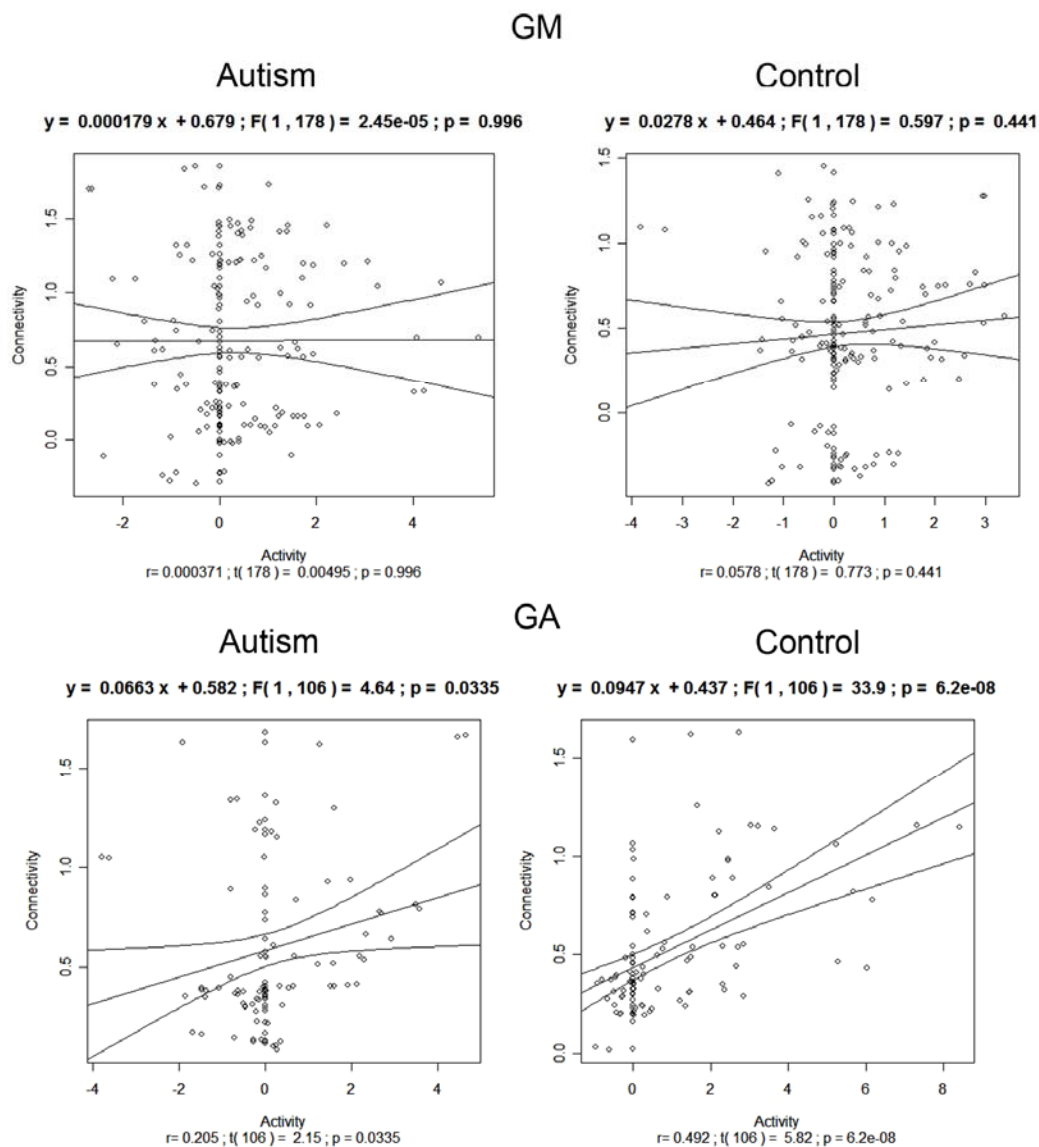
Significant autism associations were detected between fusiform neutral-rest dynamics and mean face fixation time per trial [Autism: $r(10)=-0.697$; $p=0.0118$; Control: $r(9)=0.0931$; $p=0.785$]; cingulate emotion-rest dynamics and the response time to emotional straight faces [Autism: $r(8)=-0.723$; $p=0.0182$; Control: $r(10)=0.0511$; $p=0.875$]; cingulate emotion-neutral dynamics and the difference in response time to emotional and neutral faces [Autism: $r(8)=-0.71$; $p=0.0215$; Control: $r(10)=0.258$; $p=0.419$] and fusiform emotion-neutral dynamics and age [Autism: $r(10)=0.584$; $p=0.046$; Control: $r(10)=0.104$; $p=0.748$]. Significant control associations were detected between medial lingual emotion–rest dynamics and the number of facial affects correctly identified [Autism: $r(10)=0.424$; $p=0.17$; Control: $r(10)=-0.628$; $p=0.0287$].

Supplementary Figure 8: Autism Verbal and Non-Verbal Associations with Individual Connectivity Differences.

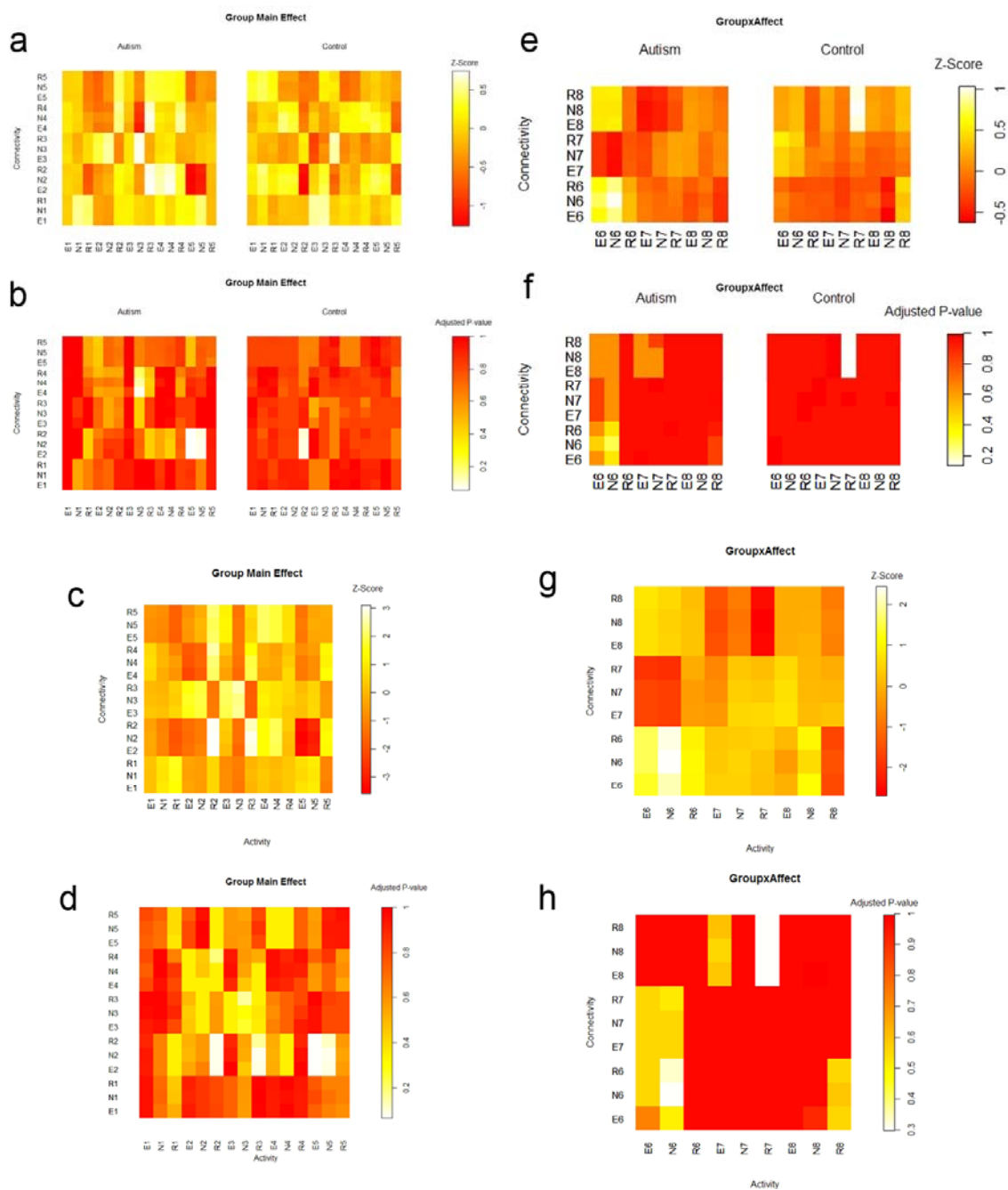
The association of individual connectivity differences with verbal and nonverbal ADI-R measures were significant in autism between the fusiform emotion-rest dynamics and nonverbal IQ [Autism: $r(5)=0.781$; $p=0.0382$] and between cingulate neutral-rest dynamics and the number of verbal items correct [Autism: $r(6)=-0.731$; $p=0.0395$].



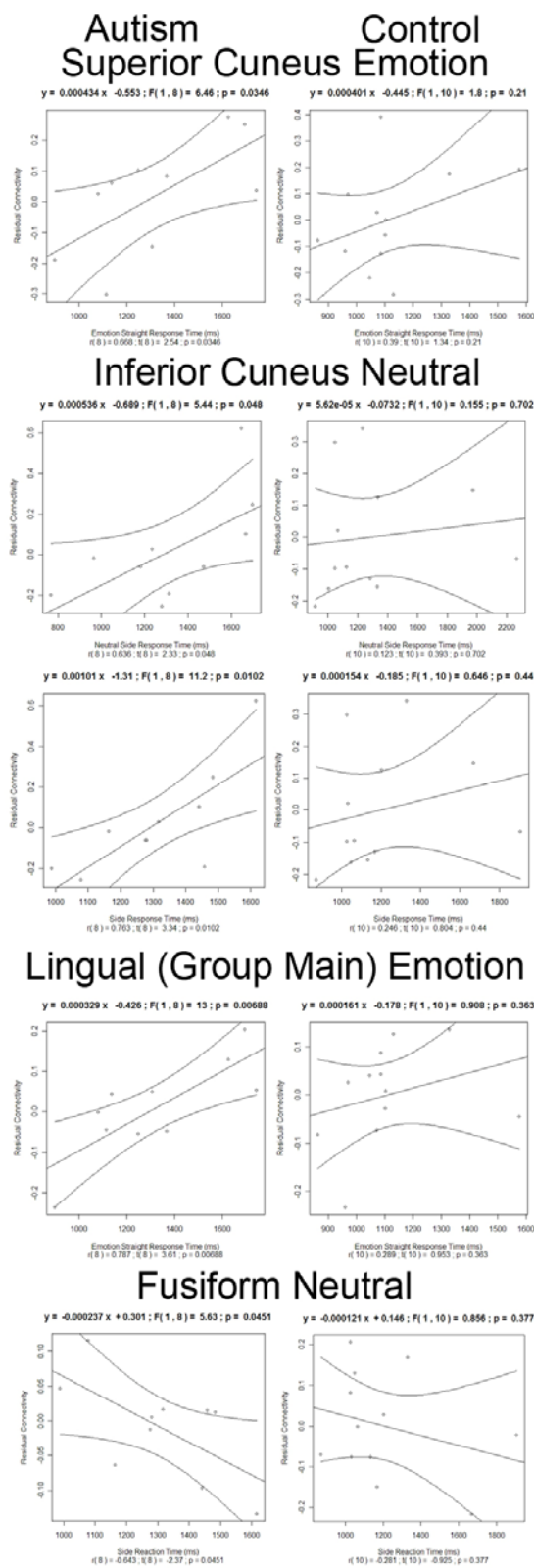
Supplementary Figure 1



Supplementary Figure 2

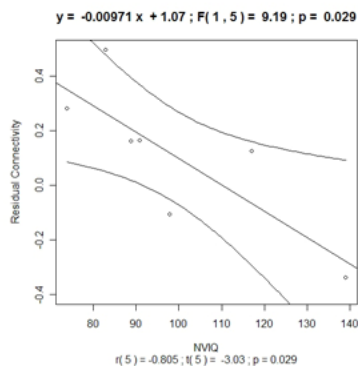


Supplementary Figure 3

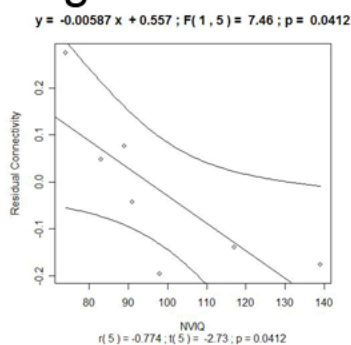


Supplementary Figure 4

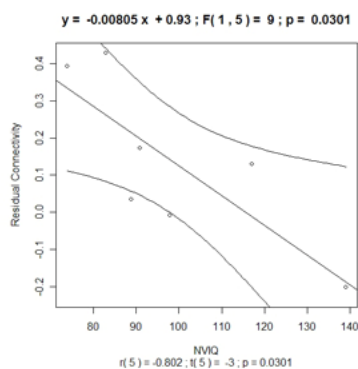
Insula Emotion



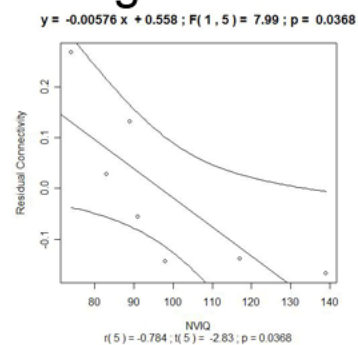
Cingulate Emotion



Insula Rest

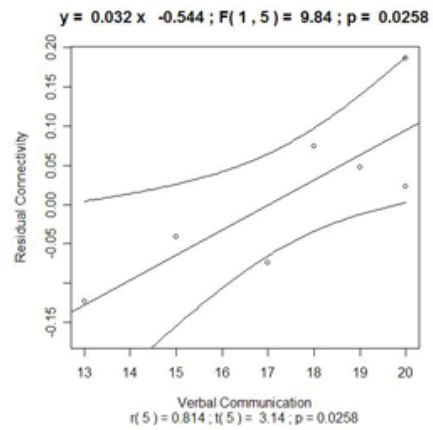


Cingulate Rest

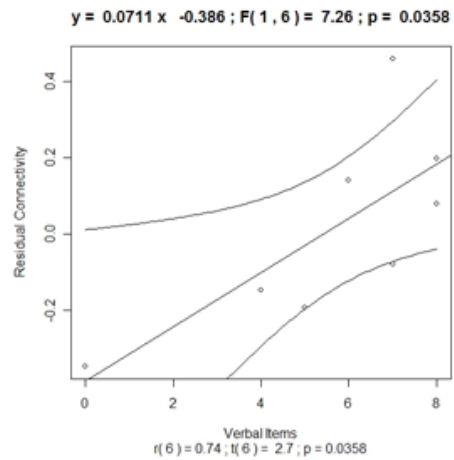


Supplementary Figure 5

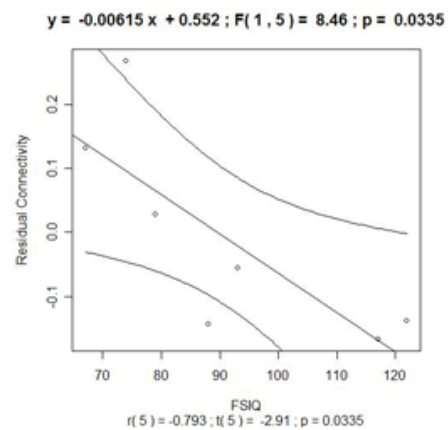
Fusiform Emotion



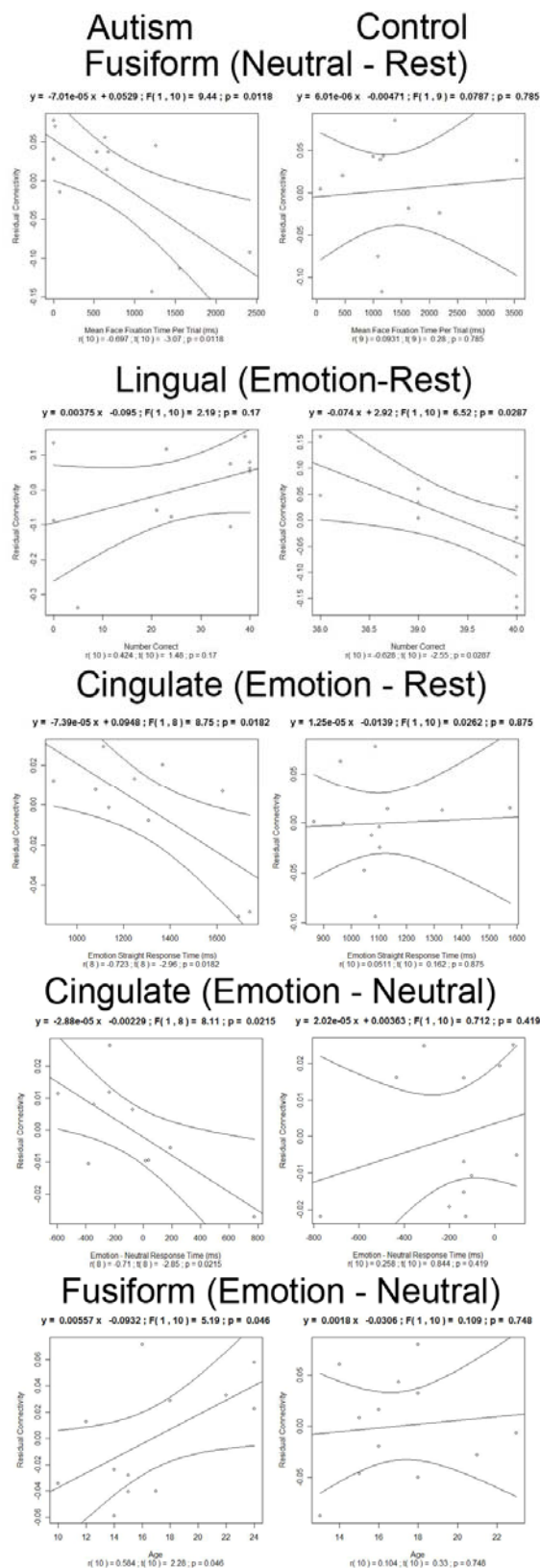
Insula Neutral



Cingulate Rest

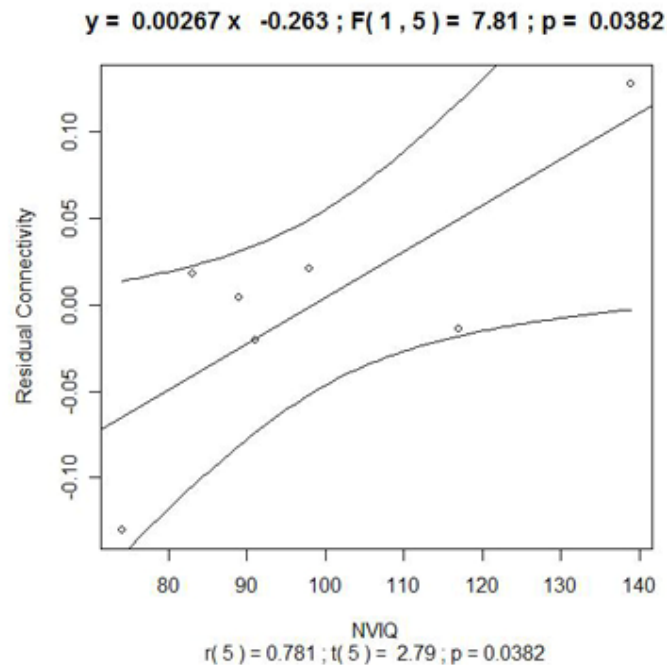


Supplementary Figure 6

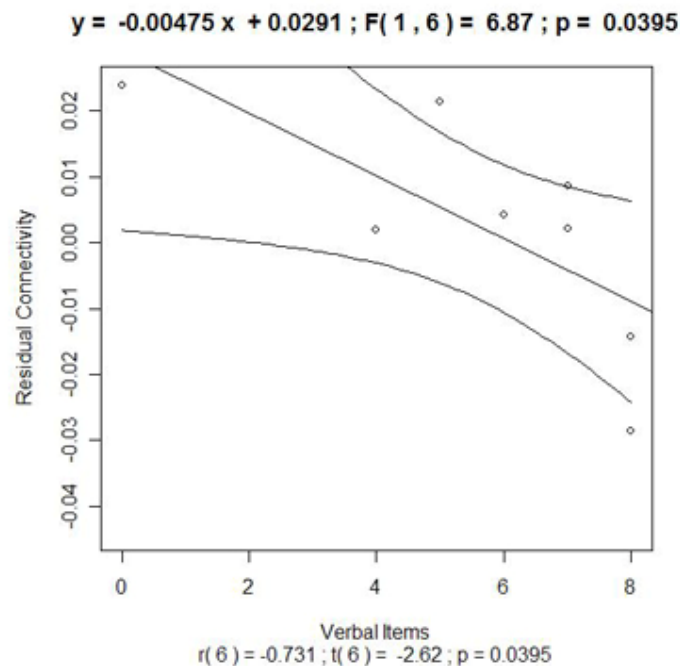


Supplementary Figure 7

Fusiform (Emotion - Rest)



Cingulate (Neutral-Rest)



Supplementary Figure 8

Chapter 5:

Pattern Visualization of Interhemispheric Affective Face Networks in Autism

Pattern Visualization of Interhemispheric Affective Face Networks in Autism

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Running Head: Interhemispheric Autism Patterns

Abstract

Autism is a disorder of neurodevelopment in which social behavior is impaired. Aberrant interhemispheric patterns of homotopic functional connectivity may contribute to the autism phenotype. However, the high dimensionality of complex functional interactions in brain makes it difficult to visualize interhemispheric network patterns. To overcome this, we applied agglomerative hierarchical analysis, undirected graph theory, and multidimensional scaling approaches to examine autism interhemispheric functional patterns captured with functional MRI during an emotional face processing task. These statistical neuroinformatic techniques provided a framework that reduced the dimensionality of functional connectivity data and visually mapped functional motifs in affective networks to distinguish autism from controls. Patterns of functional interhemispheric connectivity provided by neuroinformatic techniques may serve as a useful biomarker for the autism phenotype and are consistent with an integration model of altered metastability in autism.

Author Summary

Autism is a neurodevelopmental disorder with a deficiency in several domains including social behavior. With implications for understanding autism behavior, the effect of altered development on communication within the brain is challenging to visualize given the high dimensionality of data collected with functional MRI, a non-invasive imaging technique that records brain function as subjects perform a task. We hypothesized that the pattern of interhemispheric functional circuits would provide insight into autism brain communication based on the differences we identified in autism functional connectivity, a measure of brain communication magnitude. Profiles of functional connectivity patterns may provide further insight into the etiology of neurodevelopmental disorders by identifying underlying structure among dysfunctionally connected circuits. Here we characterized the pattern of functional connectivity differences in autism using freely available, open-source implementations of three neuroinformatic techniques including agglomerative hierarchical analysis, undirected graph theory, and multidimensional scaling. Based on their functional connectivity patterns, individuals with autism have a unique profile of interhemispheric brain communication that is visually distinct from controls. Although most commonly used to understand gene expression in microarray analysis, these neuroinformatic techniques also have utility in exploring biomarkers of communication within the brain by mapping underlying patterns among functionally connected circuits.

Introduction

The human brain connectome is complex [1]. Neuronal projections and their functional dynamics comprise system level structural and functional networks. In vivo anatomical and functional investigations of brain utilize neuroimaging to clarify how multiple brain states can emerge from a network topology with limited functional and anatomical resources [2]. Neuroimaging studies using functional MRI (fMRI) to examine brain function produce large datasets of high dimensionality that require advanced analysis techniques to characterize functional activation patterns [3,4]. Functional connectivity is an analysis technique that can be applied to fMRI data to examine the inter-regional association of brain function [5]. Neuroinformatic analysis techniques are emerging that enable pattern visualization of networks to summarize connectome topology.

Autism is a neurodevelopmental disorder with a triad of deficits in the communication, social interaction, and behavioral domains [6]. Neuronal alterations in autism contribute to system level modifications in structure and function [7,8]. In support of this, fMRI functional connectivity studies indicate that the functional network topology of autism is disrupted [For review, see 9]. Pattern characterization of interhemispheric functional autism networks would be helpful in characterizing underlying structure in differentially connected functional circuitry. However, this was challenging given the high dimensionality of fMRI data and the number of brain regions and networks under investigation.

To summarize the patterns of interhemispheric connectivity differences that characterize autism, we turned to agglomerative hierarchical cluster analysis (AHCA), undirected correlation graphs, and classical multidimensional scaling (MDS) analysis. The approaches we describe are freely available in the R software package [10,11].

Results

Agglomerative hierarchical clustering was used to examine the grouping of Group Main effect (GM) and Group x Affect (GA) interaction clusters over each affective face network by plotting the Euclidean distances between vectors of subject z-scores as dendrograms. The z-map dendrogram correctly classified 75% of autism subjects into one limb of the inverted dendrogram trees (Figure 1a). The dendrogram of pooled autism and control GM and GA z-scores identified unsupervised classification differences between autism and controls (Figure 1b). The emotional, neutral, and resting z-scores within a cluster were agglomerated except for the inferior cuneus at rest in controls. These agglomerations were the only significant classifications. Edge 45 agglomerated the superior cuneus, inferior cuneus, and lateral lingual regions for both autism and controls; however, Edges 37 and 39 showed differential autism and control classification of the lateral lingual and inferior cuneus regions. Edge 46 indicated that the insula at Edge 44 is distant from Edge 42 which agglomerated the lateral lingual, inferior parietal, and fusiform region for both autism and controls. The inferior parietal region for controls was agglomerated into Edge 42 but into Edge 44 in autism.

The autism and control z-maps and dendrograms of pooled GA and GM identified unsupervised classification differences among autism and controls (Figure 2). Edge 21

agglomerated the superior cuneus, medial lingual, and inferior cuneus regions in both autism and controls but the classification of inferior cuneus and medial lingual regions differed between autism and controls with medial lingual being closer to the superior cuneus (less distant) in autism. Edge 22 agglomerated the insula, inferior parietal, lateral lingual, cingulate, and fusiform regions in autism but did not include the insula in controls. The insula and inferior parietal regions were agglomerated in autism (Edge 19), but inferior parietal was agglomerated with fusiform in controls (Edge 18). In autism, fusiform was agglomerated with cingulate (Edge 17) and lateral lingual regions (Edge 20), but lateral lingual regions were agglomerated with cingulate (Edge 16) and more distantly with fusiform (Edge 19) in controls.

The z-maps and dendrograms of GM and GA connectivity for autism and control identified unsupervised classification differences between autism and controls (Fig. 3). For GM clusters, both the autism and control group agglomerated the superior cuneus and inferior cuneus (Autism: Edge 11; Control: Edge 12). However, Edge 13 significantly agglomerated lateral lingual, insula, and inferior parietal in autism but the agglomeration of insula with the inferior parietal and lateral lingual regions was not significant in controls. The lateral lingual region in Edge 13 was classified more closely with the superior cuneus and inferior cuneus in autism but the insula was more closely classified to inferior and superior cuneus in controls. The classification of GA clusters was comparable across affective networks, although resting networks were more distant from the emotional networks in controls.

Next we examined the thresholded ($p=0.05$, uncorrected) undirected graphs of integrative interhemispheric connectivity across cluster and affect parameter space (Fig. 4). For GM clusters, controls showed segregation of insula, inferior parietal, and lateral lingual regions but only the insula was segregated in autism. For GA clusters, the insula was associated with the medial lingual region during emotional face viewing in autism, but not controls. When GM and GA clusters were pooled, only the insula and cingulate regions were segregated in autism; but, in addition to these, lateral lingual, fusiform, and cingulate regions were segregated in controls. Within-cluster motif associations of the emotional, neutral, and resting networks were present for all brain regions in the GM, GA, and pooled GM and GA undirected graphs.

Finally we applied classical MDS to map the relationship among homotopic nodes (Figures 5 and 6). For the GM and GA result pooled across autism and controls (Figure 5), the positive and negative axis of coordinate 1 respectively corresponded to homotopic nodes with higher level affective processing areas (insula, inferior parietal, fusiform, cingulate) and medial visual processing (inferior cuneus, superior cuneus, medial lingual). Coordinate 2 can distinguish groups. Seventy-five percent of the autism and control nodes were identified by the respective positive and negative values of coordinate 2. When pooled GM and GA nodes were plotted separately for autism (Figure 6a) and controls (Figure 6b), the sign of coordinate 1 continued to distinguish affective from medial visual areas. However, compared to autism, the cingulate, fusiform, and inferior parietal regions were more centrally located in controls relative to both the insula and visual processing areas. The affective component in controls had greater spacing in the

inferior cuneus, lateral lingual, and fusiform regions along coordinate 2. In addition, the signs of medial lingual and superior cuneus visual processing nodes were reversed along component 2 in autism. Lastly, the relative position of affective processing nodes (cingulate, fusiform, inferior parietal, insula) among each other and relative to visual processing areas was altered in autism. The patterns among GM nodes for autism (Figure 6c) and controls (Figure 6d) were distinct. Positive and negative coordinate 1 values continued to respectively distinguish visual processing (inferior and superior cuneus) and affective processing (insula and inferior parietal). However, the position of insula and inferior cuneus were similar in autism along coordinate 1 but different in controls. Along coordinate 2, the spatial patterns were different between autism and controls and the affect factor for the inferior cuneus was more easily distinguished in controls compared to autism. For the GA clusters, the autism (Figure 6e) and control (Figure 6f) patterns were spatially similar along coordinate 1, but the fusiform and cingulate were inverted along coordinate 2.

Discussion

Neuroinformatic techniques that enable visualization of brain network patterns reduced the high dimensionality of fMRI data and complex functional interactions in our study. The grouping patterns of interhemispheric associations using agglomerative hierarchical cluster analysis, undirected correlation graphs, and classical MDS patterns varied between autism and controls. Seventy-five percent of autism subjects were agglomerated into one limb of an inverted tree dendrogram (Figure 1a) and were placed along the positive coordinate 2 axis in a classical MDS plot (Figure 5). The primary subdivision in the dendrograms involved agglomeration of extrastriate regions (cuneus, medial lingual)

and regions involved in downstream, higher level processing (Figure 1,2). This downstream pattern (medial lingual vs cingulate and fusiform) was also present in the GA dendrogram (Figure 3). Furthermore, coordinate 1 in classical MDS plots distinguished medial areas involved in early visual processing from downstream lateral regions involved in affective processing (Figures 5 and 6). These results indicated that interhemispheric patterns of connectivity may be useful classification biomarkers in autism. Based on AHCA and classical MDS, patterns of interhemispheric connectivity can distinguish homotopic nodes involved in early visual and affective processing.

We also identified pattern differences when grouping nodes and nodal affect. The insula was identified as an element whose agglomeration was superordinate to the cingulate, lateral lingual, inferior parietal, and fusiform regions in controls but significantly agglomerated with these other regions in autism whether GM and GA nodes were plotted together (Figure 2b) or separately (Figure 3b). Affect patterns within nodes were distinct in autism and controls. The dendrogram in Figure 1b showed that the resting medial lingual region was superordinate to the medial lingual emotional and neutral face networks in controls, but agglomerated with the medial lingual emotional and neutral networks in autism. In addition, controls had more spacing by affect within a node compared to controls using classical MDS. For example, differences in affect are easily distinguishable in the inferior cuneus and fusiform in controls but not autism (Figures 6a and 6b). Undirected graphs across the parameter space of brain region and facial affect indicated a greater similarity of brain regions across facial affect in autism compared to controls. We identified a triangular motif within homotopic nodes during emotional,

neutral, and resting states (Figure 4). Across GM and GA clusters, the nodes with triangular motifs were more segregated in controls, compared to autism, whether the GM and GA nodes were pooled or plotted separately (Figure 4). The dendrograms, undirected graphs, and MDS plots indicated that the fusiform gyrus was differentially patterned with other brain regions in autism and controls (Figures 1, 2, 4-6).

In summary, individuals with autism showed altered pattern groupings of homotopic nodes based on unsupervised classification differences (Figures 1-3), greater similarity across the parameter space of brain region and facial affect (Figure 4), and distinctive pattern maps in an arbitrary coordinate space (Figure 5,6). Based on the AHCA, undirected graph, and classical MDS patterns among brain regions that have differential functional interhemispheric connectivity with their homotope in autism and controls, we infer that metastability [12,13], the tendency of brain regions to be both segregated and integrated, is altered in autism. Further neuroimaging studies and analyses will be necessary to confirm that the interhemispheric network topology of the autism connectome is phenotypically distinct from controls.

Methods

Functional MRI Analysis

Using data from a previously reported study [14], we applied partial correlation functional connectivity analysis to identify homotopic brain regions (homologous structures in the right and left hemispheres) that showed significant functional

interhemispheric connectivity differences between autism and controls when processing emotional and neutral faces and at rest. The magnitude of functional connectivity between homotopic brain regions was quantified in affective (emotional and neutral) face processing and resting network using z-scores produced by Fisher transforming Pearson correlations of homotopic time series. These were entered into a Group x Affect x Subject(Group) ANOVA. Results indicated significant ($p \leq 0.05$, corrected) Group main (GM) effects and Group x Affect (GA) interactions in specific homotopic regions (Table 1). In this paper, we examined functional patterns in z-scores from homotopic brain regions differentially connected in autism ($n=12$) and control ($n=12$) subjects using agglomerative hierarchical analysis (AHCA), undirected graph theory, and multidimensional scaling (MDS). All fMRI statistical analyses were conducted in AFNI [15] and R [10] software packages which are respectively freely available at [<http://afni.nimh.nih.gov/afni/>] and [<http://www.r-project.org/>].

Agglomerative hierarchical cluster analysis (AHCA)

We visualized the functional patterns of several brain regions showing differential interhemispheric connectivity in autism compared to controls. AHCA is an exploratory multivariate statistical method of analysis to group elements. Given our interest in observing the unsupervised grouping of interhemispheric connectivity across the parameter space of affect and brain regions, we designated the elements of interest to be the vector of subject z-scores for a given affect at a given homotopic brain region. AHCA requires two specifications including a definition of distance and a clustering algorithm. We chose Euclidean distance (D) as the grouping statistic:

$$D(A, B) = \sqrt{\sum_{i=1}^n (a_i - b_i)^2}$$

where n is the number of subjects and A and B are vectors of respective subject z-scores, a_i and b_i , from different ROIs in a given affective face network. Euclidean distance was chosen because it preserves the magnitude of differences between negative and positive z-scores and is a grouping statistic based on the individual z-score differences, i.e. the root sum of squared z-differences. The Lance-Williams algorithm [16] was used to implement a complete linkage clustering in which distance is determined as the largest similarity between a point in the first cluster and a point in the second cluster (furthest neighbor method). Unbiased significance of edges in the resulting dendrogram was determined using multistep-multiscale bootstrap resampling [17] as implemented in the R package pvclust [18]. Functional brain connectivity heatmaps were displayed using the the Bioconductor heatmap function which was originally developed for microarray analysis [19].

Graph Theory: Undirected Correlation Graph

Graph theory provides a framework to understand functional brain connectivity [20-23] by identifying patterns such as network motifs, the non-random interconnectivity sub-patterns in graphs such as triangles [24,25]. Using graphs, brain regions are represented as nodes and associations are represented as lines, or edges, connecting nodes.

Undirected graphs (U) are defined as:

$$U = (V, E)$$

where V is a set of vertices and E is a set of edges. Unlike directed graphs which have arrows indicating association directionality on their edges, undirected graphs have edges without directionality or arrows. The `corGraph` program in the R package `pcalg` [26] calculates an undirected graph based on the correlation of nodal elements.

In our application, we tested the null hypothesis that the Pearson correlation of subjects' z-scores between one homotopic node (Table 1) and another homotopic node is zero. The Pearson correlation of z-scores corresponds to the “correlation of correlations” statistic [27] or integrative correlation [28] used in microarray analysis [29]. The computed undirected correlation graph visually displays an edge between nodes when the null hypothesis can be rejected at a given significance level ($\alpha = 0.05$). In short, groupings of clusters across the parameter space of affect and brain region were examined in autism and controls using thresholded undirected correlation graphs [30].

Classical Multidimensional Scaling

Multidimensional scaling (MDS) is a series of multivariate statistical techniques used for the reduction and visualization of high dimension data [31]. Classical MDS [32,33] decomposes a dissimilarity matrix into a set of elements whose pairwise distances are approximately equal to those in the dissimilarity matrix and maps them into a coordinate space with reduced dimensions. More specifically [34], classical MDS uses a proximity matrix of dissimilarities (D) as input to calculate a configuration matrix of mapped coordinates (X) from a scalar product matrix (B). After eigendecomposition of B into an

eigenvector matrix (Q) and a diagonal matrix of eigenvalues (V), X can be solved as follows:

$$B = XX' = QVQ' = QV^{1/2}V^{1/2}Q'$$

$$X = QV^{1/2}$$

When the dissimilarity matrix is based on Euclidean distance, this approach is equivalent to principal components analysis [11]. In our application, we used the Euclidean distance of homotopic z-scores as a dissimilarity matrix for use in classical (metric) multidimensional scaling. We reported the two dimensional solutions calculated with the program cmdscale from the R-package stats [10].

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Figures

Figure 1: Heatmap and Dendrogram of Pooled Autism and Control Group Main Effect and GroupxAffect Connectivity (z-score). (a) Z-map of interhemispheric connectivities for autism (red) and controls (black) classified according to Euclidean distances. Nine of twelve autism subjects (75%) were agglomerated together. (b) Hierarchical clustering of Euclidean distance heights identified unsupervised classification differences among autism and controls. Node labels refer to Group (A=autism, C=control), Affect (E= emotion, N=neutral, R = rest), and the node number based on Table 1 where “i” denotes interaction to indicate GA clusters.

Figure 2: Autism and Control Heatmaps and Dendograms of Pooled Group Main Effect and GroupxAffect Connectivity (z-score). (a) Z-map of interhemispheric connectivities for autism (top) and controls (bottom) classified according to Euclidean distances. (b) Hierarchical clustering of Euclidean distance heights identified significant unsupervised classification differences among autism (top) and controls (bottom). Node labels refer to Group (A=autism, C=control), Affect (E= emotion, N=neutral, R = rest), and the node number based on Table 1 where “i” denotes interaction to indicate GA clusters.

Figure 3: Autism and Control Heatmaps and Dendrograms of Group Main Effect or GroupxAffect Connectivity (z-score). Z-map of interhemispheric connectivities for (a) autism and (c) controls for GM (left) and GA (right) clusters classified according to Euclidean distances. Hierarchical clustering of Euclidean distance heights identified

significant unsupervised classification differences among (b) autism and (d) controls for GM (left) and GA (right) clusters. Significant clusters were corrected for multiple comparisons ($p=0.05$). Node labels refer to Group (A=autism, C=control), Affect (E=emotion, N=neutral, R = rest), and the node number based on Table 1.

Figure 4: Undirected Graph of Integrative Cluster Connectivity.

The eight GM and GA brain regions ($x=0..7$) from Table 1 are numbered numerically in triplicate as graphed nodes for emotional ($1+3x$), neutral ($2+3x$), and resting ($3+3x$) affective networks. For example, the graphed GM effect nodes 1, 2, and 3 refer to insula ($x=0$) during emotion, neutral, and rest conditions, respectively. Group Main Effect, $\text{Group} \times \text{Affect}$, and all clusters combined (Group main effect [nodes 1-15] and $\text{Group} \times \text{Affect}$ [nodes 16-24]) were thresholded at $p=0.05$ (uncorrected). Autism shows greater similarities across affect and cluster parameter space as indicated by the increased numbers of edges.

Figure 5: Classical Multidimensional Scaling of Homotopic Nodes in Autism and Controls.

Classical multidimensional scaling was applied to the Euclidean distance between interhemispheric z-scores from GM and GA homotopic nodes. Note that affective and visual processing nodes can be distinguished along coordinate 1 and groups can be distinguished along coordinate 2. The two dimensional solution is plotted for autism and controls in red and black, respectively. Node labels refer to Group (A=autism,

C=control), Affect (E= emotion, N=neutral, R = rest), and the node number based on Table 1.

Figure 6: Classical Multidimensional Scaling of Homotopic Nodes in Autism and Controls.

Classical multidimensional scaling was applied to the Euclidean distance between interhemispheric z-scores from GM and GA homotopic nodes. The two dimensional solution is plotted for (a,c,e) autism and (b,d,f) controls for (a,b) pooled GM and GA clusters and separately for (c,d) GM and (e,f) GA clusters. Note that affective and visual processing nodes can be distinguished along coordinate 1. Autism and control clusters are plotted in red and black, respectively. Node labels refer to Group (A=autism, C=control), Affect (E= emotion, N=neutral, R = rest), and the node number based on Table 1.

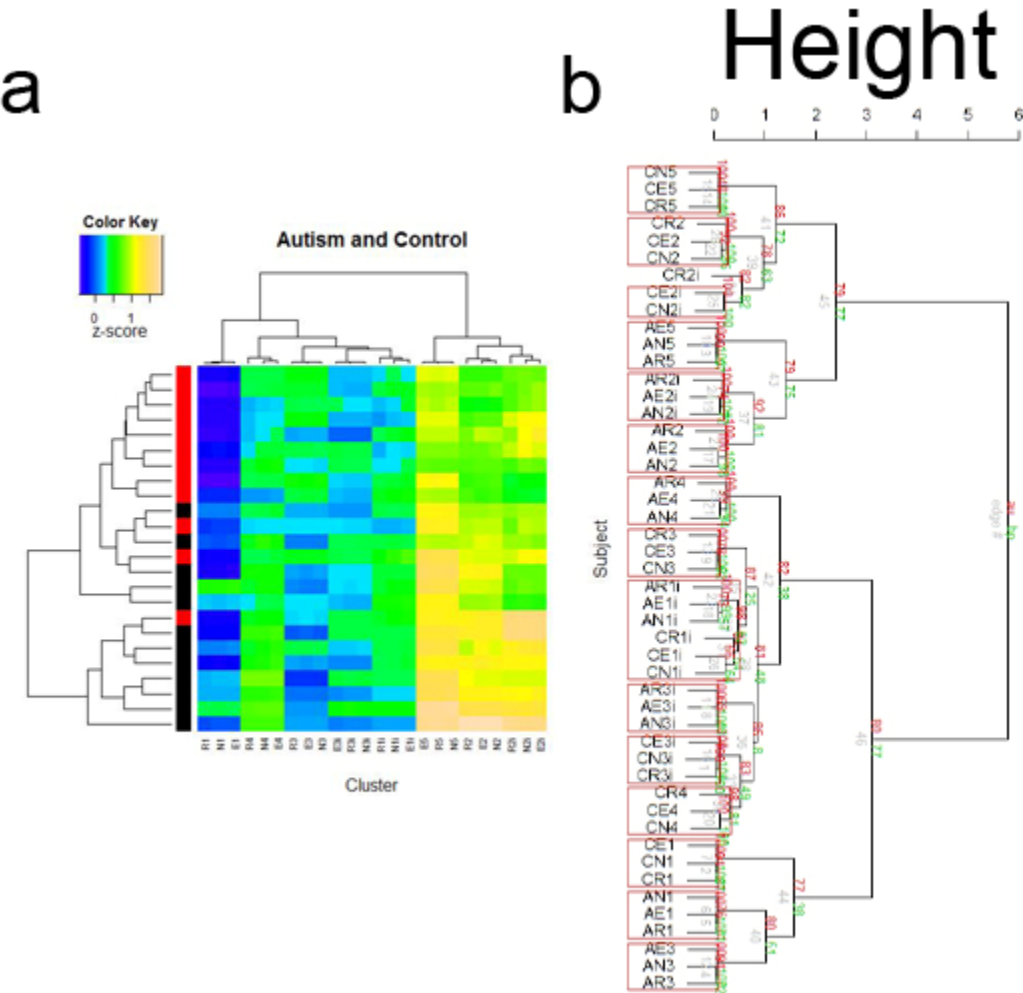


Figure 1

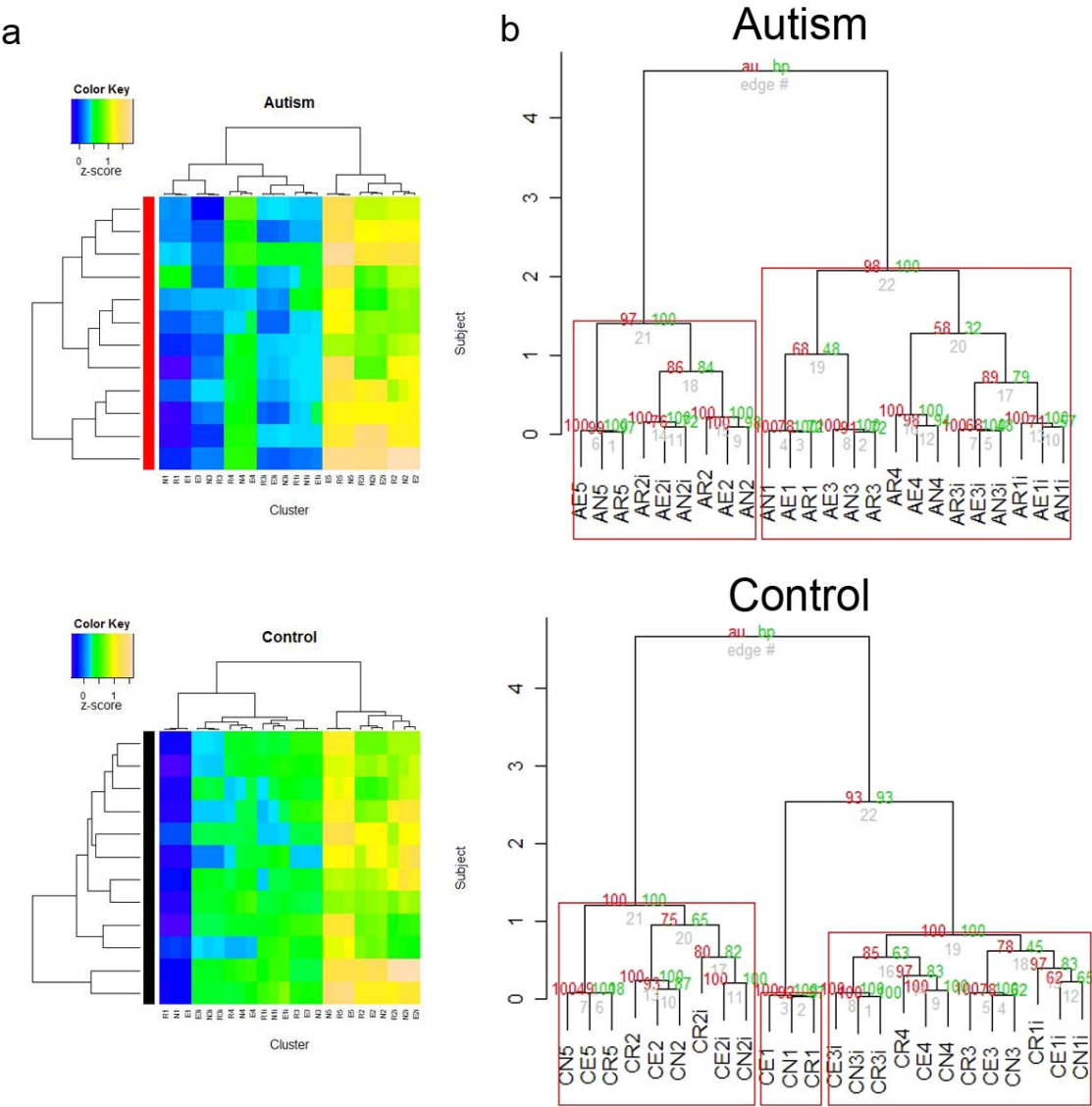


Figure 2

Figure 3

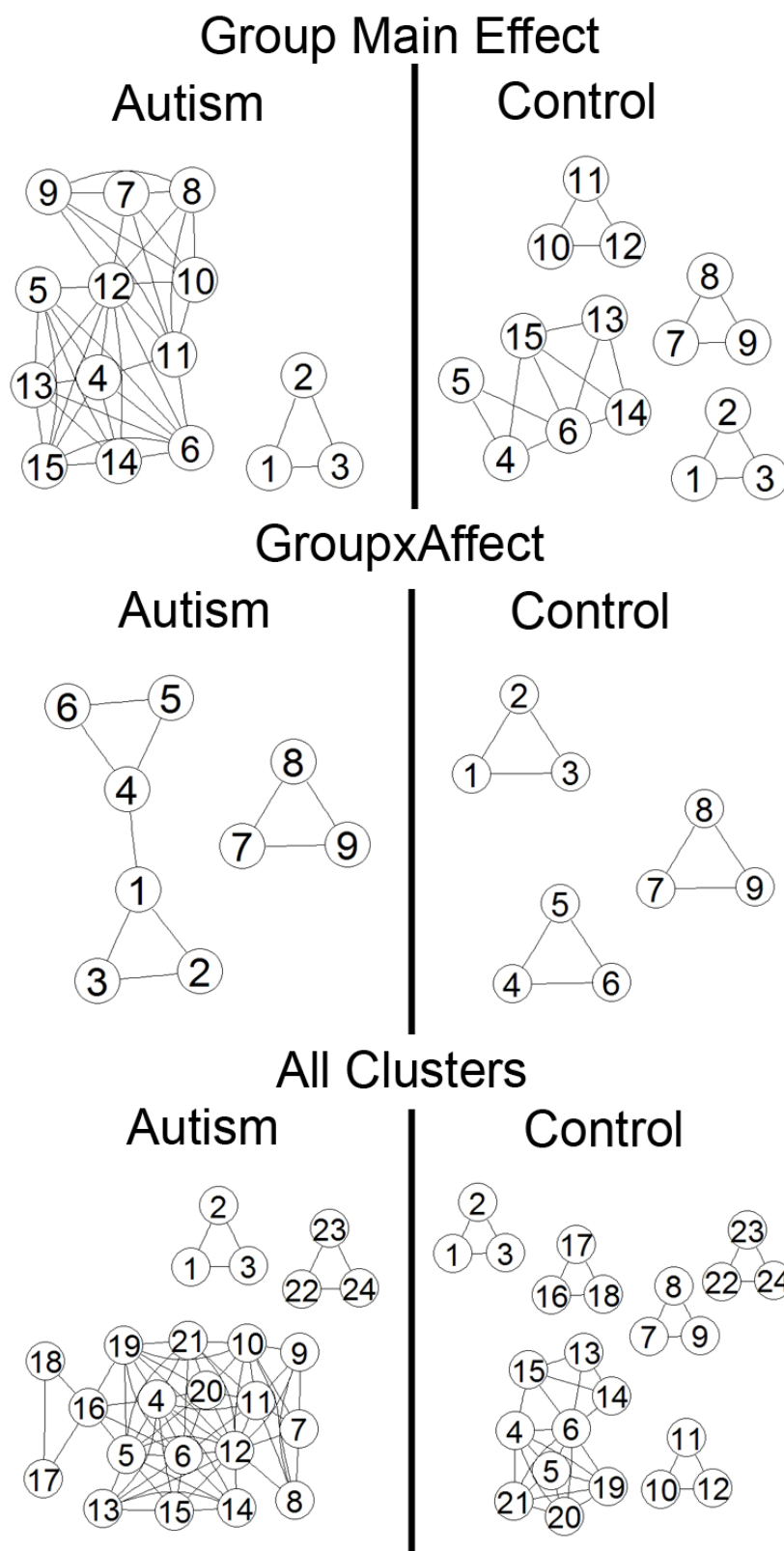


Figure 4

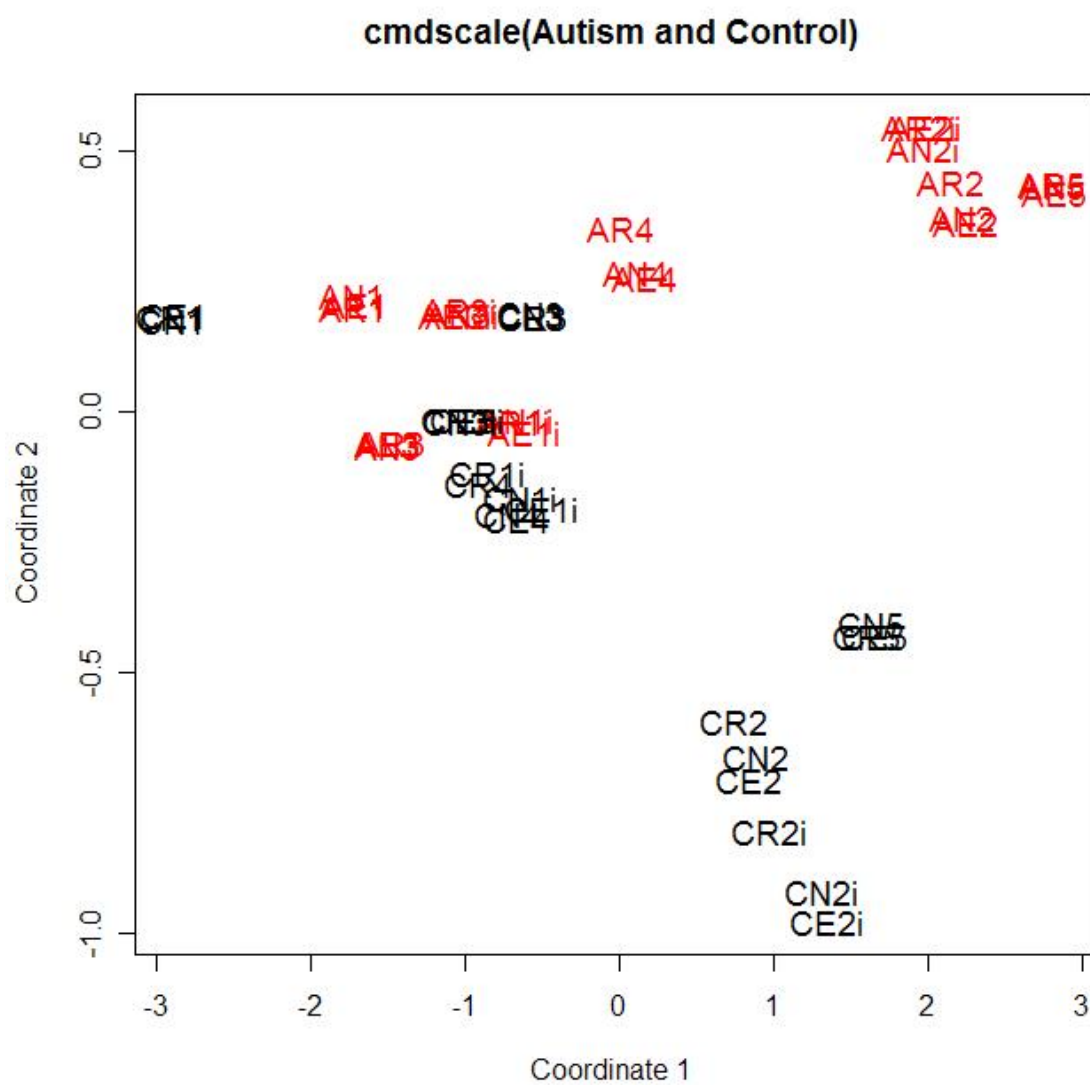


Figure 5

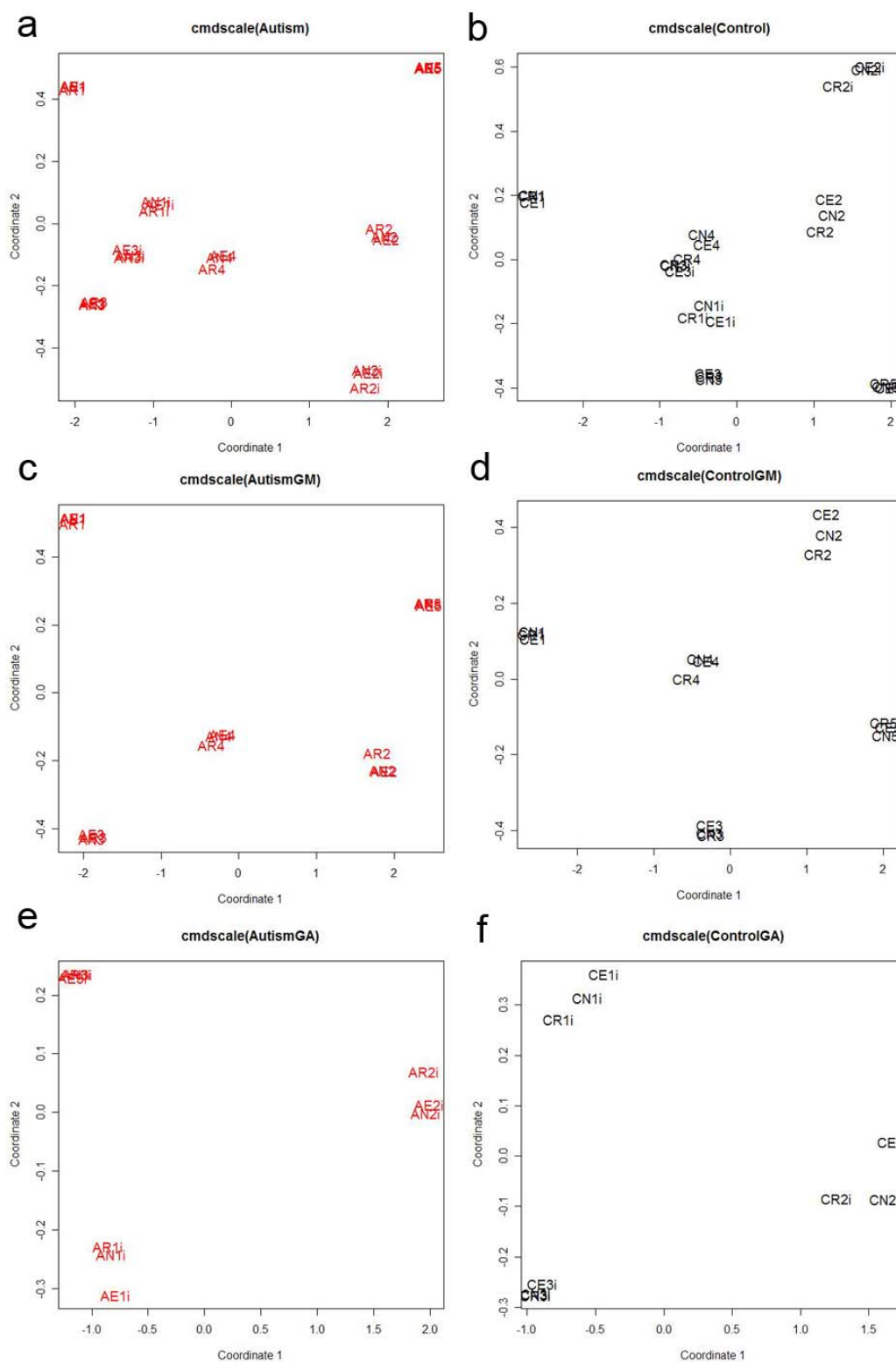


Figure 6

Tables:

Table 1: Homotopic Nodes with Functional Interhemispheric Connectivity Differences

Cluster	Group Main (GM) Effect	RPI Talarach Coordinate [x,y,z]
1	Insula	-41.9, 7.4, 2.5
2	Inferior Cuneus	0, 81.2, 12.1
3	Inferior Parietal	-36.5, 50.9, 57.3
4	Lateral Lingual	-21.3, 84.7, 0.9
5	Superior Cuneus	0, 77.6, 23.2
GroupxAffect (GA)		
1i	Fusiform	-33.8, 56.8, -8.8
2i	Medial Lingual	0, 83.8, -1.5
3i	Cingulate	-9.1, 17.4, 39.4

Chapter 6:

Automatic Physiological Waveform Processing for

fMRI Noise Correction and Analysis

Citation:

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Automatic Physiological Waveform Processing for fMRI Noise Correction and Analysis

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Abstract

Functional MRI resting state and connectivity studies of brain focus on neural fluctuations at low frequencies which share power with physiological fluctuations originating from lung and heart. Due to the lack of automated software to process physiological signals collected at high magnetic fields, a gap exists in the processing pathway between the acquisition of physiological data and its use in fMRI software for both physiological noise correction and functional analyses of brain activation and connectivity. To fill this gap, we developed an open source, physiological signal processing program, called PhysioNoise, in the python language. We tested its automated processing algorithms and dynamic signal visualization on resting monkey cardiac and respiratory waveforms. PhysioNoise consistently identifies physiological fluctuations for fMRI noise correction and also generates covariates for subsequent analyses of brain activation and connectivity.

Introduction

Physiological noise [1] describes a known low-frequency component of brain signals collected with functional MRI [2] (fMRI) that contaminates brain activation [3] and connectivity studies [4]. Retrospective removal of physiological noise from fMRI signal [5,6] requires simultaneous collection of functional brain images along with cardiac and respiratory signals, typically using a pulse oximeter and a respiratory bellows belt, respectively. Further processing of the physiological signals is necessary to remove acquisition artifact and to prepare them for use in existing fMRI noise correction and

analysis software like AFNI [7] [<http://afni.nimh.nih.gov/afni/>] . However, open-source software is not currently available to fill the gap between raw and processed physiological noise signals. We sought to fill this gap (Figure 1) by writing a fully automated, physiological noise processing program called PhysioNoise (download source code from Text S1) in the python language that enables interactive visualization of cardiac and respiratory time series, automatic detection of cardiac and respiratory peaks, and generation of processed cardiac and respiratory time series for both retrospective fMRI correction and covariates for analyses of brain activation and connectivity.

Materials and Methods

Algorithm

PhysioNoise was developed in python [8] using MacPython 2.5 [<http://www.pythonmac.org/packages/>] on the Mac OS X platform and requires scipy [9] [<http://www.scipy.org/>], numpy [10] [<http://numpy.scipy.org/>], and matplotlib [11] [<http://matplotlib.sourceforge.net/>] libraries. The program was also implemented and tested on a linux workstation running Scientific Linux [12]. PhysioNoise removes high frequency noise from both respiratory and cardiac waveforms with a user specified, zero phase, low pass Butterworth filter and calculates the residual (raw signal – Butterworth filtered signal). Acquisition artifacts are identified as time course regions with large residuals. A window around each artifact is removed and the entire filtered, cleaned dataset is fit with a spline such that clean peaks are modeled and the windowed gaps are

replaced with values from the spline interpolation to produce a clean, spline waveform (Figure 2).

Processing for Retrospective Physiological fMRI Noise

Correction

Respiratory Waveform

Respiratory peaks are detected in the respiratory spline dataset (RW) using a slightly modified version of the open-source peakdet.m algorithm of Eli Billaer [13] which we translated to python. This algorithm alternately detects peaks and troughs in a periodic signal. We adapted this algorithm to detect respiratory (RPpd) and cardiac (CPpd) peaks by adding optional, user specified thresholds to limit peak detection to physiologically relevant timings and magnitudes. The downsampled respiratory spline waveform (RW) is output for use in retrospective fMRI noise correction software such as AFNI's 3dretroicor.

Cardiac Waveform

The denoised cardiac spline was then subject to peak detection. For fMRI analysis there is a need to identify the ejection timing of the heart based on the pulse oximeter signal. The cardiac signal has several features that can be analyzed (Figure 3). A common method is to use the cardiac TTL pulse (CPttl) delivered by the scanner to estimate the R-wave, which is only detectable using EKG. Since the scanner records the peak times, PhysioNoise converts these times to a spike wave time series whose duration matches that of the fMRI brain scan. However, the CPttl peak detection contains artifactual errors of peak omission and commission and the CPttl onset time lags the R-wave [14]. Prior

studies using Doppler measures of aortic flow confirmed that the timing of ventricle contraction and the onset of systole and diastole can be detected by taking the third derivative of the pulse oximeter waveform and are identifiable as two small peaks separated by a larger trough [15]. The trough is readily detectable even in noisy data, corresponds to the time when ventricular ejection occurs, and may be a closer approximation to R-wave onset that precedes the CPttl peaks. We identified the ventricular contraction time (CPd3) as the large trough of the cardiac waveform's third derivative. After implementation we determined that smaller troughs were detected which needed to be removed from CPd3. To do so, the envelope of the cardiac waveform's third derivative peaks was used to threshold the absolute value of the third derivative's troughs. Only troughs whose absolute magnitude was greater than that of the third derivative's peak envelope and at least half the mean envelope magnitude were reported as CPd3 peaks. The R-wave estimate (CPd3R) was then determined by selecting the nearest third derivative peak that preceded the large trough. The CPttl, CPd3, and CPd3R are saved to disk as options for retrospective fMRI correction.

Processing for fMRI Functional Analysis of Activation and Connectivity

To prepare physiological noise covariates of no interest (nuisance variables) for connectivity analyses, further processing of the respiratory and cardiac peaks is required. First, the top and bottom respiratory envelopes and their difference, known as respiratory volume per time (RVT), are generated from their peaks (RPpd) as previously reported by Birn [16] using the peakdet algorithm. The cardiac volume per time curve (CVT) is also

generated using peaks (CPpd) detected with peakdet algorithm (Figure 4). Second, cardiac rate time (CRT) courses based on the TTL (CRTttl), the third derivative (CRTd3), and the third derivative's R-wave estimate (CRTd3R) are generated as the inverse change in time between CP centered points on the initial peak [14]. The respiratory rate (RRT) over time was also calculated using the RPPd in the same manner as CRT (Figure 5). Third, the RVT and CRT waveforms are sampled on the TR and half TR and output for later use in fMRI activation and connectivity analyses.

Results

With RARC approval, rhesus monkeys (n=13) were sedated and restrained during simultaneous 3.0 Tesla GE Signa scanner acquisitions of functional MRI, pulse oximetry, and respiratory bellows signals. Respiration was monitored with a respiratory belt and cardiac contraction with a pulse oximeter on a 3.0 Tesla scanner (GE Medical Systems; Waukesha, WI). Both were collected at a 1000 Hz sampling rate. Artifacts in the physiological signal were automatically detected and corrected (Figure 2) using PhysioNoise prior to identifying RPPd, CPpd, CPttl, CPd3, and CPd3R (Figure 3). The downsampled signal and peaks were then available for use in retrospective fMRI physiological noise correction in AFNI. The respiratory and cardiac envelopes derived from the peakdet peaks of the spline waveforms (Figure 4) were successfully used to generate the RVT and CVT curves. The CRT curves were also successfully generated from the CPttl, CPd3, and CPd3R peaks. In short, PhysioNoise produced useful waveforms for fMRI analysis of activation or connectivity (Figure 1). Statistical tests were conducted in SPSS 14.0 (SPSS, Inc; Chicago) and R [17].

To evaluate the peakdet algorithm, we tested the null hypothesis that the period of both the respiratory and cardiac waveforms calculated from the peakdet peaks would be comparable to the period derived from the frequency of the maximum density in their respective power spectra. The temporal period (seconds) for the respiratory waveform determined by the peakdet algorithm [mean= 2.47; SD = 1.26] was comparable to the peak power [mean= 2.06; SD = 0.532; paired $t(12)=1.08$; $p=0.301$]. The temporal period of the cardiac waveform from the power spectra [mean = 0.868; SD = 0.133] was comparable to the period using CPpd peaks determined by peakdet [mean= 0.866; SD = 0.138; paired $t(12)=-0.201$; $p= 0.844$].

To evaluate the differentiation method for improved R-wave onset approximation, we compared the period from the CPttl peaks [mean= 0.805; SD=0.225] to the period derived from the CPd3 [mean = 0.853; SD = 0.125] and CPd3R [mean = 0.853; SD = 0.133] peaks generated from the differentiation method and found them to be equivalent [$F(2,24)=0.989$; $p=0.387$] based on a one way ANOVA blocked across monkeys.

To determine whether CPd3 would provide a better estimate of R wave onset than CPttl, we evaluated the latency of CPd3 by testing the null hypothesis that the CPd3 time would be comparable to the CPttl onset. To do so, we measured the time change from CPd3R to the onset of both CPd3 [mean = 195.3 ms; SD = 51.1] and CPttl [mean = 232.2 ms; SD = 58.4] and took their difference. We determined that the CPd3 preceded CPttl by about 37

ms [mean=-36.8 ms; SD=23.1] and this time lag was significantly different from zero [paired $t(12)=-5.74$; $p=0.00$].

To quantify the spike reduction in the CRT waveforms, we tested the null hypothesis that the CRTttl, CRTd3, and CRTd3R would have comparable numbers of outliers ($SD > 4$) and those outliers would have equal variance. The mean number of outliers for these methods were not equal [$F(2,24) = 5.30$; $p = 0.012$] when tested in a one way ANOVA blocked across monkeys. The CRTttl [mean= 6895; $SD = 7228$] had significantly more outliers than the CRTd3 [mean= 1662; $SD = 3039$; paired $t(12) = 3.34$; $p = 0.006$] and marginally more significant outliers than CRTd3R [mean = 1963; $SD = 3607$; paired $t(12) = 2.13$; $p = 0.055$] methods. To demonstrate outlier dispersion, we tested the ratio of each method's outlier variances pooled across monkeys using an F-test. The CRTttl variance [mean = 0.312; $SD = 0.833$] was significantly more variable than the CRTd3 [mean = 0.036; $SD = 0.068$; $F(1,24) = 6.37$; $p = 0.019$] and the CRTd3R [mean = 0.026; $SD = 0.046$; $F(1,24) = 15.6$; $p = 0.000$].

Discussion

PhysioNoise is a fully automated physiological noise processing program in the python language that processes physiological fluctuations and fills a gap between physiological noise signal acquisition and its use in fMRI neuroanalysis. Although PhysioNoise is fully automated, users have several options to control processing. Furthermore, PhysioNoise is open-source, modifiable by the users themselves, and may serve as a base program for further algorithm implementations. The utility of the program was validated using

anesthetized monkey cardiac and respiratory signals. PhysioNoise detects and corrects acquisition artifact in the physiological signal and returns several processed waveforms suitable for fMRI correction and analysis.

PhysioNoise is available for download (Text S1) as a stand alone program. The open source algorithms we adapted as functions for use in PhysioNoise are contained within the PhysioNoise source code. Prerequisites to use PhysioNoise include a working installation of python [8] and access to scipy [9] [<http://www.scipy.org/>], numpy [10] [<http://numpy.scipy.org/>], and matplotlib [11] [<http://matplotlib.sourceforge.net/>] libraries. The PhysioNoise program was tested with physiological and fMRI signals collected from monkeys using a 3.0 Tesla GE Signa MRI scanner and a respiratory bellows belt. The algorithms we packaged into PhysioNoise are generalizable to other fMRI scanners and respiratory bellows equipment.

In summary, PhysioNoise outputs the RW, CPd3, CPd3R, and CPttl time courses as options for retrospective fMRI brain signal correction of physiological noise and the RVT, RRT, CVT, CRTd3, CRTd3R, and CRTttl curves as options for neuroanalysis. Prior studies have noted that the CPttl lags the R-wave by about 120 ms and produces CRT waves with intermittent spikes which are due to the detection of false peaks or omission of true peaks [14]. We detected the CPd3R about 230 ms prior to the CPttl R-wave estimate. This suggests that the CPd3R may precede the actual R-wave. Future studies should use PhysioNoise to directly compare the R-wave onset times from pulse oximetry using the differentiation method, the CPttl from the scanner, and the actual R-

wave from EKG measurements during fMRI acquisition. The CPd3 may be a more appropriate choice for R-wave estimation for fMRI noise correction and analysis compared to the CPttl because the CPd3 preceded the CPttl by about 37 ms and the CRTd3 has fewer outliers with less outlier dispersion.

Acknowledgements

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Figure Legends

Figure 1: PhysioNoise Fills Physiological Signal Processing Gap.

PhysioNoise processes physiological noise signals for existing software (AFNI) used in retrospective fMRI physiological noise correction and connectivity analyses. The inputs to PhysioNoise are represented by the blue arrow and the outputs from PhysioNoise are identified with the red arrows. (RW= Downsampled respiratory spline waveform; CPttl = Cardiac peak based on the TTL pulse from the scanner; CPd3 = Cardiac peak based on trough of the third derivative; CPd3R = Cardiac peak R-wave estimate based on small peak of third derivative preceding the CPd3; RVT=Respiratory volume over time based on peakdet peaks; RRT = Respiratory rate over time; CVT = Cardiac volume over time based on peakdet peaks; CRTttl=Cardiac rate over time based on the TTL pulse from the scanner; CRTd3=Cardiac rate over time based on CPd3; CRTd3R= Cardiac rate over time based on CPd3R).

Figure 2: Plot of Physiological Signal Artifact.

The raw respiratory and cardiac waveforms are plotted along with their filtered versions. Signal artifacts with large residuals (Raw Signal-Filtered Signal) are replaced with a spline interpolation. The series of green spline circles are the thick black waveform.

Figure 3: Cardiac Peaks.

The CPpd, CPttl, CPd3, and CPd3R are displayed on the cardiac spline (black) and third derivative (cyan). The envelope of the third derivative peaks is shown in yellow. The plot also shows an example of the scanner TTL error of commission in which an extra CPttl was detected during one cardiac cycle. The differentiation method correctly produced one CPd3 peak (CPttl = Cardiac peak based on the TTL pulse from the scanner; CPd3 = Cardiac peak based on trough of the third derivative; CPd3R = Cardiac peak R-wave estimate based on small peak of third derivative preceding the CPd3).

Figure 4: Respiratory and Cardiac Envelopes.

The peaks identified by peakdet (RPpd,CPpd) are shown along with the respiratory and cardiac waveforms, top envelope, bottom envelope, and the absolute value of their means.

Figure 5: Cardiac and Respiratory Rates and Volumes over Time

Representative RVT, RRT, CVT, CRTttl, CRTd3, and CRTd3R waveforms are displayed in PhysioNoise. Note that the CRTd3 contains fewer outliers than the CRTttl and the CRTd3R. Dynamic visualization of these curves in PhysioNoise is possible using the

zoom and scroll features (RVT=Respiratory volume over time based on peakdet peaks;
RRT = Respiratory rate over time; CVT = Cardiac volume over time based on peakdet
peaks; CRTttl=Cardiac rate over time based on the TTL pulse from the scanner;
CRTd3=Cardiac rate over time based on CPd3; CRTd3R= Cardiac rate over time based
on CPd3R).

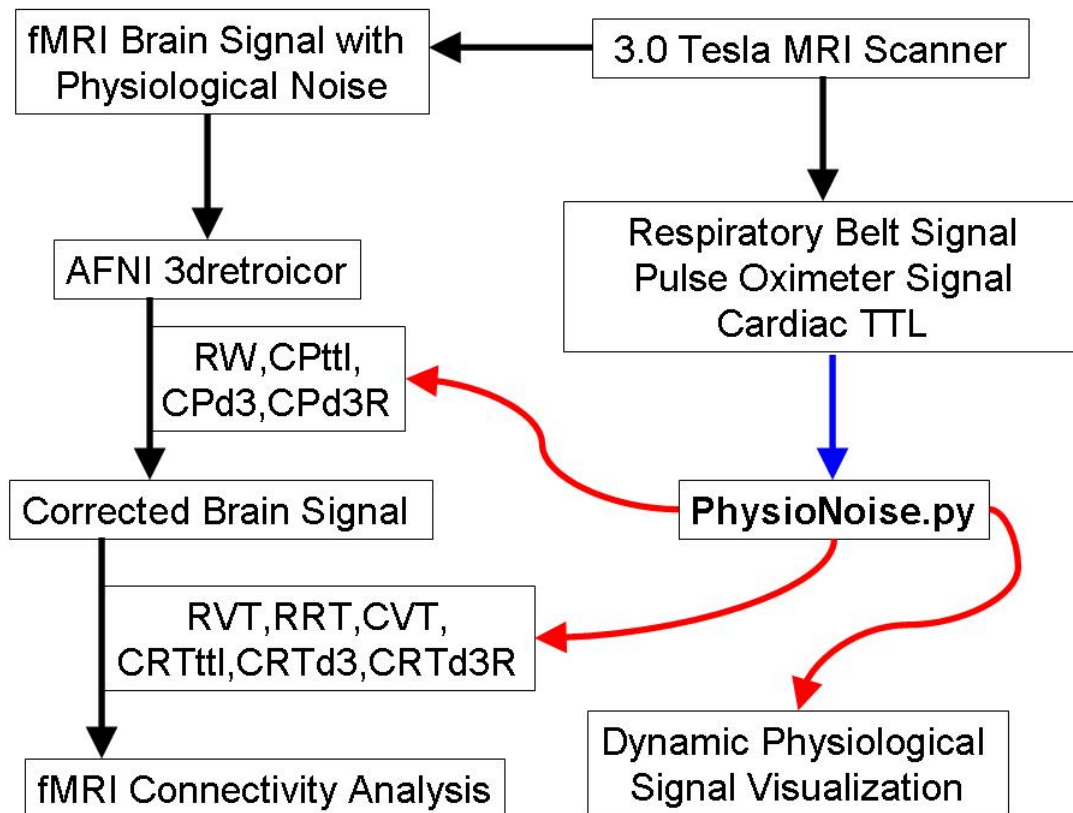


Figure 1

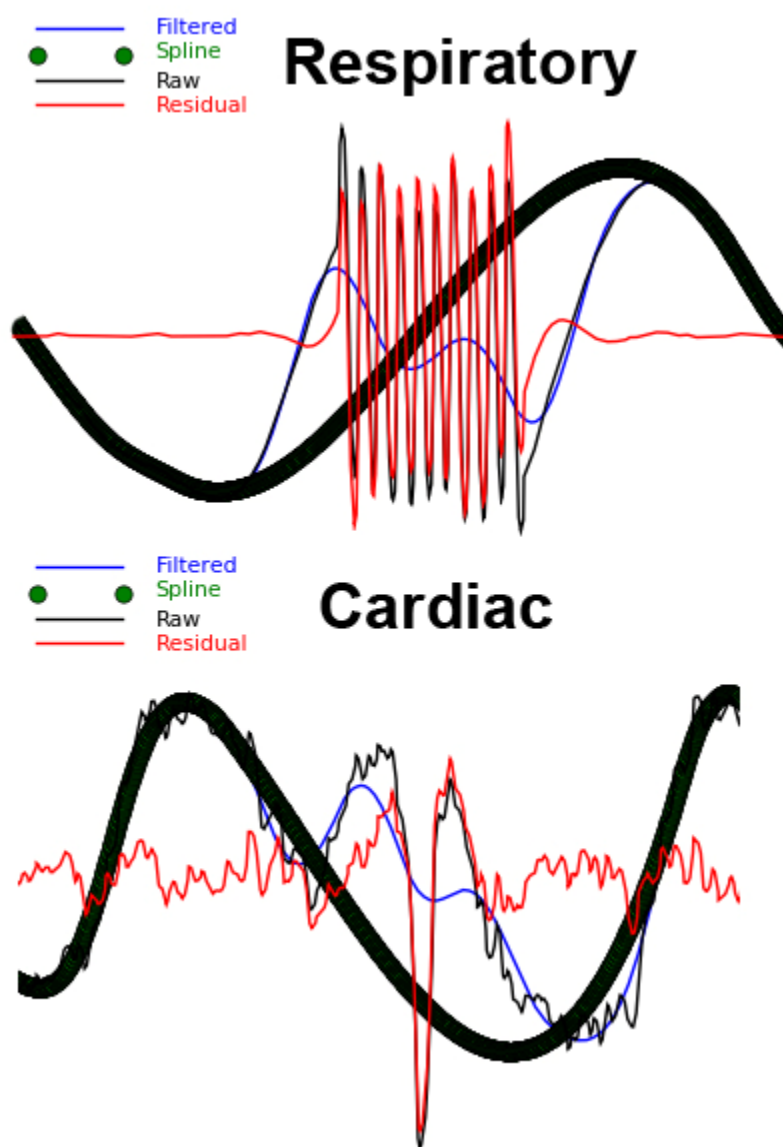


Figure 2

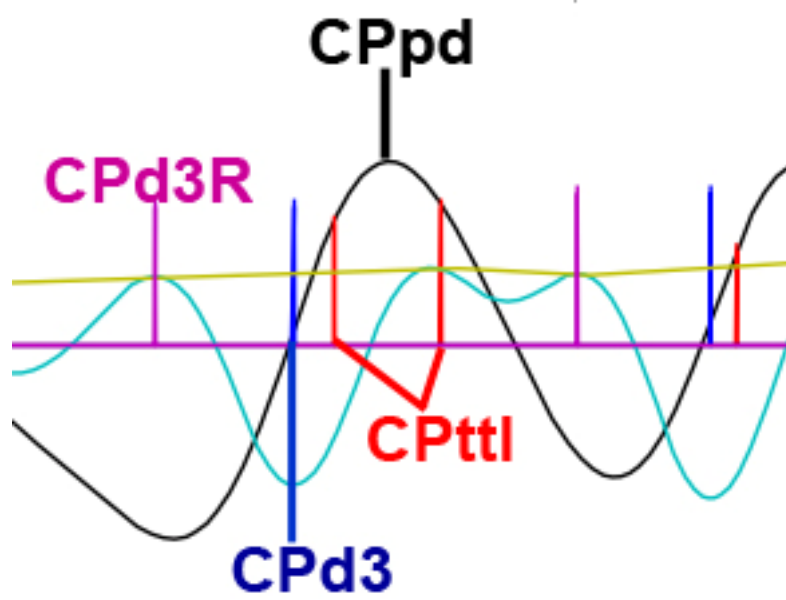


Figure 3

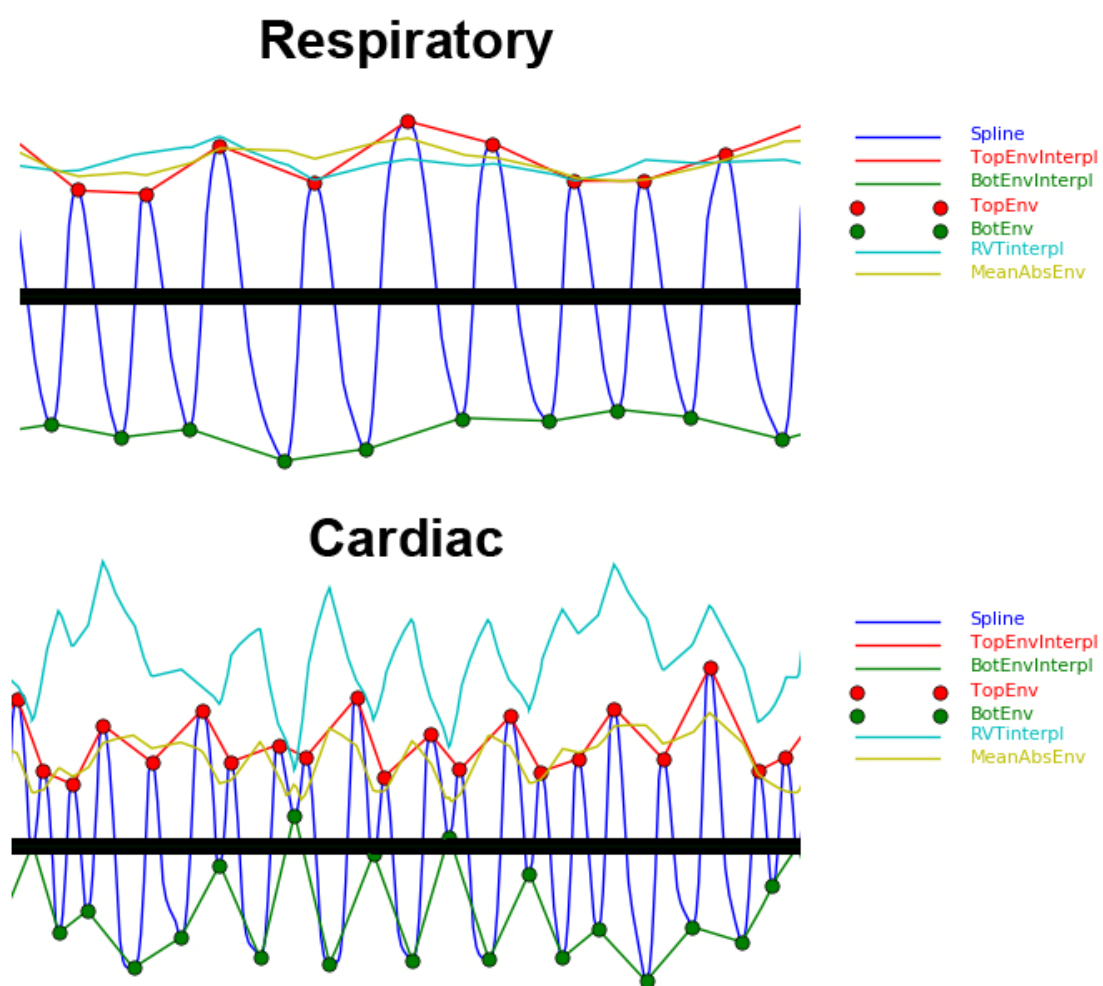


Figure 4

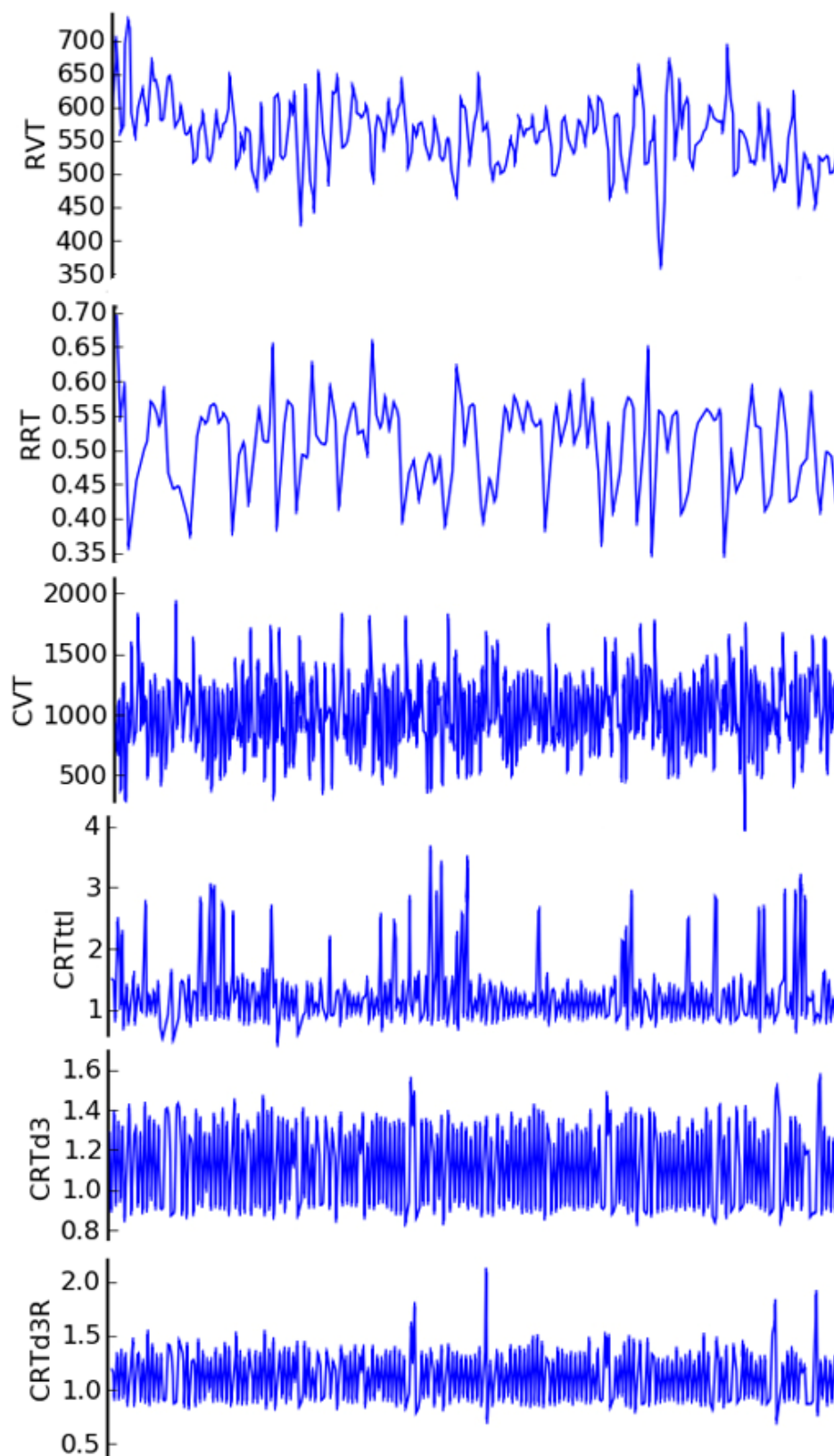


Figure 5

Supplement

```
#!/usr/local/bin/python
#from __future__ import division

"""Author: Dan Kelley, University of Wisconsin, Sept 2007
    Program: PhysioNoise.py
    Date: November 2007
    """

import string, sys
from numpy import *
import scipy as Sci
import scipy.linalg
import pytz
from pylab import *
from matplotlib import *
from scipy import linspace, polyval, polyfit, sqrt, stats,
randn, signal, interpolate
from pylab import plot, title, show, legend, figure
from scipy.signal import cspline1d, cspline1d_eval, iirdesign, lfilter,
lp2lp, freqz
import scipy.interpolate as interpolate
from timeseries.lib.moving_funcs import cmov_average
from matplotlib.mlab import psd
from scipy.signal import cspline1d, cspline1d_eval
from scipy.signal.signaltools import hilbert
from matplotlib.mlab import slopes

import matplotlib.numerix.ma as M
#####-----
-----##### Find Extrema

#Modified from peakdet.m by Eli Billauer, 3.4.05 (Explicitly not
copyrighted).
#This function is released to the public domain; Any use is allowed.
#Accessed Sept 2007

def findextrema(TS, ChangeMag, TimeConstraint):
    MinMag = Inf
    MaxMag = -Inf
    FindMaxMag = 1
    MaxCounts=zeros(len(TS))
    MinCounts=zeros(len(TS))
    MaxCountsPeak=zeros(len(TS),Float)
    MinCountsPeak=zeros(len(TS),Float)
    maxcounter=0
    mincounter=0
    maxmincounter=0
    AllExtremaPeak=zeros(len(TS),Float)
    AllExtremaPeakTime=zeros(len(TS))
    MaxPeak=0
    MaxPeakTime= -2*TimeConstraint
    MinPeak=0
```

```

MinPeakTime= -2*TimeConstraint
MinTime = -2*TimeConstraint
MaxTime = -2*TimeConstraint
constraintindex=0
AllExtremaPeakTime[0]= -2*TimeConstraint

print "Finding Extrema"
for i in xrange(len(TS)):
    TestMag = TS[i]
    if TestMag > MaxMag:
        MaxMag = TestMag
        MaxTime = i

    if TestMag < MinMag:
        MinMag = TestMag
        MinTime = i

    if FindMaxMag == 1:
        if TestMag < ( MaxMag - ChangeMag) and abs(MaxTime-
AllExtremaPeakTime[constraintindex])>=TimeConstraint:
            MaxPeak = MaxMag
            MaxPeakTime = MaxTime
            MaxCounts[maxcounter] = MaxTime
            MaxCountsPeak[maxcounter]= MaxMag
            maxcounter= maxcounter + 1
            AllExtremaPeak[maxmincounter]=MaxMag
            AllExtremaPeakTime[maxmincounter]=MaxTime
            constraintindex=maxmincounter
            maxmincounter=maxmincounter+1
            MinMag = TestMag
            MinTime = i
            FindMaxMag = 0

        elif TestMag > (MinMag + ChangeMag) and abs(MinTime-
AllExtremaPeakTime[constraintindex] )>=TimeConstraint:
            MinPeak = MinMag
            MinPeakTime = MinTime
            MinCounts[mincounter]= MinTime
            MinCountsPeak[mincounter]= MinMag
            mincounter = mincounter + 1
            AllExtremaPeak[maxmincounter]=MinMag
            AllExtremaPeakTime[maxmincounter]=MinTime
            constraintindex=maxmincounter
            maxmincounter=maxmincounter+1
            MaxMag = TestMag
            MaxTime = i
            FindMaxMag = 1

MaxCountsPeak = MaxCountsPeak[0:maxcounter]
MaxCounts = MaxCounts[0:maxcounter]
MinCountsPeak = MinCountsPeak[0:mincounter]
MinCounts = MinCounts[0:mincounter]
AllExtremaPeak= AllExtremaPeak[0:maxmincounter]
AllExtremaPeakTime=AllExtremaPeakTime[0:maxmincounter]

```

```

TopEnvTemp=zeros(size(TS))
BotEnvTemp=zeros(size(TS))
for counter in xrange(len(MaxCounts)):
    topms = MaxCounts[counter]
    TopEnvTemp[topms] = MaxCountsPeak[counter]

for counter in xrange(len(MinCounts)):
    botms = MinCounts[counter]
    BotEnvTemp[botms] = MinCountsPeak[counter]

return AllExtremaPeak, AllExtremaPeakTime, MaxCountsPeak,
MinCountsPeak, TopEnvTemp, BotEnvTemp, MaxCounts, MinCounts

#-----#
FiltFilt
from numpy import vstack, hstack, eye, ones, zeros, linalg, \
newaxis, r_, flipud, convolve, matrix, array
from scipy.signal import lfilter

def lfilter_zi(b,a):
    #compute the zi state from the filter parameters. see [Gust96].

    #Based on:
    # [Gust96] Fredrik Gustafsson, Determining the initial states in
forward-backward
    # filtering, IEEE Transactions on Signal Processing, pp. 988--
992, April 1996,
    # Volume 44, Issue 4

    n=max(len(a),len(b))
    zin = ( eye(n-1) - hstack( (-a[1:n,newaxis],
vstack((eye(n-2), zeros(n-2))))))
    zid= b[1:n] - a[1:n]*b[0]
    zi_matrix=linalg.inv(zin)*(matrix(zid).transpose())
    zi_return=[]
    #convert the result into a regular array (not a matrix)

    for i in range(len(zi_matrix)):
        zi_return.append(float(zi_matrix[i][0]))
    return array(zi_return)

def filtfilt(b,a,x):
    #For now only accepting 1d arrays
    ntaps=max(len(a),len(b))
    edge=ntaps*3
    if x.ndim != 1:
        raise ValueError, "Filtfilt is only accepting 1 dimension
arrays."

    #x must be bigger than edge
    if x.size < edge:

```

```

        raise ValueError, "Input vector needs to be bigger than 3 *
max(len(a),len(b))."
    if len(a) < ntaps:
        a=r_[a,zeros(len(b)-len(a))]

    if len(b) < ntaps:
        b=r_[b,zeros(len(a)-len(b))]
    zi=lfilter_zi(b,a)
    #Grow the signal to have edges for stabilizing
    #the filter with inverted replicas of the signal
    s=r_[2*x[0]-x[edge:1:-1],x,2*x[-1]-x[-1:-edge:-1]]
    #in the case of one go we only need one of the extremes
    # both are needed for filtfilt
    (y,zf)=lfilter(b,a,s,-1,zi*s[0])
    (y,zf)=lfilter(b,a,flipud(y),-1,zi*y[-1])
    return flipud(y[edge-1:-edge+1])

#-----
----Downsample

def DownSample(TS):
    SigDownsampleTime=range(long(len(TS)/SampRatio))
    SigDownsample=zeros(len(SigDownsampleTime),'f')
    SigDownsampleFull=zeros(len(TS),'f')
    SigDownsampleFull[0]=TS[0]
    SigDownsample[0]=TS[0]
    SigDownsampleTime[0]=0
    for counter in range(1,len(SigDownsampleTime)) :
        ms = (counter*SampRatio)-1
        SigDownsampleFull[ms] = TS[ms]
        SigDownsample[counter] = TS[ms]
        SigDownsampleTime[counter] = ms
    return SigDownsampleFull, SigDownsample, SigDownsampleTime

#-----
----TRSample
def TRSample(TS,TRFreq, SampRate):
    SigDownsampleTime=range(long(NumTR/float(TRFreq)))
    SigDownsample=zeros(len(SigDownsampleTime),'f')
    SigDownsampleFull=zeros(len(TS),'f')
    SigDownsampleFull[0]=TS[0]
    SigDownsample[0]=TS[0]
    SigDownsampleTime[0]=0
    # print "SigDownsampleTime", len(SigDownsampleTime)
    # print "NumTR", NumTR, "TRFreq", TRFreq, "No div
    T",long(NumTR/float(TRFreq))
    # print "length TS",len(TS)
    if (len(SigDownsampleTime)*TR *TRFreq *SampRate ) > len(TS):
        print "Time series is too short based on TR"
        print "Time series should be:", len(SigDownsampleTime)*TR
        *TRFreq *SampRate
        print "Time series actually is:", len(TS)
        print "Was the TR
        actually",len(TS)*(len(SigDownsampleTime)*TRFreq *SampRate)**-
        1,"not",TR,"seconds?"
    return SigDownsampleFull, SigDownsample, SigDownsampleTime
    for counter in range(1,len(SigDownsampleTime)) :
```



```

        ms = int(counter*TR *TRFreq *SampRate)-1
#       print "counter", counter, "ms", ms
        SigDownsampleFull[ms] = TS[ms]
        SigDownsample[counter] = TS[ms]
        SigDownsampleTime[counter] = ms
    return SigDownsampleFull, SigDownsample, SigDownsampleTime

#-----
-----End TRSample
def ConnectTheDots(Counts,Hills,TotPoints,RepPt):
    if len(Counts)<=3:
        print "Not enough peaks detected"
        return zeros(len(TotPoints))
    tc=long(Counts[-1]-Counts[RepPt]+1)
    Tp=interpolate.interp1d(Counts[RepPt:],Hills)
    InterpTime=range(tc)
    for i in range(tc):
        InterpTime[i]=Counts[RepPt] + i
    InterpEnv = Tp(InterpTime)
    FullInterpEnv=zeros(len(TotPoints))
    for i in range(tc):
        FullInterpEnv[Counts[RepPt] + i ]= InterpEnv[i]
    FullInterpEnv[0:Counts[RepPt]]=FullInterpEnv[Counts[RepPt]]
    FullInterpEnv[Counts[-1]:]=FullInterpEnv[Counts[-1]]
    return FullInterpEnv
#-----
EndConnectTheDots

```

```

from optparse import OptionParser, OptionGroup

usage = """
=====
=====
Usage: %prog [options] arg1 arg2
Author: Dan Kelley
        Waisman Laboratory for Brain Imaging and Behavior
        University of Wisconsin-Madison
        Last Update: November 1, 2007
This program:
1 Removes noise with a lowpass Butterworth filter with zero phase
  shift
2 Calculates the residual = Raw signal - filtered signal
3 Identifies dirty peaks with big residuals that need cleaning
4 Dirty peaks +/- a window are removed to produce filtered, cleaned
  data
5 Calculates the best fitting spline through the cleaned data
6 Spline interpolates over the removed dirty peaks
7 Downsamples the clean, spline data
8 Finds the peaks
9 Calculates the top and bottom envelopes and their difference (RVT) as
  in
        Birn RM, Diamond JB, Smith MA, Bandettini PA.
        Separating respiratory-variation-related fluctuations
        from neuronal-activity-related fluctuations in fMRI.
        Neuroimage. 2006 Jul 15;31(4):1536-48. Epub 2006 Apr 24.

```

PMID: 16632379

An alternative RVT is also returned which is the absolute value of the Hilbert transformed spline interpolation.

10 Returns the downsampled spline interpolation, the upper peaks, and the Birn and Hilbert RVT curves.
Peak finding was slightly modified from the open source peakdet.m algorithm and ported into python.

usage: %prog [options] arg1 arg2

Example usage:

```
./PhysioNoise.py -s Resp.txt -t RespTrig.txt --trimwindow 300 --plots"""
```

epilogue

```
=====
=====
"""
```

```
parser = OptionParser(usage=usage, epilog=epilogue)
parser.add_option("-r", "--resp", action="store", type="string",
dest="sigfile", help="read respiratory signal from FILE",
metavar="FILE")
parser.add_option("-c", "--card", action="store", type="string",
dest="csigfile", help="read cardiac signal from FILE", metavar="FILE")
parser.add_option("-o", "--ox", action="store", type="string",
dest="ctrigfile", help="read cardiac pulse ox trigger from FILE",
metavar="FILE")
parser.add_option("--TR", action="store", type="float",
dest="TR", help="TR is TR seconds [2.5]", metavar="TR", default=2.5)
parser.add_option("--numTR", action="store", type="int",
dest="NumTR", help="Number of TRs [480]", metavar="TR", default=480)
parser.add_option("--inputHz", action="store", type="int",
dest="OrigSamp", help="input frequency in Hz[1000]", metavar="Hz",
default=1000)
parser.add_option("--outputHz", action="store", type="int",
dest="NewSamp", help="desired output frequency in Hz[40]", metavar="Hz",
default=40)
parser.add_option("--fpass", action="store", type="int",
dest="fpass", help="Butterworth filter:Stop frequency of passband in
Hz[2]", metavar="Hz", default=2)
parser.add_option("--fstop", action="store", type="int",
dest="fstop", help="Butterworth filter:Start frequency of the stopband
in Hz[10]", metavar="Hz", default=10)
parser.add_option("--trimwindow", action="store", type="int",
dest="TrimWindow", help="Number of points PTS to clean on either side of
dirty residual peaks[650]", metavar="PTS", default=650)
parser.add_option("--plr", action="store", type="float",
dest="FwdRm", help="Percentage of points on the left of dirty peaks to
clean on the right [1.2]", metavar="Percentage", default=1.2)
parser.add_option("--statthresh", action="store", type="int",
dest="FoldSTD", help="Mark points beyond FOLD standard deviation(s) as
dirty residual peaks for cleaning [6]", metavar="FOLD", default=6)
```

```

parser.add_option("--rmagthresh", action="store", type="int",
dest="RMagThresh", help="Respiratory Peak must be RMAG units away from
nearest extrema [100]", metavar="RMAG", default=100)
parser.add_option("--rtimethresh", action="store", type="float",
dest="RTimeThresh", help="Respratory Peak must be RTIME seconds away
from the nearest extrema [0]", metavar="RTIME", default=0)
parser.add_option("--cmagthresh", action="store", type="int",
dest="CMagThresh", help="Cardiac Peak must be CMAG units away from
nearest extrema [20]", metavar="CMAG", default=20)
parser.add_option("--ctimethresh", action="store", type="float",
dest="CTimeThresh", help="Cardiac Peak must be CTIME seconds away from
the nearest extrema [0]", metavar="CTIME", default=0)
parser.add_option("--plots",
action="store_true", dest="PlotAll", help="this turns on interactive
plotting", default=False)
parser.add_option("-p", "--prefix", action="store",
type="string", dest="Prefix", help="Output prefix [PreIcor]",
default='PreIcor')

```

```

options, args = parser.parse_args()

```

```

if '-help' in sys.argv:
    parser.print_help()
    raise SystemExit()
if not (options.sigfile and options.csigfile and options.ctrigfile) or
'-help' in sys.argv:
    print "The respiratory signal file, cardiac signal file, and
cardiac trigger file are required. Try --help "
    raise SystemExit()

```

```

if options.sigfile and options.csigfile and options.ctrigfile:
    Sig = fromfile( options.sigfile, 'f', sep='\n')
    meanSig=float(mean(Sig[0:long(len(Sig)/2)]))
    Sig = Sig - meanSig
    SigT = fromfile( options.ctrigfile, 'f', sep='\n')
    CSig = fromfile( options.csigfile, 'f', sep='\n')
    meanCSig=float(mean(CSig[0:long(len(CSig)/2)]))
    CSig = CSig - meanCSig
    CSigT = fromfile( options.ctrigfile, 'f', sep='\n')

```

```

TR=options.TR
OrigSamp=options.OrigSamp
NewSamp=options.NewSamp
SampRatio=long(OrigSamp/NewSamp)
SampRatioIndex=SampRatio-1
samplerate=OrigSamp
Nyquist=long(samplerate/2)
PlotAll=options.PlotAll
MagThresh=options.RMagThresh
OrigTimeThresh=long(options.RTimeThresh*OrigSamp)
NewTimeThresh=long(options.RTimeThresh*NewSamp)
NumTR=options.NumTR
TotOutSampNew= long(TR*NumTR*NewSamp)

```

```

TotOutSampOrig= long(TR*NumTR*OrigSamp)

COrigSamp=OrigSamp
CNewSamp=NewSamp
CSampRatio=SampRatio
CSampRatioIndex=SampRatioIndex
Csamplerate=OrigSamp
CNyquist=long(Csamplerate/2)
CMagThresh=options.CMagThresh
COrigTimeThresh= long(options.CTimeThresh*OrigSamp)
CNewTimeThresh= long(options.CTimeThresh*NewSamp)
CTotOutSampNew= long(TR*NumTR*CNewSamp)
CTotOutSampOrig= long(TR*NumTR*COrigSamp)

#Trim
#Sig=Sig[0:TotOutSampOrig]
#CSig=CSig[0:TotOutSampOrig]

####-----Butter Filter  Data

fpass=options.fpass #passband edge frequency
fstop=options.fstop  #stopband edge frequency

filter_b,filter_a=scipy.signal.iirdesign(float(fpass)/Nyquist,float(fstop)/Nyquist,gpass=5,gstop=60,analog=0,ftype="butter")

FiltFiltSig=filtfilt(filter_b, filter_a, Sig)

w, h = freqz(filter_b,filter_a)
mag = abs(h)
phase = unwrap(angle(h))*180/pi
radsample=w/pi

x= int((log(OrigSamp)-log(0.01))/log(2))
NFFT=int(2**x)
nooverlap=int(0.5*(2**x))
pxx, freqs = psd( FiltFiltSig, NFFT=NFFT ,
Fs=OrigSamp,nooverlap=nooverlap , detrend=detrend_linear,
window=window_hanning)
maxfreqpt = pxx.argmax()
maxfreq =freqs[maxfreqpt]
TPeriod=1./maxfreq
PeakPower=pxx[maxfreqpt]

if maxfreq==0:
    print "Respiratory power peak at zero. Threshold
at",freqs[4],"Hz"
    Tempxx=pxx[5:]
    Tempfreqs=freqs[5:]
    maxfreqpt = Tempxx.argmax()
    maxfreq =Tempfreqs[maxfreqpt]
    TPeriod=1./maxfreq
    PeakPower=Tempxx[maxfreqpt]

print "The spectral respiratory peak is:", maxfreq,"Hz "
print "The respiratory peak power density is",PeakPower

```

```

print "The spectral respiratory period is", 1./maxfreq,"seconds"

#Cardiac
Cfilter_b,Cfilter_a=scipy.signal.iirdesign(float(fpass)/Nyquist,float(f
stop)/Nyquist,gpass=5,gstop=60,analog=0, ftype="butter")
CFiltFiltSig=filtfilt(Cfilter_b, Cfilter_a, CSig)
Cpxx, Cfreqs = psd( CFiltFiltSig, NFFT=NFFT ,
Fs=OrigSamp,noverlap=nooverlap , detrend=detrend_linear,
window=window_hanning)
Cmaxfreqpt = Cpxx.argmax()
Cmaxfreq =Cfreqs[Cmaxfreqpt]
CTPeriod=1./Cmaxfreq
CPeakPower=pxx[maxfreqpt]
if Cmaxfreq==0:
    print "Cardiac power peak at zero. Threshold at",freqs[4],"Hz"
    CTempxx=Cpxx[5:]
    CTempfreqs=Cfreqs[5:]
    Cmaxfreqpt = CTempxx.argmax()
    Cmaxfreq =CTempfreqs[Cmaxfreqpt]
    CTPeriod=1./Cmaxfreq
    CPeakPower=CTempxx[Cmaxfreqpt]

print "The spectral cardiac peak is:", Cmaxfreq,"Hz "
print "The cardiac peak power density is",CPeakPower
print "The spectral cardiac period is", 1./Cmaxfreq,"seconds"

#FinalFreq=[]
#if Cmaxfreq==0:
#    CidPeaks= nonzero(greater_equal( Cpxx,100000))
#    Cmaxfreq=Cfreqs[CidPeaks]
#    print "The spectral cardiac power densities are:",Cpxx[CidPeaks]
#    print "The spectral cardiac frequency is:", Cmaxfreq,"Hz"
#    print "The spectral cardiac period is", 1./Cmaxfreq,"seconds"

if PlotAll:
    figure(1)
    subplot(311)
    semilogy(radsample*Nyquist,mag)
    ylabel('Magnitude')
    title('Bode Plot')
    pylab.ylim((10e-4, 10e0))
    pylab.xlim((0.0, fstop))

    figure(1)
    subplot(312)
    plot(freqs,pxx)
    pylab.ylabel('Respiratory Power')
    pylab.xlim((0.0, fstop))

    figure(1)
    subplot(313)
    plot(Cfreqs,Cpxx)
    pylab.xlabel('frequency(Hz)')
    pylab.ylabel('Cardiac Power')

```

```

pylab.xlim((0.0, fstop))

#-----#
Expand Trigger File
FiltSig=FiltFiltSig
SigTime_ms = range(len(Sig))
SigTPeak=zeros(len(Sig),'int')
SigTtips=zeros(len(SigT),'f')
SigTtipsTime=zeros(len(SigT),'int')

CFiltSig=CFiltFiltSig
CSigTime_ms = range(len(CSig))
CSigTPeak=zeros(len(CSig),'int')
CSigTtips=zeros(len(CSigT),'f')
CSigTtipsTime=zeros(len(CSigT),'int')

for counter in xrange(len(CSigT)) :
    ms = CSigT[counter]
    CSigTPeak[ms] = CFiltSig[ms]
    CSigTtips[counter]=CFiltSig[ms]
    CSigTtipsTime[counter]= ms
CPttl=CSigTPeak
#-----#Remove regions of
high noise variance for interpolation
print "Finding the regions of high variance"

print "Start Resp"
ResidSig=Sig - FiltSig
FoldSTD=options.FoldSTD
OutlierThresh =float(FoldSTD)*std(ResidSig)
RemoveRange= options.TrimWindow
ExtremTime=0
FwdRm=options.FwdRm
AllOutliers=zeros(len(FiltSig))
DirtyPoints= []
SigThreshResid = abs(ResidSig) - OutlierThresh
idUglyPoints= nonzero(greater_equal(SigThreshResid,0))
for counter in idUglyPoints:
    AllOutliers[counter]=ResidSig[counter]
    for i in (range(long( counter -
RemoveRange), long(counter+FwdRm*RemoveRange))) :
        DirtyPoints.extend([i])
DirtyPoints= sort(unique(clip(unique(DirtyPoints),1, len(ResidSig))))
NoOutliers=zeros(len(FiltSig))
NoOutlierTime=zeros(len(SigTime_ms))
NoOutliers=delete(FiltSig, DirtyPoints)
NoOutlierTime=delete(SigTime_ms, DirtyPoints)

#Cardiac
print "Start Cardiac"
CResidSig=CSig - CFiltSig
CFoldSTD=options.FoldSTD
COutlierThresh =float(FoldSTD)*std(CResidSig)
CRemoveRange= options.TrimWindow
CExtremTime=0

```

```

CFwdRm=options.FwdRm
CAllOutliers=zeros(len(CFiltSig))
CDirtyPoints= []
CSigThreshResid = abs(CResidSig) - COutlierThresh
CidUglyPoints= nonzero(greater_equal(CSigThreshResid,0))
for counter in CidUglyPoints:
    CAllOutliers[counter]=CResidSig[counter]
    for i in (range(long( counter -
CRemoveRange), long(counter+CFwdRm*CRemoveRange))) :
        CDirtyPoints.extend([i])
CDirtyPoints= sort(unique(clip(unique(CDirtyPoints),1,len(CResidSig))))
CNoOutliers=zeros(len(CFiltSig))
CNoOutlierTime=zeros(len(CSigTime_ms))
CNoOutliers=delete(CFiltSig,CDirtyPoints)
CNoOutlierTime=delete(CSigTime_ms,CDirtyPoints)

```

```

print "Interpolating"
print "Respiratory"

```

```

#Generate spline
SplineEst = scipy.interpolate.splrep(CNoOutlierTime ,CNoOutliers)
BestFit = scipy.interpolate.splev(CSigTime_ms , SplineEst)
print "Cardiac"

CSplineEst = scipy.interpolate.splrep(CNoOutlierTime ,CNoOutliers)
CBestFit = scipy.interpolate.splev(CSigTime_ms , CSplineEst)

```

```

if PlotAll:
#    figure(2000)
#    plot(CNoOutlierTime,CNoOutliers,'bo',label='NoOutliers')
#    pylab.legend()

    figure(2)
    subplot(211)
    plot(CSigTime_ms,CFiltSig,'b-', CSigTime_ms,CBestFit,'go',
SigTime_ms, CSig, 'k', CSigTime_ms, CResidSig,'r' )
    pylab.legend((r'Filtered', r'Spline', r'Raw', r'Residual'),
shadow = True, loc = 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0], fontsize = 8, color = 'b')
    pylab.setp(ltext[1], fontsize = 8, color = 'g')
    pylab.setp(ltext[2], fontsize = 8, color = 'k')
    pylab.setp(ltext[3], fontsize = 8, color = 'r')
    title('Respiratory')

    subplot(212)
    plot(CSigTime_ms,CFiltSig,'b-', CSigTime_ms,CBestFit,'go',
CSigTime_ms, CSig, 'k', CSigTime_ms, CResidSig,'r' )
    pylab.legend((r'Filtered', r'Spline', r'Raw', r'Residual'),
shadow = True, loc = 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0], fontsize = 8, color = 'b')
    pylab.setp(ltext[1], fontsize = 8, color = 'g')

```

```

pylab.setp(ltext[2],  fontsize = 8, color = 'k')
pylab.setp(ltext[3],  fontsize = 8, color = 'r')
title('Cardiac')

#-----Data is now clean
CleanSig=BestFit
CCleanSig=CBestFit

AllExtrema, AllExtremaTime, AllHills, AllValleys, TopEnv, BotEnv,
TopCounts, BotCounts=findextrema(CleanSig, MagThresh, OrigTimeThresh)
CAllExtrema, CAllExtremaTime, CAllHills, CAllValleys, CTopEnv, CBotEnv,
CTopCounts, CBotCounts=findextrema(CCleanSig, CMagThresh,
COrigTimeThresh)

"""
if PlotAll:
#Resp
    figure(3)
    subplot(321); plot(SigTime_ms, Sig, 'b', SigTime_ms, FiltSig, 'g', SigTi
me_ms, ResidSig, 'r' )
    ylabel('Amplitude')
    grid(True)
    pylab.legend((r'Raw', r'Filter', r'Residual'), shadow = True, loc
= 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0],  fontsize = 8, color = 'b')
    pylab.setp(ltext[1],  fontsize = 8, color = 'g')
    pylab.setp(ltext[2],  fontsize = 8, color = 'r')
    title('Respiratory')

    figure(3)
    subplot(323)
    plot(SigTime_ms, FiltSig, 'b', SigTime_ms,
SigTPeak, 'r', linewidth=1.0)
    pylab.legend((r'Filter', r'TTLTrigger'), shadow = True, loc = 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0],  fontsize = 8, color = 'b')
    pylab.setp(ltext[1],  fontsize = 8, color = 'r')

    figure(3)
    subplot(325); plot(SigTime_ms, CleanSig, 'k', SigTime_ms, FiltSig,
'b', SigTime_ms, TopEnv, 'r', SigTime_ms, BotEnv, 'g');
    pylab.legend((r'Spline', r'Filtered', r'TopEnv', r'BotEnv'),
shadow = True, loc = 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0],  fontsize = 8, color = 'k')
    pylab.setp(ltext[1],  fontsize = 8, color = 'b')
    pylab.setp(ltext[2],  fontsize = 8, color = 'r')
    pylab.setp(ltext[3],  fontsize = 8, color = 'g')
    xlabel('Time (ms)')

#Cardiac

```



```

figure(3)
subplot(322); plot(CSigTime_ms, CSig, 'b', CSigTime_ms, CFiltSig, 'g', C
SigTime_ms, CResidSig, 'r' )
ylabel('Amplitude')
grid(True)
pylab.legend((r'Raw', r'Filter', r'Residual'), shadow = True, loc
= 1)

ltext = pylab.gca().get_legend().get_texts()
pylab.setp(ltext[0], fontsize = 8, color = 'b')
pylab.setp(ltext[1], fontsize = 8, color = 'g')
pylab.setp(ltext[2], fontsize = 8, color = 'r')
title('Cardiac')


figure(3)
subplot(324)
plot(CSigTime_ms, CFiltSig, 'b', CSigTime_ms,
CSigTPeak, 'r', linewidth=1.0)
pylab.legend((r'Filter', r'TTLTrigger'), shadow = True, loc = 1)
ltext = pylab.gca().get_legend().get_texts()
pylab.setp(ltext[0], fontsize = 8, color = 'b')
pylab.setp(ltext[1], fontsize = 8, color = 'r')


figure(3)
subplot(326); plot(CSigTime_ms, CCleanSig, 'k', CSigTime_ms,
CFiltSig, 'b', CSigTime_ms, CTopEnv, 'r', CSigTime_ms, CBotEnv, 'g');
pylab.legend((r'Spline', r'Filtered', r'TopEnv', r'BotEnv'),
shadow = True, loc = 1)
ltext = pylab.gca().get_legend().get_texts()
pylab.setp(ltext[0], fontsize = 8, color = 'k')
pylab.setp(ltext[1], fontsize = 8, color = 'b')
pylab.setp(ltext[2], fontsize = 8, color = 'r')
pylab.setp(ltext[3], fontsize = 8, color = 'g')
xlabel('Time (ms)')


#Resp
figure(4)
subplot(211);
plot(SigTime_ms, CleanSig, 'k', SigTime_ms, FiltSig, 'b', SigTime_ms,
TopEnv, 'c', SigTime_ms, BotEnv, 'm', SigTime_ms, AllOutliers, 'go',
SigTime_ms, Sig, 'y', NoOutlierTime, NoOutliers, 'ro', SigTime_ms,
ResidSig, 'r');
pylab.legend((r'Spline', r'Filtered', r'TopEnv', r'BotEnv',
r'Outliers', r'RawSignal', r'NoOutliers', r'Resid'), shadow = True, loc
= 1)

ltext = pylab.gca().get_legend().get_texts()
pylab.setp(ltext[0], fontsize = 8, color = 'k')
pylab.setp(ltext[1], fontsize = 8, color = 'b')
pylab.setp(ltext[2], fontsize = 8, color = 'c')
pylab.setp(ltext[3], fontsize = 8, color = 'm')
pylab.setp(ltext[4], fontsize = 8, color = 'g')
pylab.setp(ltext[5], fontsize = 8, color = 'y')
pylab.setp(ltext[6], fontsize = 8, color = 'r')
pylab.setp(ltext[7], fontsize = 8, color = 'r')
title('Respiratory')

```

```

#Card
    figure(4)
    subplot(212);
    plot(CSigTime_ms,CCleanSig,'k',CSigTime_ms,
CFiltSig,'b',CSigTime_ms, CTopEnv,'c', CSigTime_ms, CBotEnv, 'm',
CSigTime_ms, CAllOutliers,'go', CSigTime_ms, CSig,'y', CNoOutlierTime,
CNoOutliers,'ro',CSigTime_ms, CResidSig,'r');
    pylab.legend((r'Spline', r'Filtered', r'TopEnv', r'BotEnv',
r'Outliers', r'RawSignal', r'NoOutliers',r'Resid'), shadow = True, loc
= 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0],  fontsize = 8, color = 'k')
    pylab.setp(ltext[1],  fontsize = 8, color = 'b')
    pylab.setp(ltext[2],  fontsize = 8, color = 'c')
    pylab.setp(ltext[3],  fontsize = 8, color = 'm')
    pylab.setp(ltext[4],  fontsize = 8, color = 'g')
    pylab.setp(ltext[5],  fontsize = 8, color = 'y')
    pylab.setp(ltext[6],  fontsize = 8, color = 'r')
    pylab.setp(ltext[7],  fontsize = 8, color = 'r')
    title('Cardiac')

"""

#-----
-----Find Volume over Time
print"Calculating Envelopes"

print "Respiratory"

RWTopEnv=TopEnv
FullInterpTimeEnv=ConnectTheDots(TopCounts,diff(TopCounts) ,CleanSig,1)
FullInterpTopEnv=ConnectTheDots(TopCounts,AllHills,CleanSig,0)
FullInterpBotEnv=ConnectTheDots(BotCounts,AllValleys,CleanSig,0)
FullInterpRVTEnv=zeros(len(CleanSig), 'f')
FullInterpRVTEnv = OrigSamp*abs(FullInterpTopEnv-FullInterpBotEnv)/
(FullInterpTimeEnv)

MeanRVT=zeros(len(CleanSig), 'f')
MeanRVT = (    abs(FullInterpTopEnv)+    abs(FullInterpBotEnv))/ 2.

print "Cardiac"
CRWTopEnv=CTopEnv
CFullInterpTimeEnv=ConnectTheDots(CTopCounts,diff(CTopCounts)
,CCleanSig,1)
CFullInterpTopEnv=ConnectTheDots(CTopCounts,CAllHills,CCleanSig,0)
CFullInterpBotEnv=ConnectTheDots(CBotCounts,CAllValleys,CCleanSig,0)
CFullInterpRVTEnv=zeros(len(CCleanSig), 'f')
CFullInterpRVTEnv = COrigSamp*abs(CFullInterpTopEnv-CFullInterpBotEnv)/
(CFullInterpTimeEnv)

CMeanRVT=zeros(len(CCleanSig), 'f')
CMeanRVT = (abs(CFullInterpTopEnv)+abs(CFullInterpBotEnv))/ 2.

if PlotAll:
    figure(7)

```

```

        subplot(211)
        plot(SigTime_ms,CleanSig,'b'); hold(True);
        plot(SigTime_ms,FullInterpTopEnv, 'r', SigTime_ms ,
FullInterpBotEnv , 'g',SigTime_ms ,TopEnv, 'ro',SigTime_ms ,BotEnv,
'go',SigTime_ms , FullInterpRVTEnv , 'c', SigTime_ms, MeanRVT, 'y')
        hold(False)
        pylab.legend((r'Spline', r'TopEnvInterpl', r'BotEnvInterpl',
r'TopEnv', r'BotEnv', r'RVTEnterpl', r'MeanAbsEnv' ), shadow = True,
loc = 1)
        ltext = pylab.gca().get_legend().get_texts()
        pylab.setp(ltext[0],  fontsize = 8, color = 'b')
        pylab.setp(ltext[1],  fontsize = 8, color = 'r')
        pylab.setp(ltext[2],  fontsize = 8, color = 'g')
        pylab.setp(ltext[3],  fontsize = 8, color = 'r')
        pylab.setp(ltext[4],  fontsize = 8, color = 'g')
        pylab.setp(ltext[5],  fontsize = 8, color = 'c')
        pylab.setp(ltext[6],  fontsize = 8, color = 'y')
        title('Respiratory Data')

figure(7)
subplot(212)
plot(CSigTime_ms,CCleanSig,'b'); hold(True);
plot(CSigTime_ms,CFullInterpTopEnv, 'r', CSigTime_ms ,
CFullInterpBotEnv , 'g',CSigTime_ms ,CTopEnv, 'ro',CSigTime_ms
,CBotEnv, 'go',CSigTime_ms , CFullInterpRVTEnv , 'c',  CSigTime_ms ,
CMeanRVT, 'y')
        hold(False)
        pylab.legend((r'Spline', r'TopEnvInterpl', r'BotEnvInterpl',
r'TopEnv', r'BotEnv', r'CVTEnterpl', r'MeanAbsEnv' ), shadow = True,
loc = 1)
        ltext = pylab.gca().get_legend().get_texts()
        pylab.setp(ltext[0],  fontsize = 8, color = 'b')
        pylab.setp(ltext[1],  fontsize = 8, color = 'r')
        pylab.setp(ltext[2],  fontsize = 8, color = 'g')
        pylab.setp(ltext[3],  fontsize = 8, color = 'r')
        pylab.setp(ltext[4],  fontsize = 8, color = 'g')
        pylab.setp(ltext[5],  fontsize = 8, color = 'c')
        pylab.setp(ltext[6],  fontsize = 8, color = 'y')
        title('Cardiac Data')

#-----Find third derivatives of Cardiac signal
to create new trigger file
#CCSigBase=CCleanSig

CCSigBase= CCleanSig
print"Finding Cardiac Derivatives"
dCCleanSig1=slopes(CSigTime_ms,CCSigBase)      *(COrigSamp)
#figure(3333321)
#plot(CSigTime_ms,dCCleanSig1,'b',CSigTime_ms,CCSigBase,'r',CSigTime_ms
,CCleanSig,'c')

dCCleanSig2=slopes(CSigTime_ms,dCCleanSig1)      *(COrigSamp)
dCCleanSig3=slopes(CSigTime_ms,dCCleanSig2)      *(COrigSamp)
CSmoothCCleanSig3=cmov_average(dCCleanSig3,int(0.05*COrigSamp))
#Find large Troughs

```

```

CallExtrema, CallExtremaTime, CallHills, CallValleys, CTopEnv, CBotEnv,
CTopCounts, CBotCounts=findextrema( CSmoothCCleanSig3, MagThresh,
NewTimeThresh)

idRise=nonzero(less_equal(dCCleanSig1 ,0 ))
put(CBotEnv,idRise,0)
CPd3=-1.*CBotEnv
#CPd3 has location of the large deriv3 troughs

#use envelope of deriv3 peaks as a threshold
CPd3Threshd3 =ConnectTheDots(CTopCounts,abs(CallHills),CSig,0)

print "Thresholding CPd3"

idRise=nonzero(less_equal(CPd3-CPd3Threshd3,0))
put(CPd3,idRise,0)
idRise=nonzero(less_equal(CPd3,0.5*mean(CPd3Threshd3)))
put(CPd3,idRise,0)

# now find R waves which are the deriv3 peaks that precede the trough
which is in CPd3
CPd3Rwave=zeros(len(CCSigBase), 'f')
CRwaveCounts=array(nonzero(greater(CPd3,0)))
for peaktime in CRwaveCounts:
    tempR=nonzero(less(CTopCounts,peaktime))
    CPd3Rwave[CTopCounts[tempR[-1]]]= CPd3[peaktime]

if PlotAll:

    figure(41)
    subplot(211)
    plot(CSigTime_ms,CCSigBase*(COrigSamp),'k', CSigTime_ms,
CSigTPeak*(COrigSamp), 'r',CSigTime_ms,CPd3,'b', CSigTime_ms,
dCCleanSig1*(COrigSamp), 'g', CSigTime_ms, dCCleanSig2,
'y',CSigTime_ms, CSmoothCCleanSig3, 'c');
    pylab.legend((r'Filter', r'CPttl', r'CPd3', r'Deriv1', r'Deriv2',
r'Deriv3'), shadow = True, loc = 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0], fontsize = 8, color = 'k')
    pylab.setp(ltext[1], fontsize = 8, color = 'r')
    pylab.setp(ltext[2], fontsize = 8, color = 'b')
    pylab.setp(ltext[3], fontsize = 8, color = 'g')
    pylab.setp(ltext[4], fontsize = 8, color = 'y')
    pylab.setp(ltext[5], fontsize = 8, color = 'c')
    xlabel('Time (ms)')
    subplot(212)
    plot(CSigTime_ms,CCSigBase*(COrigSamp),'k', CSigTime_ms,
CSigTPeak*(COrigSamp), 'r',CSigTime_ms, CPd3 , 'b',CSigTime_ms,
CSmoothCCleanSig3, 'c',CSigTime_ms, CPd3Threshd3, 'y',CSigTime_ms,
CPd3Rwave, 'm');
    pylab.legend((r'Filter', r'CPttl', r'CPd3', r'Deriv3',
r'Threshold', r'Rwave'), shadow = True, loc = 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0], fontsize = 8, color = 'k')
    pylab.setp(ltext[1], fontsize = 8, color = 'r')
    pylab.setp(ltext[2], fontsize = 8, color = 'b')

```

```

        pylab.setp(ltext[3],  fontsize = 8, color = 'c')
        pylab.setp(ltext[4],  fontsize = 8, color = 'y')
        pylab.setp(ltext[5],  fontsize = 8, color = 'm')
        xlabel('Time (ms)')

#-----Calculate respiratory (RRT) and cardiac rate per
time (CRT)
print "Calculating Rates over Time"
#Calculate RRT

RCPd3SigT=array(nonzero(greater(TopEnv,0)))
RCSigTHills=COrigSamp * (diff(RCPd3SigT)**(-1.))
RCSigTTopCounts=RCPd3SigT[0:-1]
RRT=ConnectTheDots(RCSigTTopCounts,RCSigTHills,Sig,0)

CSigTHills= (diff(CSigT)**(-1))*COrigSamp
CSigTTopCounts=CSigT[0:-1]
CRTttl=ConnectTheDots(CSigTTopCounts,CSigTHills,CSig,0)

#Calculate CRTd3

CPd3SigT=array(nonzero(greater(CPd3,0)))
CSigTHills=COrigSamp * (diff(CPd3SigT)**(-1.))
CSigTTopCounts=CPd3SigT[0:-1]
CRTd3=ConnectTheDots(CSigTTopCounts,CSigTHills,CSig,0)

#Calculate CRTd3R

CPd3RSigT=array(nonzero(greater(CPd3Rwave,0)))
CSigTHills=COrigSamp * (diff(CPd3RSigT)**(-1.))
CSigTTopCounts=CPd3RSigT[0:-1]
CRTd3R=ConnectTheDots(CSigTTopCounts,CSigTHills,CSig,0)

if PlotAll:
    figure(333)
    subplot(321)
    plot(CRTd3R)
    ylabel('CRTd3R')
    subplot(322)
    plot(CRTttl)
    ylabel('CRTttl')
    subplot(324)
    plot(CRTd3)
    ylabel('CRTd3')
    subplot(323)
    plot(RRT)
    ylabel('RRT')
    subplot(325)
    plot(FullInterpRVTEEnv)
    ylabel('RVT')
    subplot(326)
    plot(CFullInterpRVTEEnv)
    ylabel('CVT')

```

```

#-----
-----DownSampling
print "Downsampling"
#Resp
SigDownSamp,SigDownSamplePt, SigDownSamplePtTime=DownSample(CleanSig)
#Cardiac
CSigDownSamp,CSigDownSamplePt,
CSigDownSamplePtTime=DownSample(CCleanSig)
"""
if PlotAll:
    figure(5)
    subplot(211)
    plot(SigTime_ms, CleanSig,'b',SigTime_ms, Sig ,'k', SigTime_ms,
SigDownSamp,'r')
    pylab.legend((r'Spline', r'Raw', r'Downsamped'), shadow = True,
loc = 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0], fontsize = 8, color = 'b')
    pylab.setp(ltext[1], fontsize = 8, color = 'k')
    pylab.setp(ltext[2], fontsize = 8, color = 'r')
    title('Downsampled Respiratory Spline')

    figure(5)
    subplot(212)
    plot(CSigTime_ms, CCleanSig,'b',CSigTime_ms, CSig ,'k',
CSigTime_ms, CSigDownSamp,'r')
    pylab.legend((r'Spline', r'Raw', r'Downsamped'), shadow = True,
loc = 1)
    ltext = pylab.gca().get_legend().get_texts()
    pylab.setp(ltext[0], fontsize = 8, color = 'b')
    pylab.setp(ltext[1], fontsize = 8, color = 'k')
    pylab.setp(ltext[2], fontsize = 8, color = 'r')
    title('Downsampled Cardiac Spline')
"""
#-----Trim length for
retroicor
print "Trim length"
SigDownSamplePt=SigDownSamplePt[0:TotOutSampNew]
RWTopEnv=TopEnv[0:TotOutSampOrig]
FullInterpRVTEEnv= FullInterpRVTEEnv[0:TotOutSampOrig]
RRT=RRT[0:TotOutSampOrig]

CleanSig=CleanSig[0:TotOutSampOrig]
CCleanSig=CCleanSig[0:CTotOutSampOrig]

CPd3=CPd3[0:TotOutSampOrig]
CPd3Rwave=CPd3Rwave[0:TotOutSampOrig]
CPttl=CPttl[0:TotOutSampOrig]

CRTd3=CRTd3[0:TotOutSampOrig]
CRTttl=CRTttl[0:TotOutSampOrig]
CRTd3R=CRTd3R[0:TotOutSampOrig]

CSigDownSamplePt=CSigDownSamplePt[0:CTotOutSampNew]
CRWTopEnv=CRWTopEnv[0:CTotOutSampOrig]
CFullInterpRVTEEnv= CFullInterpRVTEEnv[0:CTotOutSampOrig]

```

```

#-----Calculating Stats
print "Calculating Statistics for", options.Prefix
OutputStat= zeros(65,'f')

OutputStat[0]=( len(nonzero(greater(RWTopEnv ,0 ))) )
print "Peakdet detected ",OutputStat[0] , " positive respiratory
peaks"
OutputStat[1]=( mean(diff(nonzero(greater( RWTopEnv ,0 )))) )
print "The RW peakdet average respiratory interpeak time was",
OutputStat[1]
OutputStat[2]=( std(diff(nonzero(greater( RWTopEnv ,0 )))) )
print "The RW peakdet std respiratory interpeak time was",
OutputStat[2]

OutputStat[3]=( maxfreq )
print "The spectral respiratory peak is:",OutputStat[3] , "Hz "

OutputStat[4]=(PeakPower )
print "The respiratory peak power density is", OutputStat[4]

OutputStat[5]=(1./maxfreq )
print "The spectral respiratory period is",OutputStat[5] , "seconds"

OutputStat[6]=( Cmaxfreq )
print "The spectral cardiac peak is:",OutputStat[6] , "Hz "

OutputStat[7]=(CPeakPower )
print "The cardiac peak power density is", OutputStat[7]

OutputStat[8]=( 1./Cmaxfreq )
print "The spectral cardiac period is",OutputStat[8] , "seconds"

OutputStat[9]=( len(nonzero(greater( CPttl ,0 ))) )
print "The trigger detected ",OutputStat[9] , " positive cardiac peaks"

OutputStat[10]=( len(nonzero(greater(CPd3Rwave ,0 ))) )
print "CPd3R detected ",OutputStat[10] , " positive cardiac peaks"

OutputStat[11]=( mean(diff(nonzero(greater( CPttl ,0 )))) )
print "The average cardiac CPttl interpeak time was",
OutputStat[11]
OutputStat[12]=( mean(diff(nonzero(greater( CPd3Rwave ,0 )))) )
print "The average cardiac CPd3R interpeak time was", OutputStat[12]
OutputStat[13]=( std(diff(nonzero(greater( CPttl ,0 )))) )
print "The std cardiac CPttl interpeak time was", OutputStat[13]

OutputStat[14]=( std(diff(nonzero(greater( CPd3Rwave ,0 )))) )
print "The std cardiac CPd3R interpeak time was", OutputStat[14]

OutputStat[15]=( std(CRTttl) )
print "The CRTttl std is:", OutputStat[15]
OutputStat[16]=( std(CRTd3) )
print "The CRTd3 std is:", OutputStat[16]

```

```

OutputStat[17]=( std(CRTd3R) )
print "The CCRTd3R std is:", OutputStat[17]

OutputStat[18]=( mean(CRTttl) )
print "The CRTttl mean is:", OutputStat[18]
OutputStat[19]=( mean(CRTd3) )
print "The CRTd3 mean is:", OutputStat[19]
OutputStat[20]=( mean(CRTd3R) )
print "The CCRTd3R mean is:", OutputStat[20]

OutputStat[21]=( len(nonzero(greater(CPd3 ,0 ))) )
print "CPd3 detected ", OutputStat[21] ," positive cardiac peaks"

OutputStat[22]=( mean(diff(nonzero(greater( CPd3 ,0 )))) )
print "The average cardiac CPd3 interpeak time was", OutputStat[22]
OutputStat[23]=( std(diff(nonzero(greater( CPd3 ,0 )))) )
print "The std cardiac CPd3 interpeak time was", OutputStat[23]

OutputStat[24]=( mean((nonzero(greater( CPTtl ,0 )))) )
print "The avg cardiac CPTtl peak time was", OutputStat[24]
OutputStat[25]=( mean((nonzero(greater( CPd3 ,0 )))) )
print "The avg cardiac CPd3 peak time was", OutputStat[25]
OutputStat[26]=( mean((nonzero(greater( CPd3Rwave ,0 )))) )
print "The avg cardiac CPd3R peak time was", OutputStat[26]

OutputStat[27]=( std((nonzero(greater( CPTtl ,0 )))) )
print "The std cardiac CPTtl peak time was", OutputStat[27]
OutputStat[28]=( std((nonzero(greater( CPd3 ,0 )))) )
print "The std cardiac CPd3 peak time was", OutputStat[28]
OutputStat[29]=( std((nonzero(greater( CPd3Rwave ,0 )))) )
print "The std cardiac CPd3R peak time was", OutputStat[29]

# now find time between CPTtl and both CPd3 and CPd3R
PostPeakd3=array(nonzero(greater(CPd3,0)))
PostPeakttl=array(nonzero(greater(CPTtl,0)))
PeakBase=array(nonzero(greater(CPd3Rwave,0)))
InterPeakCounts3=zeros(len(PeakBase), 'f')
InterPeakCountsttl=zeros(len(PeakBase), 'f')
IPd3Mttl=zeros(len(PeakBase), 'f')
counter=0
for peaktime in PeakBase[1:-1]:
    TempPostd3=nonzero(greater_equal(PostPeakd3,peaktime))
    TempPostttl=nonzero(greater_equal(PostPeakttl,peaktime))
    if len(TempPostd3)!=0 and len(TempPostttl)!=0 :
        InterPeakCounts3[counter]=PostPeakd3[TempPostd3[0]]-
peaktime
        InterPeakCountsttl[counter]=PostPeakttl[TempPostttl[0]]-
peaktime
        IPd3Mttl[counter]=InterPeakCounts3[counter]-
InterPeakCountsttl[counter]
        counter=counter+1
OutputStat[30]= mean( InterPeakCounts3 )
print "The avg time between CPd3R and CPd3 peaks was",OutputStat[30]
OutputStat[31]= std( InterPeakCounts3 )
print "The std time between CPd3R and CPd3 peaks was",OutputStat[31]

```



```

OutputStat[32]= mean( InterPeakCountsttl )
print "The avg  time between CPd3R and CPttl peaks was",OutputStat[32]

OutputStat[33]= std( InterPeakCountsttl )
print "The std  time between CPd3R and CPttl peaks was",OutputStat[33]

OutputStat[34]= mean( IPd3Mttl )
print "The avg  time between CPd3 and CPttl peaks was",OutputStat[34]
OutputStat[35]= std( IPd3Mttl )
print "The std  time between CPd3 and CPttl peaks was",OutputStat[35]
#PostPeakBase=array(nonzero(greater(CPttl,0)))
#PeakBase=array(nonzero(greater(CPd3Rwave,0)))
#InterPeakCounts=zeros(len(PeakBase), 'f')
#counter=0
#for peaktime in PeakBase[1:-1]:
#    TempPost=nonzero(greater_equal(PrePeakBase,peaktime))
#    InterPeakCounts[counter]=PostPeakBase[TempPost[0]]- peaktime
#    counter=counter+1

OutputStat[36]= len(nonzero(greater( CRTttl ,mean(CRTttl)+3*std(CRTttl)
)))
print "The number of CRTttl peaks > 3 std",OutputStat[36]

OutputStat[37]= len(nonzero(greater( CRTd3 ,mean(CRTd3)+3*std(CRTd3)
)))
print "The number of CRTd3 peaks > 3 std",OutputStat[37]

OutputStat[38]= len(nonzero(greater( CRTd3R ,mean(CRTd3R)+3*std(CRTd3R)
)))
print "The number of CRTd3R peaks > 3 std",OutputStat[38]

OutputStat[39]= len(Sig)
print "Length of Sig",OutputStat[39]

OutputStat[40]= len(CSig)
print "Length of CSig",OutputStat[40]

OutputStat[41]= std(nonzero(greater( CRTttl ,mean(CRTttl)+3*std(CRTttl)
)))
print "The std of CRTttl peaks > 3 std",OutputStat[41]

OutputStat[42]= std(nonzero(greater( CRTd3 ,mean(CRTd3)+3*std(CRTd3)
)))
print "The std of CRTd3 peaks > 3 std",OutputStat[42]

OutputStat[43]= std(nonzero(greater( CRTd3R ,mean(CRTd3R)+3*std(CRTd3R)
)))
print "The std of CRTd3R peaks > 3 std",OutputStat[43]

OutputStat[44]= len(nonzero(greater( CRTttl ,mean(CRTttl)+1. )))
print "The number of CRTttl peaks > mean CRTttl + 1 ",OutputStat[44]

OutputStat[45]= len(nonzero(greater( CRTd3 ,mean(CRTd3)+1. )))
print "The number of CRTd3 peaks > mean CRTd3 + 1 ",OutputStat[45]

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```

OutputStat[46]= len(nonzero(greater( CRTd3R ,mean(CRTd3R)+1.)))
print  "The number of CRTd3R peaks > mean CRTd3R + 1 ",OutputStat[46]

OutputStat[47]= len(nonzero(greater( CRTttl ,mean(CRTttl)-0.4 )))
print  "The number of CRTttl peaks 0.4 Hz below mean ",OutputStat[47]

OutputStat[48]= len(nonzero(greater( CRTd3 ,mean(CRTd3)-0.4)))
print  "The number of CRTd3 peaks 0.4 Hz below mean ",OutputStat[48]

OutputStat[49]= len(nonzero(greater( CRTd3R ,mean(CRTd3R)-0.4)))
print  "The number of CRTd3R peaks 0.4 Hz below mean ",OutputStat[49]

OutputStat[50]=( len(nonzero(greater(CRWTopEnv ,0 ))) )
print  "Peakdet detected ",OutputStat[50]  ," positive cardiac peaks"

OutputStat[51]=( mean(diff(nonzero(greater( CRWTopEnv ,0 )))) )
print  "The peakdet average cardiac interpeak time was",
OutputStat[51]
OutputStat[52]=( std(diff(nonzero(greater( CRWTopEnv ,0 )))) )
print  "The peakdet std cardiac interpeak time was",  OutputStat[52]

OutputStat[53]= len(nonzero(greater( CRTttl ,mean(CRTttl)+4*std(CRTttl)
)))
print  "The number of CRTttl peaks > 4 std",OutputStat[53]

OutputStat[54]= len(nonzero(greater( CRTd3 ,mean(CRTd3)+4*std(CRTd3)
)))
print  "The number of CRTd3 peaks > 4 std",OutputStat[54]

OutputStat[55]= len(nonzero(greater( CRTd3R ,mean(CRTd3R)+4*std(CRTd3R)
)))
print  "The number of CRTd3R peaks > 4 std",OutputStat[55]

OutputStat[56]= std(nonzero(greater( CRTttl ,mean(CRTttl)+4*std(CRTttl)
)))
print  "The std of CRTttl peaks > 4 std",OutputStat[56]

OutputStat[57]= std(nonzero(greater( CRTd3 ,mean(CRTd3)+4*std(CRTd3)
)))
print  "The std of CRTd3 peaks > 4 std",OutputStat[57]

OutputStat[58]= std(nonzero(greater( CRTd3R ,mean(CRTd3R)+4*std(CRTd3R)
)))
print  "The std of CRTd3R peaks > 4 std",OutputStat[58]
print OutputStat
"-----"

print "Saving files"

#-----save files to disk

#from numpy import *
#from scipy.io import write_array
#write_array(filename , SigDownSamplePt , separator=' ', linesep='\n')
from pylab import save

```

```

filename = options.Prefix + '_OutputStatistics' + ".txt"
save(filename, OutputStat, fmt='%8.6f')

#RW
filename = options.Prefix + '_RW_Spline_' + str(NewSamp) + ".txt"
save(filename, SigDownSamplePt + abs( min(SigDownSamplePt)),
fmt='%8.6f')

SigTRfull, SigTRPt, SigTRPtTime=TRSample(CleanSig,1.0,OrigSamp)
filename = options.Prefix + '_TR_RW_Spline_' + str(OrigSamp) + ".txt"
save(filename, SigTRPt + abs(min(SigTRPt)), fmt='%8.6f')

HSigTRfull,HSigTRPt, HSigTRPtTime=TRSample(CleanSig,0.5,OrigSamp)
filename = options.Prefix + '_HalfTR_RW_Spline_' + str(OrigSamp) + ".txt"
save(filename, HSigTRPt + abs(min(HSigTRPt)), fmt='%8.6f' )

#RVT
filename = options.Prefix + '_RVT_' + str(OrigSamp) + ".txt"
save(filename, FullInterpRVTEnv + abs(min(FullInterpRVTEnv)),
fmt='%8.6f' )

SigTRfull, SigTRPt, SigTRPtTime=TRSample( FullInterpRVTEnv,1.0,OrigSamp)
filename = options.Prefix + '_TR_RVT_' + str(OrigSamp) + ".txt"
save(filename, SigTRPt + abs(min(SigTRPt)), fmt='%8.6f')

HSigTRfull,HSigTRPt, HSigTRPtTime=TRSample(FullInterpRVTEnv
,0.5,OrigSamp)
filename = options.Prefix + '_HalfTR_RVT_' + str(OrigSamp) + ".txt"
save(filename, HSigTRPt + abs(min(HSigTRPt)), fmt='%8.6f' )

#RRT
filename = options.Prefix + '_RRT_' + str(OrigSamp) + ".txt"
save(filename, RRT + abs(min(RRT)), fmt='%8.6f' )

SigTRfull, SigTRPt, SigTRPtTime=TRSample( RRT,1.0,OrigSamp)
filename = options.Prefix + '_TR_RRT_' + str(OrigSamp) + ".txt"
save(filename, SigTRPt + abs(min(SigTRPt)), fmt='%8.6f')

HSigTRfull,HSigTRPt, HSigTRPtTime=TRSample(RRT ,0.5,OrigSamp)
filename = options.Prefix + '_HalfTR_RRT_' + str(OrigSamp) + ".txt"
save(filename, HSigTRPt + abs(min(HSigTRPt)), fmt='%8.6f' )

#Cardiac Peaks
filename = options.Prefix + '_CPd3_' + str(OrigSamp) + ".txt"
save(filename, CPd3 , fmt='%8.6f' )

filename = options.Prefix + '_CPd3R_' + str(OrigSamp) + ".txt"
save(filename, CPd3Rwave , fmt='%8.6f' )

filename = options.Prefix + '_CPTtl_' + str(OrigSamp) + ".txt"
save(filename, CPd3 , fmt='%8.6f' )

```

```
#CRTd3 trough
```

```
filename = options.Prefix + '_CRTd3_' + str(OrigSamp) + ".txt"
save(filename, CRTd3 + abs(min(CRTd3)), fmt='%8.6f' )
```

```
SigTRfull, SigTRPt, SigTRPtTime=TRSample( CRTd3,1.0,OrigSamp)
filename = options.Prefix + '_TR_CRTd3_' + str(OrigSamp) + ".txt"
save(filename, SigTRPt + abs(min(SigTRPt)), fmt='%8.6f' )
```

```
HSigTRfull,HSigTRPt, HSigTRPtTime=TRSample(CRTd3 ,0.5,OrigSamp)
filename = options.Prefix + '_HalfTR_CRTd3_' + str(OrigSamp) + ".txt"
save(filename, HSigTRPt + abs(min(HSigTRPt)), fmt='%8.6f' )
```

```
#CRTd3Rwave
```

```
filename = options.Prefix + '_CRTd3R_' + str(OrigSamp) + ".txt"
save(filename, CRTd3R + abs(min(CRTd3R)), fmt='%8.6f' )
```

```
SigTRfull, SigTRPt, SigTRPtTime=TRSample( CRTd3R,1.0,OrigSamp)
filename = options.Prefix + '_TR_CRTd3R_' + str(OrigSamp) + ".txt"
save(filename, SigTRPt + abs(min(SigTRPt)), fmt='%8.6f' )
```

```
HSigTRfull,HSigTRPt, HSigTRPtTime=TRSample(CRTd3R ,0.5,OrigSamp)
filename = options.Prefix + '_HalfTR_CRTd3R_' + str(OrigSamp) + ".txt"
save(filename, HSigTRPt + abs(min(HSigTRPt)), fmt='%8.6f' )
```

```
#CRTttl
```

```
filename = options.Prefix + '_CRTttl_' + str(OrigSamp) + ".txt"
save(filename, CRTttl + abs(min(CRTttl)), fmt='%8.6f' )
```

```
SigTRfull, SigTRPt, SigTRPtTime=TRSample( CRTttl,1.0,OrigSamp)
filename = options.Prefix + '_TR_CRTttl_' + str(OrigSamp) + ".txt"
save(filename, SigTRPt + abs(min(SigTRPt)), fmt='%8.6f' )
```

```
HSigTRfull,HSigTRPt, HSigTRPtTime=TRSample(CRTttl ,0.5,OrigSamp)
filename = options.Prefix + '_HalfTR_CRTttl_' + str(OrigSamp) + ".txt"
save(filename, HSigTRPt + abs(min(HSigTRPt)), fmt='%8.6f' )
```

```
#-----
-----
```

```
if PlotAll:
    show()
```

Chapter 7:

Discussion, Future Studies, and Conclusion

Chapter 7: Discussion, Future Studies, and Conclusion

Discussion

In this thesis, we tested the hypothesis that the functional connectivity of affective face processing circuitry is dysfunctional in autism. By employing fMRI and a facial affect discrimination task that probes one of the core features of autism social behavior, we were able to identify functional connectivity alterations that may impair the flow of social information and contribute to aberrant social behavior in autism. While other studies have used functional connectivity to investigate various autism cognitive and social domains, we believe these are the first studies of autism using voxelwise functional amygdala and interhemispheric connectivity analyses to assess the integrity of circuits in the emotional face processing network. By extending investigations from altered activity in specific brain regions to dysfunctional connectivity between brain regions, our novel findings quantified functionally overconnected and underconnected circuits of interregional subcortico-cortical and cortico-cortical brain communication in patient-volunteers with autism. Furthermore, we demonstrated significant group differences between autism and controls in the integrity of functional connectivity associations with behavioral measures of task performance and significant autism functional connectivity associations with behavioral measures of clinical severity. These connectivity-behavior associations were circuit and affect specific and suggested that the altered functional connectivity of amygdala and interhemispheric circuits in affective face processing networks contribute to the core features of the behavioral phenotype of autism.

Our findings in the first study (Chapter 2) suggested that individuals with autism showed an altered orchestration of activity in several brain regions involved in processing facial affect. In autism, the right amygdala demonstrated overconnectivity with parahippocampal and insular regions, while at the same time displayed underconnectivity with prefrontal and parietal regions. The altered balance between overconnectivity and underconnectivity with the amygdala indicated that the underconnectivity hypothesis (Just, Cherkassky et al. 2004) was not generalizable to affective face processing and that autism affective face networks had reduced network metastability (Bressler 2003; Fingelkurts and Fingelkurts 2004), the tendency of brain regions to be both functionally segregated and integrated.

In study two, by isolating functional amygdala connectivity resting networks, emotional face processing networks, and neutral face processing networks using partial correlation functional connectivity analysis (Chapter 3), we identified the amygdalae as behavioral catalysts (McIntosh 2004), nodes involved in transitioning between brain states, that have impaired functional catalysis of affective network behaviors in autism. Evidence was based on the reduced changes between emotional, neutral, and resting functional amygdalae (left and right) connectivity magnitudes for Group x Affect clusters in cerebellum and left superior temporal gyrus (Chapter 3; Figure 7b,c). In addition to reduced metastability in autism affective face processing networks, we identified reduced autism multivariability (Fingelkurts and Fingelkurts 2004), the ability to form multiple brain states. Controls demonstrated significant differential amygdala connectivity that was either altered or lacking in autism (Chapter 3; Figure 7 d,e). For example, group

differences in Group x Hemisphere clusters were present for connectivity with the right amygdala to the left middle frontal gyrus, right insula, right middle temporal gyrus, right fusiform gyrus, right posterior cingulate, and right precentral gyrus and for connectivity with the left amygdala to the left insula and claustrum, left lingual gyrus, and left fusiform (Chapter 3; Figure 7d). The amygdalae are behavioral catalysts with reduced functional catalysis in autism based on the reduced metastability and reduced multivariability of autism functional amygdala networks.

After identifying subcortico-cortical connectivity alterations in autism functional amygdala networks, we used partial correlation functional connectivity analysis in the third study to test whether cortico-cortical functional interhemispheric connectivity was altered in autism at rest and when processing emotional and neutral faces. By including multiple affective processing states in our functional network analysis, we could examine group differences in affective connectivity dynamics. Our results indicated that deficits in autism affective social behavior were related to an imbalance in affective network dynamics in the functional interhemispheric connectivity of homotopes, homologous brain regions in different hemispheres, and their association with density measures of the corpus callosum body (Chapter 4). The interhemispheric autism networks we described warrant a reexamination of a prior underconnectivity hypothesis (Just, Cherkassky et al. 2004) which did not consider the altered connectivity dynamics of facial affect discrimination in autism. The reduced dynamics of affective face processing networks in autism suggested that a more general “dynamic network framework” in addition to overconnectivity or underconnectivity may be beneficial in understanding autism

affective networks. Metastability (Bressler 2003; Fingelkurts and Fingelkurts 2004) was altered in autism based on the patterns of both overconnectivity and underconnectivity we identified. We identified behavioral catalysts (McIntosh 2004) that were impaired in autism based on the reduced interhemispheric connectivity capacity dynamics of autism. In autism, fusiform, medial lingual, and cingulate regions did not significantly change the extent of their homotopic connectivity between the processing of facial affects, but these behavioral catalysts had a greater dynamic capacity in the control group (Chapter 4; Figure 5d). These results provided further evidence that autism has reduced network multivariability (Fingelkurts and Fingelkurts 2004). Affective connectivity dynamics were differentially associated in autism and control groups with corpus callosum density (Chapter 4; Figure 9) and suggested that structural changes to corpus callosum contributed to the altered capacity of interhemispheric communication in autism. In union, study three results indicated that affective face processing produces over and underconnected homotopic networks in autism with reduced metastable dynamics, reduced multivariability, and reduced dynamic connectivity capacity.

In study four, we formally examined patterns, or signatures, among dysfunctionally connected homotopic nodes using neuroinformatic techniques including agglomerative hierarchical clustering analysis, graph theory, and multidimensional scaling (Chapter 5). The pattern grouping of connectivity among homotopic nodes provided a signature of the autism phenotype based on the unsupervised classification differences (Chapter 5; Figures 1-3), the greater similarity of nodes with increased connectivity among triangular motifs in the parameter space of brain region and facial affect (Chapter 5; Figure 4), and

the distinctive pattern maps in an arbitrary coordinate space (Chapter 5; Figures 5 and 6).

In union, this study provided further evidence for reduced metastability in autism and enabled visualization of signatures of connectivity unique to the autism phenotype.

In study five, to advance future fMRI connectivity analyses, we tested the hypothesis that the temporal third derivative of the pulse oximetry signal (Cardiac Peak derivative 3, CPd3) would improve estimation of the cardiac R-wave for removal of physiological noise from fMRI signal (Chapter 6). We developed an open-source software package in the python programming language (van Rossum and Drake 2001) called PhysioNoise to test this hypothesis. Compared to the scanner estimation of the R-wave peak (Cardiac Peak transistor transistor logic, CPttl), the CPd3 may be a more appropriate choice for fMRI noise correction and analysis for R-wave peak estimation because the CPd3 preceded the CPttl by about 37 ms and the CRTd3 had fewer outliers with less outlier dispersion. The use of CPd3 in retrospective physiological noise correction would improve fMRI functional activation and connectivity signal detection.

We identified several regions in autism with changes to the underlying correlation structure of communication in neural circuitry (Salinas and Sejnowski 2001). In study 1, the autism amygdala was dysfunctionally connected in the facial affect network to the insula, middle frontal gyrus, parahippocampus, superior parietal lobule, and superior temporal gyrus (Chapter 2). In study 2, the autism amygdala was dysfunctional connected in emotional, neutral, and resting networks to the angular gyrus, cerebellum declive, cingulate, cerebellar vermis, claustrum, fusiform, insula, inferior frontal gyrus, inferior

parietal lobule, lingual gyrus, middle frontal gyrus, middle temporal gyrus, postcentral gyrus, posterior cingulate, precentral gyrus, precuneus, superior frontal gyrus, and superior temporal gyrus (Chapter 3). In study 3, autism interhemispheric connectivity was altered for the following homotopes: cingulate, cuneus, fusiform, inferior parietal lobule, insula, and lingual gyrus (Chapter 4). In study four, insula and fusiform had distinctive interhemispheric connectivity signatures in autism (Chapter 5). Autism regions consistently dysfunctionally connected in at least two of our studies 1 through 4 included the amygdala, cingulate, fusiform, inferior parietal lobule, insula, lingual gyrus, middle frontal gyrus, superior parietal lobule, and the superior temporal gyrus. The aggregated list of dysfunctionally connected regions in autism suggested that functional amygdala connectivity alterations may be related to the aberrant interhemispheric functional connectivity of cingulate, fusiform, the inferior parietal lobule, insula, and lingual gyrus. Further studies in autism will be necessary to clarify the effective influence of intrahemispheric amygdala connectivity on interhemispheric networks, or vice versa, and the impact of these network dynamics on behavior.

Behavioral associations with functional connectivity confirm the hypothesis that autism is a disorder of altered neural and social communication (Minshew and Williams 2007). For several circuits in the affective face processing network, the association of both functional subcortico-cortical and cortico-cortical connectivity, and their dynamics, with behavioral measures of task performance differentiated the autism and control groups (For details, see Chapters 2-4). In addition, clinical measures of autism severity were associated with the integrity of functional connectivity in specific circuits of specific

affective face networks and with the dynamics of functional connectivity circuit integrity across affective face processing states (For details, see Chapters 3 and 4). Although autism is considered a distributed disorder with multiple affected networks (Muller 2007) and affective face processing invokes a distributed network (Adolphs 2002; Haxby, Hoffman et al. 2002), the specificity of functional connectivity associations to behaviors that we report indicated that specific nodes may be held in common across networks but were dysfunctionally connected in autism. In particular, the reduced behavioral catalysis of specific nodes may contribute to the reduced metastability and reduced multivariability of multiple distributed autism networks.

In keeping with the conclusion that specific nodes of altered connectivity could influence multiple networks, our results indicated that the nodes of altered affective face processing in autism were consistent with those activated in cortical and subcortical visual processing, the social brain, the mirror neuron system, and the empathy network. The functional connected circuits we reported as aberrant in autism correspond to those previously reported in activation networks of affective visual processing. Modulation of cortical activity levels in the ventral stream, comprised of the amygdala, insula, ventral striatum, ventral anterior cingulate, and prefrontal cortex, and the dorsal stream, comprised of the hippocampus, dorsal anterior cingulate, and prefrontal cortex, permit differential emotion perception through production of an identified affective state and its regulation, respectively (Phillips, Drevets et al. 2003). The connectivity within and between streams is equally important to activity regulation (Goodale 1998) and our findings suggested a dorsal-ventral stream imbalance was present in autism during facial

affect processing (For further discussion, see Chapter 2). In support of the need for dorsal-ventral stream balance, the processing of fearful and angry emotional face stimuli in controls was distributed across a network of regions in communication with the amygdala including the insula, parahippocampal gyrus, orbitofrontal cortex, and the subgenual, posterior, and supragenual cingulate (Stein, Wiedholz et al. 2007). In autism, further effective connectivity studies would provide insight into the directional influence of the amygdala within the dorsal and ventral streams.

In addition to connectivity of the amygdala with cortical visual streams, amygdala subcortical processing of faces may also contribute to altered social processing in autism. High and low spatial frequencies of face stimuli are processed in distinct visual pathways involving a parvocellular and magnocellular pathway, respectively (Vuilleumier, Armony et al. 2003). The fine resolution details of a face, which make facial identification possible, are contained in higher spatial frequencies and primarily carried through the parvocellular visual pathway to the ventral visual stream. Facial form elements, such as an open mouth or open eyes, are contained in lower spatial frequencies, and conveyed to the dorsal stream, superior colliculus, and pulvinar regions through the magnocellular visual pathway (Johnson 2005). The retinal–superior colliculus-pulvinar circuit may rapidly provide the amygdala with low spatial frequency facial affect (fear) information and provide a subcortical route for affective face processing that bypasses primary visual cortex (Vuilleumier, Armony et al. 2003). In autism, amygdala modulation through this subcortical circuit may influence social behavior by impacting amygdala communication with the fusiform (Schultz 2005) and, more generally, affect the ability of the autism

social brain to process low frequency components of facial affect (Johnson 2005).

Although our results indicated that the amygdala-fusiform and interfusiform connectivity were altered in autism affective face processing networks (Chapters 3-5), studies using facial affect probes with high and low spatial resolution would be necessary to directly examine the influence of the subcortical visual pathway on autism behavior and on the functional connectivity of facial affect processing.

Aberrant autism functional connectivity occurred in regions that comprise the social brain. The social brain (Brothers 1990) describes a neural cognition network, comprised of the amygdala, anterior insula, medial prefrontal cortex, temporoparietal junction, posterior superior temporal sulcus, inferior frontal gyrus, interparietal sulcus, and anterior cingulate cortex, that enables social interaction through the recognition and understanding of others (Blakemore 2008). Components of the social brain network describing impaired autism social processing were hypothesized to include the amygdala, orbitofrontal gyrus, anterior cingulate, fusiform, superior temporal area, inferior frontal gyrus, and posterior parietal cortex (Amaral, Schumann et al. 2008). Except for the orbitofrontal gyrus which may not have adequate coverage in our fMRI studies, the regions affected in the autism social network correspond to the nodes of altered functional connectivity in the autism affective face processing network which we reported in studies 1 through 5. Furthermore, our results of altered connectivity among the amygdala, inferior parietal lobule, and insula in autism may indicate an altered role of amygdala-pulvinar-supramarginal gyrus and amygdala-insula connectivity in attentional modulation of somatosensory and visual processing in the autism social brain (Adolphs

and Spezio 2006). Using the pulvinar region as a seed and a facial affect task, future functional connectivity studies may provide insight into functional thalamic connectivity (Mizuno, Villalobos et al. 2006) with the amygdala and both subcortical visual and social processing in autism.

Several of the dysfunctionally connected nodes that we reported in autism are part of the mirror neuron and empathy network of facial affect processing (Hadjikhani, Joseph et al. 2006). In humans, the mirror neuron system is comprised of the inferior frontal cortex and the rostral inferior parietal lobule (Iacoboni 2005) and may contribute to autism social behavior through deficits in the mirror neuron system itself or deficits in communication with the mirror neuron system such as the imitation network comprised of the superior temporal area-parieto-frontal circuit (Williams, Whiten et al. 2001; Iacoboni and Dapretto 2006). As part of the mirror neuron system, the pars opercularis of the inferior frontal gyrus showed reduced activation in an fMRI study of autism subjects during the viewing of facial affect (neutral, happy, fear, anger, sad) and facial imitation compared to controls and provided evidence that deficits in the mirror neuron system may contribute to deficits in autism social behavior (Dapretto, Davies et al. 2006). The processing of facial expressions may occur through empathy in which an emotion is understood through the nonconscious, imitative action of the perceived emotion (Carr, Iacoboni et al. 2003). Neuroanatomically, the imitative action is represented in a recursive loop between the superior temporal gyrus and mirror neurons in the inferior frontal and parietal regions. Action coding interacts with the limbic system through the insula which has reciprocal connections with the superior temporal, inferior frontal, and

parietal regions. Support for this theory, comes from a block-design fMRI study in which the premotor face area, the dorsal sector of pars opercularis, the superior temporal sulcus, the insula, and the amygdala were active during passive emotional face viewing and modulated their activity during the imitation of facial emotions (Carr, Iacoboni et al. 2003). Given that the nodes of aberrant affective face processing networks we described in autism correspond to nodes in the mirror neuron system and empathic network, further studies with a larger sample size and more face trials will be necessary to localize group differences in functional connectivity within the empathy system and pars opercularis of the mirror neuron system in particular.

Future Studies

Further studies are needed to characterize the autism connectome. In autism, altered neuronal packing in the amygdala (Bauman and Kemper 1985) may influence the intrinsic connectivity within the amygdala required for proper information transfer (Bonda 2000; Amaral and Corbett 2003) that could contribute to altered ectoamygdala communication. The integrative nature of amygdala regions into functional columns (see Fig. 14 (Bonda 2000)) suggests that neuronal connectivity between nuclei within the amygdala (Pitkanen and Amaral 1998) may impact the functional integrity of intraamygdala circuits to influence functional ectoamygdala circuits. Using the COSLOF index (Li, Li et al. 2002) discussed in Chapter 8.5, a voxelwise intranodal functional connectivity analysis of the amygdala could be used to test the hypothesis that functional coupling within the amygdala is altered in autism. Alternatively, structural equation or dynamic causal models, discussed in Chapter 8.5, could be used to test the hypothesis

that social inputs alter dynamic communication between nodes to a greater degree in autism than controls. With these approaches, the condition specific neuronal dynamics of effective amygdala connectivity in autism could be quantified while taking into account the intrinsic connectivity of the amygdala.

Although functional interactivity can be assessed with fMRI, the structural integrity of fiber tracts in human brain requires an alternative methodology known as diffusion tensor imaging (DTI), an application of MRI and an extension of diffusion weighted imaging (For brief discussion, see Chapter 8.4). With this technique both the magnitude and direction of water diffusion can be quantified in vivo (LeBihan, Mangin et al. 2001). The DTI technique has been implemented in both clinical (Sundgren, Dong et al. 2004) and neurodevelopment studies (Watts, Liston et al. 2003; Nagy, Westerberg et al. 2004). By combining DTI measures of fractional anisotropy (FA), an index of structural integrity, and fMRI measures of connectivity (Toosy, Ciccarelli et al. 2004), whole brain maps could be generated to examine brain communication relationships with in vivo brain structure and development (Werring, Clark et al. 1998; Koch, Norris et al. 2002; Guye, Parker et al. 2003; Olesen, Nagy et al. 2003; Johansen-Berg, Behrens et al. 2004; Baird, Colvin et al. 2005). A voxelwise correlation analysis of whole brain FA with functional connectivity maps could test the hypothesis that altered structural integrity underlies defective functional amygdala connectivity in autism by contrasting whole brain maps of structure-function integrity across autism and control groups. The relationship between FA and functional connectivity measures may be directly related. FA could impact functional connectivity by influencing neurotransmission efficiency; in like fashion, the

communication between regions may induce myelination and enhance structural integrity (Demerens, Stankoff et al. 1996; Olesen, Nagy et al. 2003). Based on studies showing alterations in FA (Barnea-Goraly, Eliez et al. 2003; Barnea-Goraly, Kwon et al. 2004), myelin (Dong and Greenough 2004), and the connectivity alterations in affective face processing networks we described in autism, combined DTI-fMRI studies could test the hypothesis that FA and functional connectivity (Belmonte, Allen et al. 2004) measures are directly related and that the structure-function integrity is altered in the affective face processing circuitry of autism. The path of white matter projections between regions could be determined using DTI-based tractography (Melhem, Mori et al. 2002) to form maps of structural connectivity in human brain (Jellison, Field et al. 2004; Wakana, Jiang et al. 2004). The interhemispheric structure-connectivity associations examined through combination of DTI tractography and fMRI would also clarify the role of corpus callosum in producing altered affective processing networks and impaired social behavior in autism.

Analytical approaches to examine temporal associations deserve further development. Brain regions with group differences in connectivity to a functional seed may result from group differences in temporal fluctuations either in the seed region or in the region connected to the seed. The group differences in lateralization of network connectivity with the amygdalae in study 2 (Chapter 3; Figure 7e), for example, may reflect hemispheric differences in the time courses of amygdala neurofluctuations, which has previously been reported for fearful faces (Phillips, Medford et al. 2001). The intraregional correlation methods and the neuroinformatic approaches described in

Chapter 5 could be applied to the time courses themselves in order to examine underlying signatures in amygdalae neurofluctuations.

To advance functional connectivity methodology for applications in affective and cognitive neuroscience, future studies should use PhysioNoise to directly compare the R-wave onset times from pulse oximetry using the differentiation method, the CPttl from the scanner, and the actual R-wave from EKG measurements during fMRI acquisition (Chapter 6). Furthermore, the utility of physiological signals in fMRI functional connectivity analysis requires further study. PhysioNoise outputs the RW, CPd3, CPd3R, and CPttl time courses as options for fMRI retrospective brain signal correction of physiological noise and the RVT, RRT, CVT, CRTd3, CRTd3R, and CRTttl curves as options for neuroanalysis (Chapter 6). These measures may be useful in identifying brain regions susceptible to physiological fluctuations, improve upon global brain estimates of physiological noise (Birn, Diamond et al. 2006), and enable correction of respiratory and cardiac fluctuations by incorporating these covariates of no interest within the general linear model.

Future studies will need to address the potential for therapeutic interventions to normalize functional connectivity alterations and improve behavior. Evidence is emerging that functional connectivity can be modulated in specific circuits by hormonal, pharmacological, and alternative therapies. In a randomized, double-blind, crossover baseline EEG study, testosterone administration increased functional connectivity of the left prefrontal and right parietal cortex (Schutter, Peper et al. 2005). During a visuomotor

and emotion discrimination task, progesterone administration to women significantly decreased fMRI functional amygdala connectivity with the fusiform gyrus and marginally increased functional amygdala connectivity with the dorsal anterior cingulate cortex (van Wingen, van Broekhoven et al. 2008). Cabergoline, a dopamine receptor (D2) agonist, modulated the extent of fMRI connectivity in frontostriatal circuitry between putamen and both ventral and anterior prefrontal cortex (Cohen, Krohn-Grimberghe et al. 2007). Sulpiride, a D2 receptor antagonist, increased functional connectivity between caudate and thalamus during an fMRI object–location learning task (Honey, Suckling et al. 2003). A randomized, double-blind, crossover fMRI study of facial affect processing showed that levodopa decreased functional interamygdala connectivity during facial affect (fear, anger) recognition (Delaveau, Salgado-Pineda et al. 2007). Acupuncture in the right leg (Hegu accupoint ST.36) modulated the fMRI functional connectivity of the left amygdala with the anterior frontal cortex, anterior cingulate, thalamus, nucleus accumbens, hypothalamus, brainstem, periaqueductal grey mater, and cerebellum (Qin, Tian et al. 2006). Initial reports suggest that acupuncture may help improve autism behavior (Ma, Yuan et al. 2006; Wang, Liu et al. 2007; Yan, Wei et al. 2007; Allam, Eldine et al. 2008). Imitation therapy (Ingersoll and Schreibman 2006; Ingersoll and Gergans 2007) has also shown some success and may be mediated through modulation of connectivity with the mirror neuron system (Iacoboni and Mazziotta 2007). Although our studies establish functional connectivity alterations as a pathofunctional mechanism in autism, further studies using functional or effective connectivity alterations pre- and post-therapy would be necessary to establish the therapeutic efficacy of functional connectivity modulation in autism treatment.

Conclusion

Using functional connectivity analyses, these studies aimed to characterize the functional connectivity of affective face processing in autism. Functional connectivity was a useful analytical approach to identify underlying correlation structure in spatiotemporal patterns of affective face processing circuitry. The integrity of functional amygdala and interhemispheric connectivity was both overconnected and underconnected in autism and could be explained by network reductions in behavioral catalysis, metastability, and multivariability. The dysfunctionally connected nodes in autism affective facial processing networks correspond to those previously reported in activation networks of affective visual processing, of the social brain, and of the empathy and mirror neuron networks. Future connectivity studies should incorporate diffusion tensor imaging, neuroinformatic techniques, and pulse oximetry derived R-wave estimates when characterizing the autism connectome.

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Chapter 8:**Appendix**

The Cyclic AMP Phenotype of Fragile X and Autism

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Abstract

Cyclic AMP (cAMP) is a second messenger involved in many processes including mnemonic processing and anxiety. Memory deficits and anxiety are noted in the phenotype of fragile X (FX), the most common heritable cause of mental retardation and autism. Here we review reported observations of altered cAMP cascade function in FX and autism. Cyclic AMP may be a useful biochemical marker to distinguish autism comorbid with FX from autism per se and the cAMP cascade may be a viable therapeutic target for both FX and autism.

Keywords: Autism, Fragile X, Cyclic AMP, cAMP, signal transduction, FMRP, FMR1

Introduction

Fragile X (FX) syndrome, or Martin-Bell syndrome, is the most common inheritable cause of mental retardation and autism, with a full mutation prevalence of 1:2000-4000 for males and in 1:4000-8000 for females (Oostra and Willemsen 2003; Tsiouris and Brown 2004). The genotype is a CGG trinucleotide amplification on the X chromosome (Xq27.3) in the 5' untranslated region of the fragile X mental retardation-1 (FMR1) gene that suppresses production of fragile X mental retardation protein (FMRP) (Kaufmann, Cohen et al. 2002; Todd and Malter 2002). FX premutation carriers have more than 55 CGG repeats and those with more than 200 repeats have a full FX mutation (Fu, Kuhl et al. 1991). The comorbidity of autism and FX was initially reported to be around 18% (Brown, Jenkins et al. 1982) and a lower percentage of those with autism have FX (about 7%) (Brown, Friedman et al. 1982; Brown, Jenkins et al. 1986; Loesch, Bui et al. 2007). Although reported incidences may vary, FX is known to be the most common inherited cause of autism. Autism is a behaviorally defined neurodevelopmental disorder with a characteristic triad of deficient diagnostic domains: social, behavioral, and communicative (Rapin 1997). Anxiety-related social behavior and memory deficits are reported in both the autism (Williams, Goldstein et al. 2006; Gillott and Standen 2007) and FX behavioral phenotypes (Cornish, Munir et al. 2001; Hagerman 2002). Signaling deficiencies in mGluR and cyclic AMP (cAMP) mediated mechanisms are central to FX pathophysiology (Berry-Kravis 1990; Berry-Kravis, Hicar et al. 1995; Miyashiro and Eberwine 2004). In this paper, we examine the evidence for the contributions of defects in signaling pathways in FX and autism behavior by reviewing the cAMP cascade and its

role in stress, anxiety, and memory and relate these findings to the potential for cAMP mediated therapy for FX and autism.

Cyclic AMP Cascade

The cAMP cascade is a ubiquitous, prototypical second messenger signaling system that transduces extracellular neurotransmitter or hormone messages that bind to a receptor. Adenylate cyclase (AC) is an enzyme that catalyzes cAMP formation from ATP in the presence of Mg^{2+} . Of the ten adenylate cyclase isozymes, all membrane-bound AC isoforms (AC1-9) are expressed in the brain. Their regional distribution is isoform dependent (Chern 2000) and their regulation is complex and isozyme specific (Tang and Hurley 1998). Serotonin, adrenergic, dopaminergic, adenosine, vasoactive intestinal peptide, muscarinic, GABA, and opioid receptors, among others, signal through the cAMP cascade (Lauder 1993) via specific heterotrimeric G proteins. G proteins are guanine nucleotide binding proteins that act as signal transducers by mediating the signal of receptor activation across the membrane to effectors, like adenylate cyclase, that can alter levels of second messengers, like cAMP. Each G protein is a heterotrimer consisting of three subunits: α, β, γ . Based on α subunit similarity, there are four families of G proteins: Gs, Gi, Gq, and G₁₂ (Cabrera-Vera, Vanhauwe et al. 2003). Gs activates all membrane bound AC isoforms and forskolin, an AC activator (Seamon, Padgett et al. 1981), is able to activate all but AC9 (Cumbay and Watts 2004). Gi regulates cAMP levels through inhibition of adenylate cyclase and Gq activates phospholipase C to cleave phosphatidylinositol bisphosphate (PIP₂) into both inositol triphosphate (IP₃), which releases Ca^{2+} from internal stores, and diacylglycerol (DAG), which activates protein

kinase C (PKC). Additional regulators of AC include Ca^{2+} , G protein subunit $\beta\gamma$, protein kinase A (PKA), PKC, and calmodulin dependent kinase (Chern 2000), making it possible for participants in the Gq cascade, like Ca^{2+} and PKC, to regulate cascades mediated by Gs and Gi. For example, mGluR1 stimulation can enhance cAMP production (Aramori and Nakanishi 1992) and stimulation of Gq linked receptors potentiates AC6 and AC9 activity but is inhibitory to AC1 and AC3 (Beazely and Watts 2005; Cumbay and Watts 2005).

Cyclic AMP has emerged as a tightly regulated second messenger with multiple downstream effectors (Gilman 1995). One known cAMP function is to serve as a second messenger that can activate cAMP-dependent PKA, an enzyme that phosphorylates tertiary messengers, which themselves can signal subsequent messengers. Alternatively cAMP can act directly as a transmitter by binding cyclic nucleotide gated channels (Matulef and Zagotta 2003). Cyclic AMP may produce short term or long term changes in neuron function by acting respectively through PKA or an EPAC-Rap-MAPK cascade involving an exchange protein activated by cAMP (EPAC), a small GTPase (Rap1), and a mitogen-activated protein kinase (MAPK) (Neves, Ram et al. 2002). Cyclic AMP subcellular distribution is not uniform (Rich, Fagan et al. 2000) and its levels are tightly regulated by phosphodiesterases (PDEs). Regulation of cAMP levels is important because cAMP has several mechanisms to manipulate cellular function.

Phosphodiesterases provide the only means of degrading cAMP levels and maintaining cAMP homeostasis. Several isoforms of PDE are known to exist (Francis, Turko et al.

2001). PDE4 is a cAMP-specific PDE that is prevalent in the brain and is the target of antidepressant and cognitive enhancing pharmacotherapeutics. With activity being regulated by both cAMP and extracellular signal-regulated kinase (ERK) signaling, PDE4 is a key component in NMDA-mediated memory processes (Zhang, Zhao et al. 2004). The PDE4 subfamilies PDE4A, PDE4B, PDE4C, and PDE4D are highly expressed in human brain regions, whereas rat brain primarily expresses PDE4A, B, and D (Cherry and Davis 1999; Takahashi, Terwilliger et al. 1999; Fujita, Zoghbi et al. 2005; Parker, Matthews et al. 2005). Cyclic AMP levels regulate the activity of PDE4 through cAMP-dependent PKA phosphorylation of PDE4 in the short term (Manganiello 2002) and regulate the density of PDE4 through a positive feedback mechanism on PDE4 transcription in the long term (Beavo and Brunton 2002; D'Sa, Tolbert et al. 2002; Conti, Richter et al. 2003; Houslay and Adams 2003). Cyclic AMP itself can upregulate the expression of certain PDE4 variants (Conti and Jin 1999; Houslay 2001). PDE4 and cAMP levels are regulated through a feedback loop in which cAMP levels regulate PDE4 levels and PDE4 regulates cAMP levels.

cAMP in Stress and Anxiety

Mounting evidence from behavioral and pharmacological studies indicates that stress and anxiety are mediated through the cAMP second messenger system. While neural substrates of anxiety are known to involve the prefrontal cortex and the amygdala (Davidson 2002), the importance of intracellular functional activity in these regions remains to be elucidated in humans. In rhesus monkeys, administration of diazepam, a benzodiazepine anxiolytic known to increase cAMP by inhibiting PDE cAMP hydrolysis

(Cherry, Thompson et al. 2001), produces increased activity in parietal areas and activation in frontal areas lateralized to the left (Davidson, Kalin et al. 1992; Davidson, Kalin et al. 1993). Asymmetric distribution of activity reflects a role for diazepam-induced cAMP level increases in behavioral inhibition, increased positive affect, and decreased anxiety (Davidson, Kalin et al. 1992; Davidson, Kalin et al. 1993). In the rat response to stress, intra and interhemispheric associations of intracellular functional activity in the hypothalamus, hippocampus, frontal cortex, and amygdala can be drawn from mapped increases of *in vivo* brain cAMP levels (Egorova, Stepanichev et al. 2003). Rat pituitary increases cAMP production to multiple stressors (cold, forced running, formalin injection, immobilization, electric footshock) and the extent of cAMP increases corresponds to the intensity of the stressor (Kant, Meyerhoff et al. 1982). Based on microdialysis studies in rat, the brain contributes cAMP to plasma during stress (Stone and John 1992). The cAMP cascade mediates stress and anxiety in localized brain regions and the levels of cAMP are proportional to the intensity of the behavioral stressor.

The pharmacological mechanism of drug-induced anxiolysis can provide insight into the intracellular components contributing to behavioral anxiety. Several anxiolytic drugs exert their effect through the cAMP cascade. Diazepam, an anxiolytic benzodiazepine, and rolipram, a pyrrolidinone antidepressant with anxiolytic properties, mediate their anxiolytic effect through PDE inhibition, as do the methyl xanthines like caffeine. *In vitro* potency of several PDE inhibitors show a positive correlation with the anxiolytic efficacy of rat performance on the Vomer conflict task (Beer, Chasin et al. 1972). *In vivo* anxiolytic pharmacologic studies show a similar PDE inhibition mechanism. For

example, diazepam inhibits mouse brain PDE4 subtypes transfected to human embryonic kidney cells (Cherry, Thompson et al. 2001). Behavioral testing of the methyl xanthines, caffeine and pentoxifylline (a PDE4 inhibitor), confirms their effect as anxiolytics (Rao, Santos et al. 1999). During an elevated plus-maze test, the selective PDE4 inhibitor rolipram produces anxiolytic-like behavior in rats independent of simultaneous locomotor behavior (Silvestre, Fernandez et al. 1999). Thought to originate as intracellular cAMP, extracellular cAMP levels detected by microdialysis in rats increase in right medial prefrontal areas in response to a restraint stressor or peritoneal saline injection stressor (Stone and John 1992). This effect was enhanced using rolipram, a selective inhibitor of PDE4 known to increase intracellular cAMP levels. The anxiolytic pharmacologic response appears to be mediated through a stress-reducing cAMP elevation. One possible pathway for increased cAMP levels to ultimately decrease anxiety is by directly upregulating expression of the cAMP response element (CRE) binding protein (CREB) (Nibuya, Nestler et al. 1996). CREB is a transcription factor that binds CRE and increases the expression of neuropeptide Y (Higuchi, Yang et al. 1988), a peptide mediating anxiolysis (Pandey 2003) found in corticolimbic structures like the amygdala (Millan 2003). Pharmacologic studies indicate that the cAMP cascade is one mechanism that mediates anxiety and therapy that enhances the cAMP cascade in specific brain regions like the amygdala may be anxiolytic.

cAMP in Memory

Deficiencies in the cAMP cascade result in memory deficits. This role has been elucidated by research in many systems including the gill withdrawal reflex of *Aplysia*;

rutabaga, turnip, and dunce *Drosophila* mutants; and type 1a pseudohypoparathyroidism in humans (Berry-Kravis and Huttenlocher 1992). In a mouse study, rolipram-induced increases of cAMP reduce the spatial memory defects associated with aging (Bach, Barad et al. 1999). The cAMP cascade is one of several implicated in studies of long term potentiation (LTP), a neuronal model of memory (Lynch 2004). LTP may be considered a form of synaptic plasticity based on synaptic strengthening which is known to involve cAMP (Frey, Huang et al. 1993; Weisskopf, Castillo et al. 1994), AMPA receptors, and NMDA receptors. For LTP, high frequency trains are required for glutamate to stimulate AMPA receptors to depolarize the post-synaptic cell and clear the magnesium block of NMDA channels to permit calcium flux. Insertion and recycling of AMPA receptors to the synapse requires cAMP-dependent PKA phosphorylation of the receptor (Esteban 2003). In response to repetitive high frequency stimulation, NMDA receptors flux Ca^{2+} which can activate calcium dependent AC1 and AC8 to increase cAMP production (Wong, Athos et al. 1999). LTP can be divided into an early, short-term phase and a late, long term phase. The early phase is independent of cAMP and protein levels are unaltered whereas the late phase is cAMP dependent and is associated with protein synthesis (Ma, Zablow et al. 1999). The involvement of the cAMP cascade in memory and LTP is well characterized in transgenics overexpressing AC1 (Wang, Ferguson et al. 2004) and AC1-AC8 double knockouts (Wong, Athos et al. 1999). Pharmacologically targeting the cAMP system may enhance LTP and improve memory related processes.

FX Memory and Anxiety

Fragile X Patients

Phenotypically, FX is characterized by mental retardation, cognition and memory deficits, autistic and stereotypic behaviors, developmental delays, hyperactivity, attention deficit disorder, and seizures (Reiss and Freund 1990; Berry-Kravis 2002). FX premutation humans older than 50 carrying unmethylated (CGG)_{50–200} repeats may have a tremor-ataxia disorder with an associated decline in memory. The human phenotype includes ubiquitin-positive intranuclear inclusions, elevated FMR1 mRNA, and reduced FMRP expression (Greco, Hagerman et al. 2002). Memory deficits and anxiety-related social behavior are central to the FX full mutation phenotype (Cornish, Munir et al. 2001; Hagerman 2002). For example, the FMRP defect impacts the anatomy (Reiss, Lee et al. 1994) and function of memory related areas like the hippocampus. Reduced hippocampal and basal forebrain activation patterns are reported in a functional MRI (fMRI) study of FX patient-volunteers encoding a visual memory task (Greicius, Boyett-Anderson et al. 2004). In another fMRI study, lymphocytic FMRP levels are shown to have a direct relationship with regions of brain activated during a working memory task (Menon, Kwon et al. 2000). Anxiety is commonly featured with memory deficits in FX individuals (Hagerman 2002; Tsiouris and Brown 2004). Although anxiety is difficult to detect in individuals with intellectual disability, behavioral equivalents of anxiety have been reported in FX (Sullivan, Hooper et al. 2007) and are likely related to the hypersensitivity and hyperarousal (Cohen 1995) described in electrodermal studies of FX individuals (Miller, McIntosh et al. 1999). As Hagerman notes (Hagerman 2002), pharmacologic inhibition of the cAMP second messenger system prevents normal sensitization to

sensory stimuli in *Aplysia* (Kandel and Schwartz 1982) which may have implications for the abnormal sensitization seen in FX individuals.

Fragile X Fly

Drosophila melanogaster has an FMR1 homologue, *dfmr1*, in their genome. *Drosophila* express the *dfmr1* protein, *dfmrp*, in the central nervous system and specifically in mushroom bodies, key neural structures in learning and memory (Schenck, Van de Bor et al. 2002). The *dfmr1* mutant, an FX fly model, has an altered mushroom body structure which may contribute to the FX memory phenotype (Michel, Kraft et al. 2004; Pan, Zhang et al. 2004; Restifo 2005). One example of an altered FX fly memory phenotype comes from assessment using conditioned courtship behavior, a model for memory in *Drosophila* (McBride, Giuliani et al. 1999). Briefly, a male fly will court a virgin female fly without training. Conditioned courtship suppression occurs when male flies suppress their courting behavior with virgin females after training in which the male, paired with a mated female, learns to reduce courtship activity. Although *dfmr1* mutants learn the paradigm when paired with mated females, the memory of training is absent when paired with virgin females. This memory defect in FX *Drosophila* behavior is improved with lithium and mGluR2 antagonists (LY341495, MPPG, MTPG), pharmaceuticals known to increase cAMP cascade function in flies (McBride, Choi et al. 2005). Based on the *dfmr1* mutant, impaired FX anxiety and memory behavior may be improved through cAMP pharmacotherapy.

Fragile X Mouse

A knockout mouse model of FX syndrome was generated by insertion of a neomycin resistance cassette into exon 5 of the FMR1 gene (Bakker, Verheij et al. 1994). The FMR1 knockout mouse exhibits deficits in neuronal pruning and alteration of FMRP-regulated protein synthesis (Comery, Harris et al. 1997; Nimchinsky, Oberlander et al. 2001). Since FMRP is thought to repress protein synthesis (Laggerbauer, Ostareck et al. 2001; Li, Zhang et al. 2001), a loss of function mutation of the FMR1 allele is expected to enhance protein synthesis of FMRP-repressed proteins. Consistent with this view, radiotracer autoradiography indicates enhanced protein expression in hypothalamus, thalamus, basolateral amygdala, hippocampus, frontal association, and posterior parietal cortex of FMR1 knockout mice (Qin, Kang et al. 2005).

Targeted disruption of FMRP results in several mouse phenotypes that share commonalities with human FX and autism behavior, but there are also some potential differences (For further review, see Bernardet and Crusio 2006). Depending upon genetic background, behavioral and synaptic models of memory in mice are consistent with the human FX phenotype (For background, see Lombroso 2003; Hessler, Rivera et al. 2004). Measures of learning and memory in a fear based paradigm were generally comparable for targeted and wild type alleles on a C57BL/6 background (Peier, McIlwain et al. 2000), though these mice expressed reduced memory for avoidance in a leverpress paradigm compared to wild type (Brennan, Albeck et al. 2006). Lack of functional FMR1 alleles on an FVB/NJ background resulted in deficits in a passive avoidance memory task (Qin, Kang et al. 2002). The memory defect in gene-targeted mice C57BL/6J is not due

to an FMRP-reduction of CREB, since CREB levels are comparable for targeted and wild type alleles (Li, Pelletier et al. 2002). However, if cAMP levels are reduced in FX, then CREB activity might be attenuated, resulting in downstream memory deficits. Since LTP is a synaptic model of memory dependent upon cAMP expression, FMR1 knockout mice were also evaluated for late-phase LTP in the hippocampus. No differences in LTP were identified between targeted and wild-type alleles in hippocampus for mice with the C57BL/6 (Paradee, Melikian et al. 1999) or C57BL/6J (Li, Pelletier et al. 2002) background. However, FMR1 knockouts (C57BL/6J) expressed reduced LTP in cortex relative to wild type, possibly due in part to significantly reduced cortical expression of the AMPA receptor GluR1 subunit (Li, Pelletier et al. 2002). A recent patch clamp study of trace fear memory in the FMR1 knockout mouse (FVB.129P2-*Fmr1*^{tm1CgR}) found reduced LTP in anterior cingulate cortex and lateral amygdala (Zhao, Toyoda et al. 2005). Taken together, the differences in memory behavior and LTP among studies of knockout strains reflect characteristics of the experimental paradigm, brain region dependence, and could also reflect alterations in cAMP cascade function across brain regions.

Anxiety is commonly featured in human FX and has been extensively reviewed (Hagerman 2002; Tsiouris and Brown 2004). However, behavioral and physiological studies of mouse anxiety suggest abnormal, but not typically heightened anxiety responses in FMR1 knockout mice (For thorough review, see Bernardet and Crusio 2006). For example, in the FVB/NJ strain, knockout mice show reduced anxiety-related behavior in an open field test compared to wild type (Qin, Kang et al. 2002). FMR1

knockouts on a C57BL/6 background, however, express heightened open field exploration and light-dark exploration relative to wild-type controls (Peier, McIlwain et al. 2000). In the C57BL/6 background, anxiety-related behaviors associated with non-functional FMR1 alleles can be rescued via introduction of a yeast artificial chromosome (YAC) bearing a functional copy of the human FMR1 gene (Peier, McIlwain et al. 2000).

While several behavioral phenotypes of the FMR1 knockout mouse are consistent with human autism (Bernardet and Crusio 2006), the abnormal anxiety phenotype suggests that the FX model may not completely model the human condition. Several explanations could account for a possible discrepancy, which we would like to address since it is critical to our model for cAMP's role in the modulation of FX anxiety. One possible explanation is that the existing mouse behavioral tests for anxiety do not represent features of human anxiety. The open field test is commonly employed to measure anxiety-like behaviors but several mechanisms could motivate a rodent to withdraw from an open field environment. For example, open exposure to a light source might feel aversive to a mouse without provoking anxiety. Alternatively, areas adjacent to walls might feel more rewarding. Outstanding empirical and theoretical treatments on the influence of subjective experience on seeking and avoidance behaviors is provided by Theodore Schneirla (Maier and Schneirla 1942; Schneirla 1959). Importantly, mouse anxiety is only one of many subjective states that may explain open field avoidance. Rigorous theoretical and experimental models of the underlying neurobiology of subjective experience are required to gain a critical comparative understanding of affective phenotypes (Panksepp 1998). More novel work with mirrored chambers

(Spencer, Alekseyenko et al. 2005) may provide greater insight into the motivations that modulate mouse behavioral phenotypes.

Another possible explanation is that cyclic AMP does not modulate anxiety phenotypes in FMR1 knockout mice in the same fashion that anxiety is modulated in humans.

Perhaps we can gain insight into FMR1 knockout anxiety from studies of mouse social behavior. Anxiety has been shown to diminish social interaction (File and Hyde 1978; File and Seth 2003). Some studies suggest that social behaviors of FX targeted alleles can be similar to or even enhanced relative to wild type alleles, depending upon the specifics of test design (Spencer, Alekseyenko et al. 2005; Brennan, Albeck et al. 2006), indicating again that lack of a functional FMR1 allele does not enhance social anxiety. However, more recent work, that employed behavioral measures of social amnesia (Ferguson, Young et al. 2000), finds diminished social approach responses of the FMR1-targeted mice (Mineur, Huynh et al. 2006), suggesting the possibility of deficits within the social domain. While levels of social approach can be inversely associated with anxiety levels, they can also be associated with differences in social reward (Panksepp and Lahvis 2007). Since the neural pathways for the subjective experiences of reward and fear/anxiety are respectively supported, in part, by striatal nuclei, such as the nucleus accumbens (Panksepp, Normansell et al. 1994; Kelley, Schiltz et al. 2005), and the amygdala (Rodrigues, Schafe et al. 2004), then primarily cortical alterations to the cAMP system in FMR1 knockout mouse (Figure 1) may have a lesser impact on the subcortical mediation of mouse social or anxiety behaviors.

As a final explanation, the gene targeting and genetic background of the knockout mouse may preserve some aspects of the phenotype, but not all of them. A neomycin resistance cassette is in the FMR1 gene construct (Bakker, Verheij et al. 1994) and its location and orientation may modulate the function of neighboring genes. Orientation of the neomycin resistance cassette, for example, can modify phenotypes through *cis* effects (Lahvis and Bradfield 1998). Genetic background can also modulate phenotypes. Since FMR1-targeted mice are primarily evaluated on only two backgrounds (C57BL/6 and FVB), more representative models of human FX may be generated on other genetic backgrounds. Other gene targeting strategies and use of different genetic backgrounds might generate anxiety phenotypes without discrepancies from the human FX phenotype.

In this sense, more recent gene targeting strategies provide exciting new mouse models of fragile X syndrome. Knock-in mice with an expansion of CGG repeats in the FMR1 promoter have been developed to understand the human FX premutation and its expansion. Mice engineered using a yeast artificial chromosome (YAC) with a (CGG)₉₈ human premutation (Bontekoe, Bakker et al. 2001) have several features in common with the human FX premutation tremor-ataxia phenotype. Compared to wild type mice, the FX premutation knock-in mouse model expresses ubiquitin-positive intranuclear inclusions, elevated FMR1 mRNA levels, and, unlike humans, equivalent FMRP expression compared to wild-type (Willemsen, Hoogeveen-Westerveld et al. 2003). An FX knock-in premutation mouse engineered using serial ligation (SL) methodology (Grabczyk and Usdin 1999), rather than with a YAC, has reduced FMRP expression that is associated with repeat length and varies in its extent across the brain. Unlike the YAC knock-in

mouse (Brouwer, Mientjes et al. 2007), the SL knock-in mouse can also expand its repeat length to a full mutation in one generation (Entezam, Biacsi et al. 2007). Premutation knock-in mice may prove useful to model the cognitive decline of memory in the FX premutation and full mutation phenotype. To assess the value of using FX targeting to generate useful mouse models of autistic phenotypes, it would be very interesting to examine the social and anxiety-related behaviors in these mouse models.

Fragile X Signaling Theories

mGluR Theory of Fragile X

Signaling alterations are central to FX pathophysiology (Miyashiro and Eberwine 2004). Using the FX animal model, mGluR1 and mGluR5 mediated long term depression (LTD), a model for the weakening of synapses, was found to be enhanced and has been formulated into the mGluR theory of FX (Bear, Huber et al. 2004). In this theory, overstimulation of mGluR1 and mGluR5 leads to enhanced synthesis of proteins normally repressed by FMRP through enhanced PKC activity. Mechanistically, the binding of glutamate to mGluR1 and mGluR5 receptors activates the Gq cascade in which phospholipase C cleaves phosphatidylinositol bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 releases calcium from internal stores and DAG activates PKC to increase production of proteins normally repressed by FMRP. This FX theory may not be specific to the mGluR1 and mGluR5 receptors but may be generalizable to a PKC mediated defect (Weiler, Spangler et al. 2004) or to other receptors signaling through Gq (Volk, Pfeiffer et al. 2007). Due to mGluR1 and mGluR5 overstimulation, AMPA and NMDA receptors are thought to be withdrawn from the

neuronal membrane producing weakening of the synapse and LTD. The basis for FX treatment with AMPAkinases is to enhance the open duration of the remaining AMPA receptors to promote strengthening at the synapse (Berry-Kravis and Potanos 2004). Results from a recent clinical trial of AMPAkinases were inconclusive based on low dosing (Berry-Kravis, Krause et al. 2006). Pharmacologic rescue of FX *Drosophila* behavior by an mGluR1 antagonist supports the mGluR theory (McBride, Choi et al. 2005). The consequences of FMRP loss on signaling through mGluR1 and mGluR5 receptors, and more generally through the Gq cascade, contributes to the FX phenotype.

Cyclic AMP Theory of Fragile X

Berry-Kravis and colleagues argue that altered FX cAMP metabolism contributes to the FX neurobehavioral phenotype based on studies of several non-neural cell types which showed that the alteration in cAMP induction is FMRP dependent and occurs at the level of adenylate cyclase or its regulation (Berry-Kravis 1990; Berry-Kravis, Hicar et al. 1995; Berry-Kravis and Ciurlionis 1998). We have termed this line of thought the cAMP theory of FX (Kelley, Davidson et al. 2006; Kelley, Davidson et al. 2007) to distinguish it from the mGluR theory (Bear, Huber et al. 2004).

Decreased cAMP levels are noted in human FX through investigation of various cell types used to model intracellular neuronal activity (Stahl 1977; Camacho and Dimsdale 2000). In platelets, induced cAMP production is significantly lower in patients with FX relative to non-FX controls (Berry-Kravis and Huttenlocher 1992) and also relative to patients with mental retardation, autism, and normal intelligent controls (Berry-Kravis

and Sklena 1993). Although not statistically significant, basal platelet cAMP production tended to be reduced in FX to about 72% that of controls (Berry-Kravis 1992). In lymphoblastoid cell lines of FX patients, a significant negative correlation exists between levels of induced nonreceptor-mediated cAMP production and trinucleotide repeat amplification (Berry-Kravis, Hicar et al. 1995). Overexpression of FMR1 in HN2 mouse neural cells increases levels of cAMP and shows a positive correlation between FMRP and cAMP levels (Berry-Kravis and Ciurlionis 1998). These results suggest that FMRP regulates cAMP levels and, in FX, reduced FMRP levels correspond to reduced stimulated cAMP levels (Berry-Kravis and Ciurlionis 1998). While the experimental manipulations of cAMP levels and the overexpression of FMRP in a cell culture system may fall outside the range of normal cell physiology, the results from these studies point to an alteration in cAMP cascade function that is FMRP dependent. In support of these cellular studies, FX *Drosophila* behavior is improved with lithium and an mGluR2 antagonist, a pharmaceutical known to increase cAMP cascade function (McBride, Choi et al. 2005). Therefore, a relationship between cAMP, FMRP, and FX behavior was established in non-neural cells and FX fly but remained to be tested in brain tissue.

Cyclic AMP production in the FX brain was examined for the first time (Kelley, Davidson et al. 2007) by comparing cAMP levels across fly heads, mouse cortex, and human neural cell lines (Bhattacharyya, McMillan et al. 2008). Induced cAMP levels are reduced in FX and all three models have deficient stimulated cAMP production. This study suggests that neurotransmitters and receptors signaling through cAMP would be functionally deficient in FX as would the influence of cAMP on cAMP-gated channels,

GEFs, and EPAC signaling (Lauder 1993; Neves, Ram et al. 2002; Matulef and Zagotta 2003). If cAMP induction is reduced across neural cell types, all members of the tripartite synapse (Araque, Parpura et al. 1999) could be compromised with respect to cAMP signaling. In neurons, the consequences of this FX cAMP induction impairment must be considered not only postsynaptically, but also presynaptically. Cyclic AMP has a known role in neurotransmitter release from the presynaptic terminal (Kaneko and Takahashi 2004). In FX, reduced cAMP induction at the presynaptic terminal would presumably reduce the inhibition of group II and III mGluR and, depending on splice variant, the group 1 family which would result in a reduced inhibition of glutamate release from the presynaptic terminal (Serajee, Zhong et al. 2003). Taken together, the cAMP cascade is an important signaling pathway deficient in FX and further studies are necessary to characterize cAMP deficiencies at multiple loci of the tripartite synapse.

According to the cAMP theory of fragile X, a loss of FMRP may produce cAMP dependent alterations in functional neurophysiology. Cyclic AMP signal transduction, which is involved in neurotransmission (Florendo, Barnett et al. 1971), neuroplasticity, and mnemonic processes (MacKenzie, Baillie et al. 2002), is a molecular mechanism (Walton and Rehfuess 1990) by which FMRP can affect protein expression (Kaufmann and Reiss 1999). If altered, the production of intracellular cAMP would be disproportional to that demanded by hormones or extracellular neurotransmitters, like epinephrine, dopamine, and serotonin (Lauder 1993). The consequence of cAMP dysregulation in FX would produce a loss of a neurotransmitter's ability to control

postsynaptic neuronal cAMP activity. This, in turn, may ultimately produce dysfunctionally connected neural circuits with behavioral consequences.

Lower levels of PDE4 may be required in FX to maintain cAMP homeostasis because PDE4 levels are regulated by cAMP levels with expression of some PDE4 subgroups potentially being mediated through a cAMP dependent CREB-promoter interaction (Houslay 2001). The levels of cAMP and PDE4 may vary with FMRP across the brain because the level by which FMRP expression is reduced can vary among FX brain regions (Taylor, Tassone et al. 1999) and there is a direct correlation between levels of cAMP and FMRP (Berry-Kravis and Ciurlionis 1998). The extent to which cAMP and PDE4 levels vary with FMRP levels across brain regions could be studied with C¹¹-rolipram using PET (DaSilva, Lourenco et al. 2002).

The FX neurophenotype has distinctive features which may be explained in part by cAMP alterations during neural differentiation. The developmental responsiveness to adenylate cyclase stimulation may be a useful biochemical marker to monitor neural maturation (Seed and Gilman 1971). The cAMP cascade is known to be developmentally regulated in mouse and rat brain (Schmidt, Palmer et al. 1970; Rius, Streaty et al. 1991; Rius, Mollner et al. 1994). Studies examining development of the cAMP cascade in normal mice indicate that the inhibitory influence on adenylate cyclase (AC) early in development is followed by stimulatory AC responsiveness later in development (Rius, Streaty et al. 1991; Rius, Mollner et al. 1994). Developmentally, dysregulation of the cAMP cascade is known to impact growth cone motility and the structure of terminal

varicosities in *Drosophila* (Kim and Wu 1996). Cyclic AMP plays an important role in AMPA trafficking (Lu, She et al. 2003), cell survival and neurogenesis (Nakagawa, Kim et al. 2002), neural induction (Otte, van Run et al. 1989), neurite formation, and synaptogenesis (Takuro Tojima 2003). Cyclic AMP also regulates spine density by gating brain derived neurotrophic factor (BDNF) signaling, a neurotrophin implicated in dendrite formation (Ji, Pang et al. 2005). Several of these features are altered in FX. In addition, EphB2, a key component in dendritic spine formation that may contribute to abnormal pruning in FX (Ethell, Irie et al. 2001; Henkemeyer and Frisen 2001; Irie and Yamaguchi 2004), is a likely candidate to influence development of cAMP responsiveness to forskolin (Ibrisimovic, Bilban et al. 2007). The mechanism for the distinctive development of the FX cAMP response remains unknown but the maturation of AC responsiveness in FX is likely related to FMRP loss or its downstream consequences.

A potential explanation for how reduction of FMRP leads to decreased cAMP production could center on FMRP's role as an mRNA binding protein that shuttles mRNA between the nucleus and cytoplasm (Warren 1997; Weiler, Spangler et al. 2004). FMRP is known to associate with mRNA containing sequences similar to AC types 3, 5, and 6 (Brown, Jin et al. 2001; Kaytor and Orr 2001; Miyashiro, Beckel-Mitchener et al. 2003). Also, FMRP stress granules contain AC type 3 mRNA (Kim, Weiler et al. 2005). Therefore, a lack of FMRP interaction with AC mRNA in FX may explain the reductions in cAMP production because mistranslocation of these mRNAs would reduce the functional availability of AC proteins and impair cAMP production from ATP (Kelley, Davidson et

al. 2007). The cAMP defect would be expected to occur more in the cortex compared to other brain regions based on data from the Allen Brain Atlas (Lein, Hawrylycz et al. 2007) which shows that FMR1 is primarily colocalized with AC type 5 in cortex (Figure 1).

In the context of the mGluR theory (Bear, Huber et al. 2004), it is of interest to note that Ca^{2+} and PKC, both of which are part of the Gq cascade, are able to regulate certain AC isozymes and impair their functional ability. For instance, phosphorylation of AC2 and AC4 by PKC may weaken hippocampal synaptic plasticity by reducing the ability of these ACs to integrate signal from $\text{Gs}\alpha$ and $\text{Gi}\alpha$ receptors (Chern 2000). The mGluR1 and mGluR5 oversignaling through PKC proposed in the FX mGluR theory may enhance the activity of certain AC isozymes, such as type 1 ACs through Gq related calcium increases, to increase cAMP counter to the cAMP theory of FX (Chern 2000). Clearly there is evidence that cyclic AMP and mGluR may interact because a given G protein has the potential to act through a network of multiple overlapping messengers (Neves, Ram et al. 2002). Several lines of evidence support the view that mGluR and cAMP mediated pathways interact in platelets (Moos and Goldberg 1988), lymphocytes (Pacheco, Ciruela et al. 2004), neurons (Otte, van Run et al. 1989; Pilc, Legutko et al. 1996; Cartmell, Schaffhauser et al. 1997; Azad, Monory et al. 2004; Warwick, Nahorski et al. 2005) and astrocytes (Balazs, Miller et al. 1998). This may mean that the impaired cAMP signaling in the cAMP theory of FX may be related to the Gq signaling described in the mGluR theory. The group I mGluR family may interact with the cAMP cascade through a G protein mechanism (Thomsen 1996; Balazs, Miller et al. 1998) involving Gs and Gq

(Tateyama and Kubo 2006). The coupling of the mGluR1 receptor to a given cascade has been localized to the carboxy terminal (Gabellini, Manev et al. 1993) and to intracellular loops two and three (Francesconi and Duvoisin 1998). Further studies are necessary to determine whether the cAMP and mGluR mechanisms occur in series or in parallel (Kelley, Davidson et al. 2007).

cAMP in Autism

The high comorbidity of autism and FX (Brown, Jenkins et al. 1982; Brown, Jenkins et al. 1986; Loesch, Bui et al. 2007) warrants a discussion of cAMP function in autism. To date, no studies of the neuronal cAMP cascade are available in autism or autism comorbid with FX. However, cAMP levels in cerebrospinal fluid (CSF) and peripheral blood levels of cAMP in platelets and plasma are documented in autism. In autism, probenecid induced increases in CSF cAMP levels in children are not different from previously reported baseline levels in adults (Winsberg, Sverd et al. 1980). Autism and controls had comparable levels of stimulated cAMP production in platelets (Berry-Kravis 1992; Berry-Kravis and Sklena 1993), potentially useful models of neural signal transduction (Stahl 1977; Camacho and Dimsdale 2000). Plasma cAMP levels have been used as a physiologic indicator of sympathetic arousal (Huhman, Hebert et al. 1991), emotional stress (Arnetz 1979), noise related stress-induced anxiety (Iwamoto, Ishii et al. 1995), childhood psychoses (Goldberg 1984), and hyperkinetic behavior of autism and mental retardation (Hoshino, Kumashiro et al. 1980). Plasma levels of cAMP are elevated in medicated and unmedicated autistic children relative to controls, but plasma cAMP levels are reduced relative to mentally retarded patients with hyperkinetic disorder

(Hoshino, Kumashiro et al. 1980; Cook 1990). Increased plasma cyclic AMP levels in autism are significantly and positively associated with hyperkinesia and serum serotonin levels (Hoshino, Kumashiro et al. 1979). However, since a variety of tissues contribute to plasma cAMP levels, the interpretation of plasma cAMP studies is nonspecific to brain (Goldberg 1984) and could be explained by alternative pathways such as hepatorenal plasma cAMP signaling (Ahloulay, Dechaux et al. 1996). Although CSF and peripheral blood measures indicate that cAMP levels are normal to high in autism, the cAMP cascade remains to be studied in autistic brain.

Pharmacological studies based on theoretical models of autism provide further evidence for the potential role of cAMP pathology in autism. There are several agents known to affect cAMP that are therapeutic in autism. To account for the altered social and emotional behavior in autism, the opiate theory of autism proposes that autists have a hyperactive opiate system (Panksepp 1979). Opioids are known to reduce the ability of adenylate cyclase to produce cAMP (Koski and Klee 1981; Childers, Nijssen et al. 1986; Childers 1988; Prather, Tsai et al. 1994). Several autism studies indicate that naltrexone, an opioid antagonist which would improve adenylate cyclase function, may be beneficial to some aspects of autism behavior in some patients (Kolmen, Feldman et al. 1995; Riddle, Bernstein et al. 1999; Elchaar, Maisch et al. 2006) but not all behaviors (Zingarelli, Ellman et al. 1992; Willemsen-Swinkels, Buitelaar et al. 1995; Feldman, Kolmen et al. 1999). The fetal valproate model of autism was based on case reports of the increased incidence of autism in individuals with fetal exposure to the maternal anti-epileptic valproate (Williams, King et al. 2001). Valproate has several mechanisms of

action and is known to reduce stimulated, but not basal, cAMP levels (Montezinho, Mork et al. 2007). Mice exposed to valproate during development exhibit functional deficits that parallel autism (Ingram, Peckham et al. 2000; Wagner, Reuhl et al. 2006). The impact of valproate on the functional overconnectivity of cortical microcircuitry (Rinaldi, Silberberg et al. 2007) has implications for the system level functional connectivity alterations noted in autism. Secretin is a neuropeptide that increases intracellular cAMP levels by activating adenylate cyclase upon binding its type II Gs coupled receptor, which has been linked to social behavior in mice (Nishijima, Yamagata et al. 2006; Siu, Lam et al. 2006). A case study (Horvath, Stefanatos et al. 1998) reported improvement in autism behavior with infusion of secretin, although further clinical trials do not support the efficacy of this approach (Esch and Carr 2004; Sturmey 2005; Williams, Wray et al. 2005). Based on these theories and the studies testing them, elevated cyclic AMP in CSF and peripheral blood may reflect a compensatory mechanism to increase low cAMP levels.

Screens of genes associated with susceptibility to autism have also implicated cAMP signaling in the autism phenotype. Variants in the cAMP guanine nucleotide exchange factor II (cAMP-GEFII) gene are associated with the autism phenotype (Bacchelli, Blasi et al. 2003). cAMP-GEFII codes for a regulator of G proteins that is functionally dependent on cAMP levels and is involved in neuronal development and exocytotic processes. The mGluR8 receptor has an inhibitory effect on cAMP signaling; and, variants in the mGluR8 gene, GRM8, at 7q31 are associated with an increased susceptibility to autism (Serajee, Zhong et al. 2003). The cAMP cascade remains to be

studied directly in autism brain and may also prove to be a useful target to enhance memory for behavioral therapies.

cAMP Therapy

Treatment through the cAMP cascade may be beneficial in FX. Cyclic AMP was thought to regulate FMRP expression through an FMR1 enhancer (Hwu, Wang et al. 1997).

However, a recent PC12 study showed that FMR1 is not cAMP inducible, presumably due in part to the absence of a TATA box in the FMR1 promoter region, even though FMR1 is bound by phospho-CREB/ATF (Smith, Nicholls et al. 2006). Nevertheless, therapies working through cyclic AMP transduction may be beneficial through indirect effects on FX neurophysiology. In particular, FX memory deficits (Greicius, Boyett-Anderson et al. 2004) and hypersensitivity to stress (Barad, Bourtchouladze et al. 1998) may show improvement by enhancing cAMP levels.

With pharmaceutical calibration of FX cAMP levels, the intracellular cAMP signaling cascade may be able to properly regulate the cAMP second messenger in response to extracellular signals (Chen, Hasanat et al. 1999) to improve FX behavior. For example, the mood stabilizer lithium increases cAMP production (Chen, Hasanat et al. 1999) and is known to improve FX *Drosophila* memory behavior, as have mGluR2 antagonists which elevate cAMP levels (McBride, Choi et al. 2005). Enhancing cAMP production strengthens glutamatergic synapses by augmenting the function of AMPA receptors (Chavez-Noriega and Stevens 1992; Tseng and O'Donnell 2004). AMPA receptors are reduced in FX and are pharmaceutical targets of interest (Bear, Huber et al. 2004; Berry-

Kravis and Potanos 2004). Among pharmaceuticals, forskolin (Seamon and Daly 1986) is a diterpine extracted from the root of *Coleus forskohlii*, a member of the mint family, that is a reversible activator of adenylate cyclase (AC) in a number of cells including neurons (Seamon, Padgett et al. 1981). Forskolin is a useful tool in characterizing AC function and the AC response to extracellular or intracellular modulators (Insel and Ostrom 2003). In a step-down active avoidance task, long term memory in rats improved when forskolin was directly infused into hippocampus (Bernabeu, Bevilaqua et al. 1997). As an over-the-counter herbal, forskolin may act as a nootropic to enhance cAMP-mediated memory processes in FX. Rolipram, 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone, is a potent PDE4 inhibitor, a class that shows clinical potential in the treatment of depression. Through cAMP-mediated mechanisms, pharmacotherapy that inhibits PDE4 may increase the transcription and function (Miller, Vogt et al. 2002; Pace, Hu et al. 2007) of reduced numbers of FX dendritic glucocorticoid receptors (Miyashiro, Beckel-Mitchener et al. 2003) involved in hypothalamic-pituitary-adrenal (HPA) regulation (Hessl, Rivera et al. 2004). A clinical trial of rolipram reported acceptable tolerance levels at clinically relevant doses (Fleischhacker, Hinterhuber et al. 1992). In clinical and preclinical trials, the main human PDE4 inhibitor side effect, thought to be due to PDE4D activity (Robichaud, Savoie et al. 2002; Robichaud, Stamatiou et al. 2002; Conti, Richter et al. 2003), is emesis at clinical doses while rats are more susceptible to toxic effects like arteriopathy. This may be due to reduced PDE4 enzyme activity in humans relative to monkeys and rats (Bian, Zhang et al. 2004).

The prospect of using PDE4 inhibitors like rolipram is particularly appealing because memory enhancement through facilitation of cAMP signaling may be possible without changes in basal cAMP levels (Barad, Bourtchouladze et al. 1998). Several new PDE4 inhibitors are or are likely to become available and may provide new therapies for patients with FX. HT0712 (Inflazyme PDE4 Library IPL455,903) is a PDE4 inhibitor produced by Helicon Therapeutics that is currently in Phase 1 human clinical trials. In a mouse model of Rubinstein-Taybi mental retardation, both HT0712 and rolipram rescued object recognition memory processes which were deficient relative to wild-type (Bourtchouladze, Lidge et al. 2003). MEM1018 and MEM1091 are PDE4 inhibitors that behave much like rolipram with comparable PDE4 inhibitory activity profiles. MEM1018, MEM1091, and rolipram can restore pharmacologically induced impairment of long term memory and potentiate NMDA-stimulated cAMP production (Zhang, Huang et al. 2005). Based on the cAMP theory, pharmacotherapy may act as both a nootropic to improve mnemonic processing and an anxiolytic.

Conclusion

The cAMP system is known to be involved in mnemonic processing and anxiogenesis. Defects in the cAMP cascade may contribute to the phenotypic features of these behaviors in FX and autism. Cyclic AMP signaling is reduced in FX but may be normal or enhanced in autism brain. Cyclic AMP may be a useful biochemical marker in distinguishing autism per se from the autism noted in patients with FX. In patients with FX comorbid with autism, reduced cAMP signaling would suggest that the autism noted in FX is unique whereas a normal to high cAMP level would indicate that autism may be

a compensatory process to increase cAMP levels. Further studies are needed in brain to test this hypothesis and to determine the efficacy of treatment through the cAMP cascade. Treatment approaches through pharmacologic manipulation of the cAMP cascade may benefit FX and autism individuals by alleviating anxiety and improving mnemonic processing.

Acknowledgements

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Figures:

Figure 1: Expression Summary of FMR1 and Adenylate Cyclase Type V. In normal mice, cortical expression of FMR1 is greatest in cortex but adenylate cyclase expression is ubiquitous. Data available from [<http://www.brain-map.org>]. CB, cerebellum; CTX, cerebral cortex; HIP, hippocampal region; HPF, hippocampal formation; HY, hypothalamus; LSX, lateral septal complex; MB, midbrain; MY, medulla; OLF, olfactory areas; P, pons; PAL, pallidum; sAMY, striatum-like amygdalar nuclei; STR, striatum; STRd, striatum dorsal region; STRv, striatum ventral region; TH, thalamus; RHP, retrohippocampal region.

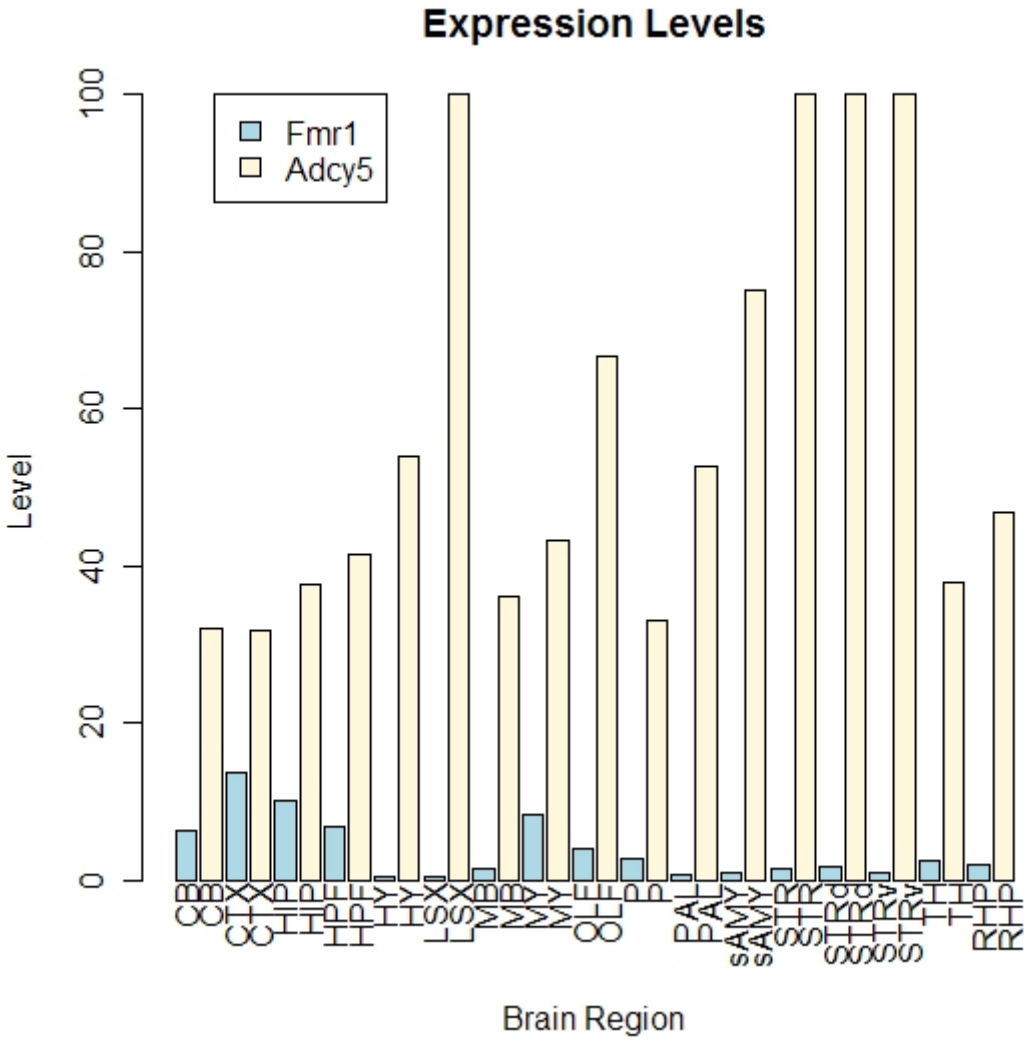


Figure 1

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Title

The Cyclic AMP Cascade is Altered in the Fragile X Nervous System.

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Abstract

Fragile X syndrome (FX), the most common heritable cause of mental retardation and autism, is a developmental disorder characterized by physical, cognitive, and behavioral deficits. FX results from a trinucleotide expansion mutation in the *fmr1* gene that reduces levels of fragile X mental retardation protein (FMRP). Although research efforts have focused on FMRP's impact on mGluR signaling, how the loss of FMRP

leads to the individual symptoms of FX is not known. Previous studies on human FX blood cells revealed alterations in the cyclic adenosine 3', 5'-monophosphate (cAMP) cascade. We tested the hypothesis that cAMP signaling is altered in the FX nervous system using three different model systems. Induced levels of cAMP in platelets and in brains of *fmr1* knockout mice are substantially reduced. Cyclic AMP induction is also significantly reduced in human FX neural cells. Furthermore, cAMP production is decreased in the heads of FX *Drosophila* and this defect can be rescued by reintroduction of the *dfmr* gene. Our results indicate that a robust defect in cAMP production in FX is conserved across species and suggest that cAMP metabolism may serve as a useful biomarker in the human disease population. Reduced cAMP induction has implications for the underlying causes of FX and autism spectrum disorders. Pharmacological agents known to modulate the cAMP cascade may be therapeutic in FX patients and can be tested in these models, thus supplementing current efforts centered on mGluR signaling.

Introduction

Fragile X syndrome (FX) is characterized by physical, cognitive, and behavioral deficits (Hagerman 2002). A CGG trinucleotide expansion in the *fmr1* gene suppresses production of the FX mental retardation protein (FMRP), an RNA binding protein that regulates translation through interaction with dendritic ribosomes and has a role in mRNA shuttling (Kremer, Pritchard et al. 1991; Pieretti, Zhang et al. 1991; Verkerk, Pieretti et al. 1991; Bagni and Greenough 2005). Both mouse and *Drosophila* models of FX have been characterized (Kooy 2003; Zhang and Broadie 2005). Rodent models are surprisingly mild in their phenotypes compared to the severity of the human disease. *Drosophila* models show stronger phenotypes with defects in brain development, axon

pathfinding, and effects on courtship-based learning and memory formation and circadian rhythms. A major advance occurred when Huber and Bear reported deficits in synaptic plasticity in FX mice. Their “mGluR theory of FX” proposes that there is excessive signaling through the mGluR5 subtype of glutamatergic receptor (Bear, Huber et al. 2004). Experiments in hippocampus show that a form of synaptic plasticity, long-term depression (LTD), can be induced with either type I mGluR (mGluR1 and mGluR5) agonists or with low frequency electrical stimulation (Huber, Gallagher et al. 2002). LTD persistence requires de novo protein synthesis and, remarkably, involves FMRP. FX mice show excessive LTD, suggesting that FMRP is normally involved in a pathway terminating this type of LTD. Contemporaneously, FX mice were shown to be sensitive to audiogenic seizures, a striking phenotype similar to some FX patients who also suffer seizures (Musumeci, Bosco et al. 2000). Key pharmacological experiments in both models show that mGluR5 antagonists can reverse most, but not all, of the endophenotypes (Bear, Huber et al. 2004; McBride, Choi et al. 2005; Yan, Rammal et al. 2005). It is therefore possible that there are other defects within the FX brain that are not reversible when mGluR signaling is inhibited.

In the early 1990s, Berry-Kravis and colleagues postulated that another intracellular signaling pathway, cyclic AMP (cAMP), contributes to FX neuropathophysiology (Berry-Kravis and Huttenlocher 1992). Cyclic AMP is produced by adenylate cyclase (AC), degraded by phosphodiesterase (PDE), and can bind PKA, GEFs, and CNG channels. AC can be stimulated by G proteins or forskolin and modulated by Ca^{2+} , G proteins, PKA, PKC, and CaMK (Gilman 1995). The cAMP system is critical in many aspects of neural development (Beavo and Brunton 2002) and

may be important in autism susceptibility (Bacchelli, Blasi et al. 2003; Serajee, Zhong et al. 2003). Cyclic AMP production is decreased in non-neural cells from FX patients and this alteration is FMRP dependent (Berry-Kravis, Hicar et al. 1995; Berry-Kravis and Ciurlionis 1998). This “cAMP theory of FX” posits that alterations to the cAMP pathway may result from dysregulated expression of proteins involved in the cAMP cascade either (1) in parallel with the mGluR theory using a mechanism independent of mGluR or (2) in series with the mGluR theory through a mechanism downstream of mGluR overactivity.

The variable clinical presentation of FX and the partial reversal of phenotypes achievable using mGluR antagonists suggest that excessive mGluR signaling is difficult to correct within the mGluR pathway or that parallel pathways are involved. Therefore, we investigated the cAMP theory in the FX central nervous system. We tested whether the cAMP defect is conserved across species, thereby indicating a common mechanism for the cAMP defect. If FMRP loss limits cAMP production in the FX brain *in vivo*, pharmacologic agents known to act through the cAMP cascade may be therapeutic to FX patients.

Results

To test the hypothesis that there is a highly conserved relationship between FMRP and cAMP activity, we examined cAMP production in mouse, fly and human systems containing a non-functional *fmr1* gene. Forskolin is a general activator of adenylate cyclases. Berry-Kravis and Sklena reported that normal human platelets show a greater forskolin-mediated increase in cAMP than platelets from individuals with FX (Berry-Kravis and Sklena 1993). We investigated this effect in wild-type and *fmr1* knockout

mouse platelets using a fluorescence-based assay, where raw fluorescence (RF) decreases when cAMP levels increase. Figure 1a shows that untreated platelets have similar levels of RF, but forskolin treated wild-type platelets show a greater decrease than comparably treated *fmr1* knockout platelets. When the fractional decrease in RF (FDRF) is calculated, *fmr1* knockout mouse platelets show less of a decrease (indicating reduced cAMP induction) compared to wild type platelets [$t(6)=3.46$; $p=0.014$] (Figure 1b).

To extend these results to brain, we measured cAMP levels from wild-type and *fmr1* knockout mouse cortical membranes. Although the RF was comparable between groups in the absence [$t(4)=1.22$; $p=0.29$] or presence [$t(4)=-1.92$; $p=0.13$] of forskolin (Figure 1c), the FDRF is significantly less in *fmr1* knockout tissue than wild-type tissue, indicating that the FX cortical tissue shows reduced cAMP induction [$t(4)=3.01$; $p=0.04$] (Figure 1d). To determine whether the cAMP defect is a ubiquitous feature of the FX knockout mouse phenotype, cAMP responses to forskolin from platelets and brain cortex from wild-type and *fmr1* knockout mice were compared as a contrast within a three way ANOVA (Group $\times$ Stimulus $\times$ OrganSystem). The *fmr1* knockout brain and blood consistently produce less cAMP than wild-type tissue based on RF measures [Group $\times$ Stimulus: $F(1,20)=10.50$; $p=0.004$] and FDRF [$F(1,10)=14.92$; $p=0.003$].

Using the *Drosophila* model, we varied the gene dosage of the fly FX gene (*dfmr1*) (0=FX, 1=FXR+, 2=FXR++), and measured cAMP production from head membrane preparations in the presence or absence of forskolin. RF-based cAMP measures are comparable among wild-type, FX, FXR+, and FXR++ flies in the absence of forskolin [$F(3,21)=1.96$; $p=0.15$]. However, in the presence of forskolin [$F(3,21)=6.23$; $p=0.003$], FX flies have a larger RF compared to all other groups taken

collectively, $[t(21)=-4.1;p=0.000]$, or individually (wild-type $[t(21)=-3.16;p=0.005]$, FXR+ $[t(21)=-3.74;p=0.001]$, and FXR++ $[t(21)=-4.10;p=0.001]$). In the presence of forskolin, the wild-type flies are indistinguishable from the rescue flies $[t(21)=1.61;p=0.123]$ with a single [FXR+ FDRF: $t(21)=1.09;p=0.29$] or multiple transgenes [FXR++ FDRF: $t(21)=1.58;p=0.13$] (Figure 2a). cAMP production, as assessed using FDRF, is comparable among wild-type and the group of rescued flies [FDRF: $t(21)=-0.155;p=0.88$] with one [FXR+ FDRF: $t(21)=0.485;p=0.632$] or two transgenes [FXR++ FDRF: $t(21)=-0.74;p=0.46$]. However, FDRF and thus cAMP induction, is diminished in FX relative to wild-type membranes [FDRF: $t(21)=3.85;p=0.001$], or relative to the group of rescued FX flies [FDRF: $t(21)=4.03;p=0.001$] with a single [FXR+ FDRF: $t(21)=3.23;p=0.004$] or multiple transgenes [FXR++ FDRF: $t(21)=4.13;p=0.000$] (Figure 2b). Therefore, FX mutant flies show less of an induction in cAMP in response to forskolin, and either one or two rescue transgenes alleviate this effect.

To extend these experiments to human cells, we examined forskolin's effects on cultures of human FX and unaffected control neural cells. Both control and FX cultures contain neurons and astrocytes (Figure 3a), but the FX cells express markedly less FMRP than control cells (Figure 3b). Levels of cAMP in FX human neural cells are comparable to control cells in the absence of forskolin [RF: $t(6)=0.82;p=0.44$]. However, when the cultures are treated with forskolin, FX cultures produce lower levels of cAMP than controls [RF: $t(6)=-3.40;p=0.015$] (Figure 3c). The fractional change in cAMP production is differentially reduced in FX compared to both controls [FDRF: $t(6)=3.43; p = 0.01$] (Figure 3d) indicating that the FX neural cell show reduced cAMP induction.

Discussion

Although FX etiology is well defined, individual differences in syndrome expression and symptoms complicate current options for treatment. To define therapeutic pathways to target, it is important to identify cellular processes affected in the FX brain. Based on previous work, we compared the inducibility of cAMP across neural models and found that cAMP induction is decreased in the FX fly, mouse and human nervous system.

The alterations we detect in FX nervous tissue have broad implications, since cAMP signaling has widespread cellular impact, potentially affecting channel function, signal transduction (GEFs and EPAC) and gene expression (CREB) (Beavo and Brunton 2002). The conservation of the cAMP defect in fly, mouse and human species suggests that cAMP metabolism may serve as a useful biomarker in the human disease population, and may account for some of the differences in clinical endophenotypes. An important corollary is that increasing cAMP levels could improve neuronal function and behavior in FX patients. Precedent exists for pharmacological rescue of FX behavior, since lithium or mGluR5 antagonists improve behavior in *Drosophila* memory formation (McBride, Choi et al. 2005). However, it is not known if these treatments affect cAMP inducibility, or otherwise target the cAMP pathway. The prospect of using PDE4 inhibitors, such as rolipram or HT0712, is particularly appealing, since memory enhancement through facilitation of cAMP signaling may be possible without changing basal cAMP levels (Barad, Bourtchouladze et al. 1998; Bourtchouladze, Lidge et al. 2003). There are numerous candidate mechanisms that might contribute to cAMP rescue of FX neuronal

defects, including different effects on cAMP signaling or cross talk with the MAPK, PKC, and/or IP3/Ca pathways.

How does a decrease in FMRP lead to alterations in cAMP? FMRP is an mRNA binding protein that shuttles subsets of mRNA between the nucleus and subcellular compartments such as neuronal dendrites. Gene expression analyses in FX models suggest that the mRNAs for adenylate cyclase types 3, 5 and 6 might be decreased in FX tissue (Brown, Jin et al. 2001; Kaytor and Orr 2001; Miyashiro, Beckel-Mitchener et al. 2003). The decrease in steady-state concentrations of other candidate mRNAs could also contribute to the alterations in cAMP metabolism that we observe.

In support of the cAMP theory, our results identify cAMP alterations in the FX nervous system that may account for some of the neuropathophysiology of FX. Several lines of evidence support the view that mGluR and cAMP mediated pathways functionally interact in platelets (Moos and Goldberg 1988), lymphocytes (Pacheco, Ciruela et al. 2004), neurons (Otte, van Run et al. 1989; Pilec, Legutko et al. 1996; Cartmell, Schaffhauser et al. 1997; Azad, Monory et al. 2004; Warwick, Nahorski et al. 2005) and astrocytes (Balazs, Miller et al. 1998). However, further studies are required to clarify the series or parallel relationship between the cAMP and mGluR defects in FX. For example, rescue of cAMP deficits in FX with mGluR inhibitors would suggest that the pathways operate in series, with the cAMP defect presumably downstream of excessive mGluR activity. Lack of, or incomplete rescue, would support a parallel mechanism where proteins that are directly involved in, or that interact with, the cAMP cascade are dysregulated by FMRP loss either through basal or non-mGluR mechanisms. The specific mechanism of FMRP interaction with the cAMP cascade and the conserved

upstream, downstream, and behavioral consequences of reduced cAMP induction can be studied within and across FX mouse, fly, and human models. Most importantly, this work lays the groundwork for future therapeutic options on FX, thus complementing current approaches focused on the mGluR pathway.

Materials and Methods

Mouse Platelet AC Membrane Preparation

Whole mouse blood (~0.75 ml/mouse) was collected from the first systolic ejection upon decapitation and collected in EDTA solution (1mg EDTA/ml whole blood). Within 2 hours of collection, blood samples were centrifuged at 700 x g for 10 minutes at room temperature (Hines and Tabakoff 2005). The upper platelet-rich plasma layer (UPRP) was transferred to a fresh centrifuge tube and centrifuged at 700 x g for 10 minutes at room temperature. The UPRP plasma was centrifuged at 2800 x g for 15 minutes at room temperature. The platelet pellet was stored at -70°C until assayed. Platelets were thawed on ice and centrifuged at 17000 x g in a microfuge for 10 minutes. Protein levels were determined with the Lowry protein assay (Bio-Rad Laboratories, Hercules, CA). 100ug protein was incubated in KRB-IBMX at 37°C for 20 minutes in the presence of ATP +/- forskolin (5 μM) (Sigma, St. Louis, MO), solubilized in DMSO or DMSO alone and then assayed for cAMP.

Mouse Brain Cortical Membrane Preparation

Cerebral cortices were harvested from three-month old wild type and age-matched FX (*fmr1* knockout) male C57/Bl6 mice. Tissue was homogenized in 25 mM HEPES, 2

mM EGTA, pH 7.4, and centrifuged at 750 x g for 10 minutes at 4°C. The supernatant was centrifuged at 45,000 x g for 60 minutes at 4°C (Ortinu, Lin et al. 2005). Pellets were resuspended in HEPES buffer and stored at -80° C until assayed. Cortical membranes were thawed and centrifuged at 17000 x g in a microfuge for 10 minutes at 4°C. The pellet was resuspended in 1X KRB-IBMX solution. 300ug of protein was incubated in KRB-IBMX at 37°C for 20 minutes in the presence of ATP +/- forskolin (5 µM) or DMSO, and then assayed for cAMP.

Drosophila Head Membrane Preparation

Wild-type, FX, FX plus a dfmr rescue transgene (FXR+), and FX plus a double dfmr transgene (FXR++) male *Drosophila Melanogaster* were frozen, heads isolated, and stored at -70°C (Running Deer, Hurley et al. 1995). Fifty heads were homogenized with a motorized pellet pestle (Kontes; Vineland, NJ) in 100 uL ice-cold homogenization buffer (15mM HEPES pH 7.5, 10mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, 5% sucrose, Roche Complete Protease Inhibitor cocktail tablet). The suspension was allowed to settle 5 min on ice to remove the chitin exoskeleton. To remove eye pigment, 5mg acid rinsed charcoal was added (Zhang, Friedman et al. 2005) and the suspension was centrifuged three times at 200 x g for 2 minutes to remove charcoal. The supernatant was centrifuged at 14,000 x g for 15 minutes and resuspended in 50uL of 1X KRB-IBMX. 2 ug *Drosophila* head membranes was incubated in KRB-IBMX at 37°C for 20 minutes in the presence of ATP +/- forskolin (5 µM) or DMSO, and then assayed for cAMP.

Human Neural Cells

Human FX and unaffected neural cells were differentiated from FX and unaffected human neural progenitor cells (hNPCs) (Bhattacharyya et al., unpublished data (Svendsen, ter Borg et al. 1998)). Human neural progenitor cells were derived from post-mortem cerebral cortices of 3 control fetuses (gestational ages 81-98 days) and a full mutation male FX fetus (gestational age approximately 98 days). Human fetal tissue was obtained according to the guidelines recommended by National Institutes of Health for the collection of fetal tissue and set out by the University of Wisconsin-Madison. Institutional Review Board approval was obtained for all of these studies. FX and control hNPCs were plated onto laminin/poly-L-lysine flasks in DMEM/Ham's F12 media (Invitrogen, Carlsbad CA) with penicillin, streptomycin, amphotericin B (PSA, 1%; Invitrogen, Carlsbad CA) and supplemented with B27 (2%; Invitrogen, Carlsbad CA) and NT4 (20ng/ml; Peprotech) for 7 days (Caldwell, He et al. 2001). Cells were then dissociated and grown at a density of 5000 cells/ul for 9-12 weeks in Neurobasal media (Invitrogen, Carlsbad CA) with penicillin, streptomycin, amphotericin B (PSA, 1%; Invitrogen, Carlsbad CA) and glutamine (Sigma, St. Louis, MO) and supplemented with fetal calf serum (10%; Invitrogen, Carlsbad CA), B27 (2%; Invitrogen, Carlsbad CA) and NT4 (20ng/ml; Peprotech). For cAMP assays, cells were enzymatically dissociated and suspended to a final concentration of 5×10^5 cells/ml in Krebs-Ringer Bicarbonate buffer (KRB) with 3-isobutyl-1-methylxanthine (IBMX; a non-selective PDE inhibitor). After 15 minutes, cells were stimulated with 50uM forskolin or DMSO alone for 30 minutes at room temperature and assayed for cAMP levels.

Immunocytochemistry: Cultures were fixed in 4% paraformaldehyde and washed in PBS. Fixed cells were blocked and permeabilized in 5% goat serum with 0.2% Triton

X-100 and subsequently incubated with antibodies to MAP2 (1:1000; Sigma, St. Louis, MO) or FMRP (1:1000; Chemicon/Millipore, Billerica, MA) followed by incubation with a fluorescent secondary antibody. Hoechst 33258 (Sigma, St. Louis, MO) was used as a nuclear stain.

Western blots: FX and control neural cells were harvested in lysis buffer (20mM Tris, pH 8, 137mM NaCl, 1% NP-40, 10% glycerol, protease inhibitors) and cleared by centrifugation. Protein extracts (25ug) and molecular weight standards were size fractionated with SDS-PAGE and immunoblotted with antibodies to FMRP (Chemicon/Millipore, Billerica, MA) and actin (Sigma, St. Louis, MO). Proteins were visualized by incubation with horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence (Amersham Biosciences), followed by exposure onto x-ray film.

Flourescent cAMP Assay

The Bridge-It cAMP designer fluorescence assay system (Mediomics, LLC, St. Louis, MO) was used to measure cyclic AMP levels in all samples (Heyduk, Fei et al. 2003). cAMP is quantified as a reduction in fluorescence when CAP (cAMP Receptor Protein), a bacterial transcription factor whose DNA binding affinity is high in the presence of cAMP and low in cAMP's absence, binds to DNA fluorescently tagged at the CAP binding site. Binding quenches fluorescence. This fluorescence assay has a 5nM cAMP detection level and was carried out according to manufacturer's specifications. Fluorescence intensity was evaluated by using a Safire II plate reader (Tecan, Inc., St. Louis, MO). Raw fluorescence (RF) was used as an index of static cAMP levels at rest

and after stimulation. The change in cAMP production was assessed using the fractional decrease in RF ($FDRF = (No\ Forskolin - Forskolin) / (No\ Forskolin)$), a statistic that measures the percentage of fluorescence quenching and is both reproducible for a given cAMP level and independent of the instrument used to measure fluorescence.

Statistical analyses were conducted in SPSS 14.0 (SPSS, Inc, Chicago, IL) and evaluated for two-tailed significance. All fluorescence trials were replicated in triplicate unless otherwise noted. Human cell studies were blinded with respect to FX status and unblinded for analysis.

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Figure Legends

Figure 1: cAMP production is decreased in FX mice. Platelets (Control n=3, FX n=5) and cortical membranes (n=3 for each group) from wildtype and *fmr1* knockout mice were stimulated with forskolin and levels of cAMP were measured (each n averaged in triplicate). Raw fluorescence (RF) is an inverse index of cAMP levels. The change in cAMP production was assessed using the fractional decrease in RF (FDRF) = (No Forskolin - Forskolin) / (No Forskolin). (a) Mouse platelet RF is comparable in the absence of forskolin [$t(6)=0.99$; $p=0.36$] but significantly different between groups (Group $\times$ Stimulus: [$F(1,12)=7.56$; $p=0.018$]) in the presence of forskolin [$t(6)=-2.62$; $p=0.040$]. (b) Mouse platelet FDRF shows reduced cAMP induction in FX platelets [$t(6)=3.46$; $p=0.014$]. (c) Mouse cortex RF is comparable between groups in the absence [$t(4)=1.22$; $p=0.29$] or presence [$t(4)=-1.92$; $p=0.13$] of forskolin. (d) Mouse cortex FDRF shows reduced FX cAMP induction [$t(4)=3.01$; $p=0.04$]. Error bars are SEM.

Figure 2: cAMP production is decreased in FX fly. Head membranes from wildtype, FX, FXR+, FXR++ flies were stimulated with forskolin and levels of cAMP were measured. Raw fluorescence (RF) is an inverse index of cAMP levels. The change in cAMP production was assessed using the fractional decrease in RF (FDRF) = (No Forskolin - Forskolin) / (No Forskolin). (a) RF-based cAMP measures in the fly head (Control n=10, FX n=3, FXR+ n=6; FXR++ n=6 sets of 20 heads) are comparable between groups in the absence of forskolin [$F(3,21)=1.96$; $p=0.15$], but not its presence [$F(3,21)=6.23$; $p=0.003$], since RF intensity in FX flies is larger than the others [$t(21)=-4.10$; $p=0.000$]. (b) Fly head FDRF shows reduced FX cAMP induction relative to control membranes [FDRF: $t(21)=3.85$; $p=0.001$] and to the FX flies rescued with a single or multiple transgenes [FXR+FDRF: $t(21)=3.23$; $p=0.004$; FXR++FDRF: $t(21)=4.13$; $p=0.000$]. Error bars are SEM.

Figure 3: cAMP production is decreased in human FX neural cells. Unaffected and FX human neural cells were stimulated with forskolin and levels of cAMP were measured. Raw fluorescence (RF) is an inverse index of cAMP levels. The change in cAMP production was assessed using the fractional decrease in RF (FDRF) = (No Forskolin - Forskolin) / (No Forskolin). (a) Neurons (MAP2, green) are present among cells (Hoechst stain, nuclei in blue) in 9 week FX cultures. (b) Extracts of human neural cells were immunoblotted with antibodies to FMRP and actin. FX cells (FX) have reduced of FMRP compared to 3 controls (control 1, 2, 3). Similar levels of actin are detected in each sample. (c) Human neural cell RF (2 Controls, each with n=3; FX, n=3) is comparable between groups in forskolin's absence [$t(6)=0.82$; $p=0.44$] but FX cAMP production is lower than controls in the presence of forskolin [RF: $t(6)=-3.40$; $p=0.015$]. (d) Human neural cell FDRF was reduced in FX [$t(6)=3.43$; $p=0.01$]. Error bars are SEM for FX and pooled SEM for controls. $(\text{Pooled SEM})^2 = \text{SEM}_1^2 + \text{SEM}_2^2 - 2 * \text{SEM}_1 * \text{SEM}_2$.

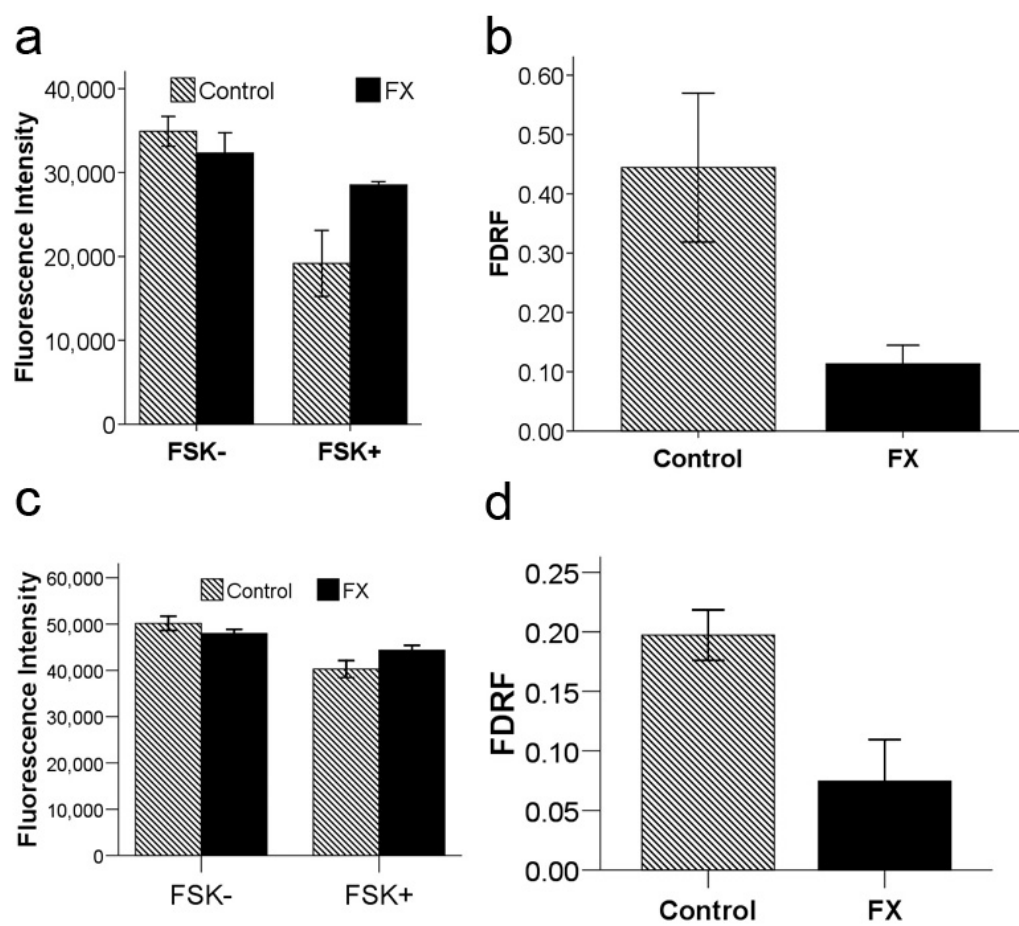


Figure 1

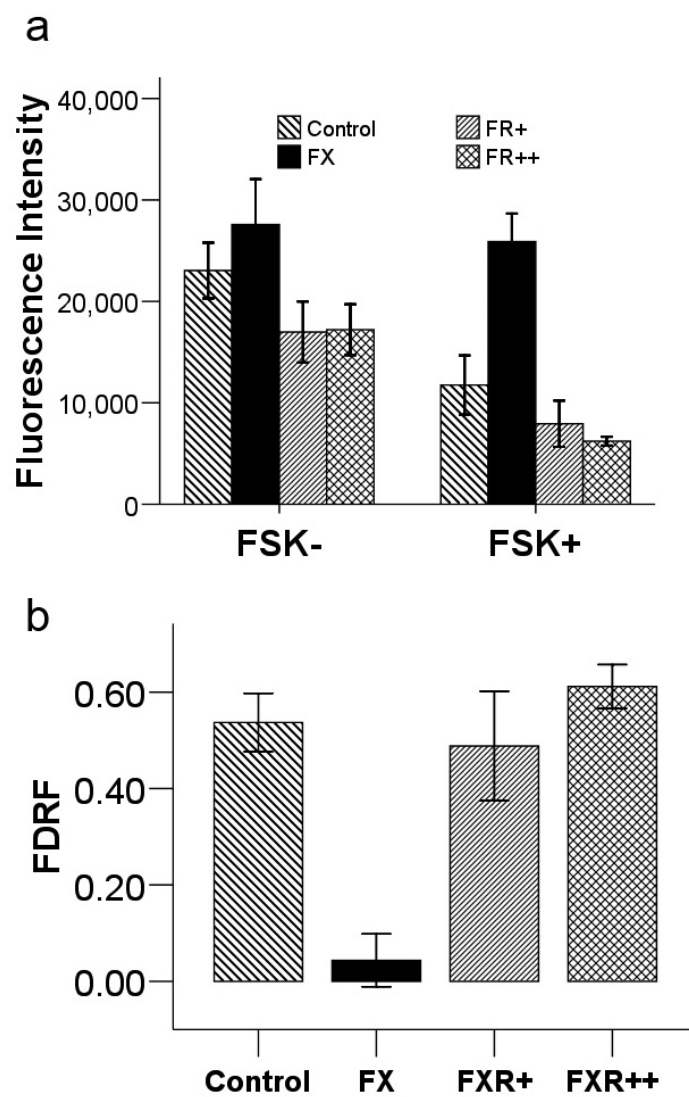


Figure 2

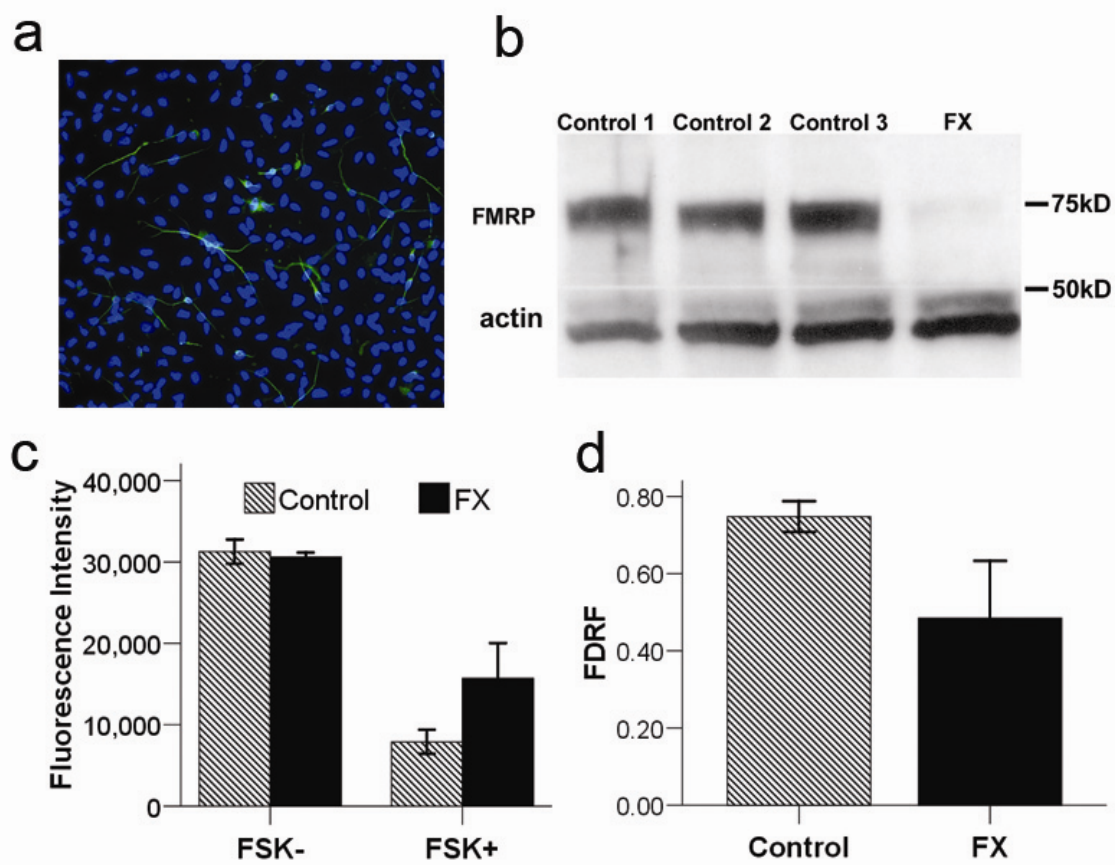


Figure 3

Cyclic AMP in Fragile X Neural Development: Implications for Prenatal Therapy

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Abstract

Fragile X is a developmental disorder caused by a trinucleotide repeat expansion in the FMR1 gene that results in loss of fragile X mental retardation protein (FMRP). We recently identified cyclic AMP (cAMP) cascade alterations in the fragile X (FX) central nervous system. Here we investigated whether cAMP alterations were detectable in FX neural development by assessing cAMP levels, a marker of neural maturation, in FX and control neural progenitor cells. FX neural progenitor cells with reduced FMRP produced comparable cAMP levels upon stimulation as control neural progenitors. However, cAMP induction in differentiated neural cells was greater than neural progenitor cells for controls but comparable in FX. Impaired maturation of post-differentiation cyclic AMP responsiveness is a biochemical marker of impeded neurodevelopment that can distinguish FX from controls. The unique trajectory of FX cAMP responsiveness suggests that prenatal treatment recalibrating the cAMP cascade may benefit FX neurodevelopment.

Introduction

Fragile X syndrome, or Martin-Bell syndrome, is the most common inheritable cause of mental retardation and autism, with a full mutation prevalence of 1:2000-4000 for males and in 1:4000-8000 for females [1,2]. The genotype involves a defect of the CGG trinucleotide amplification on the X chromosome (Xq27.3) in the 5' untranslated region of the fragile X mental retardation-1 gene (FMR1) that suppresses production of FMRP

[3,4]. According to the cAMP theory of fragile X, a loss of FMRP produces cAMP dependent alterations in functional neurophysiology. In support of the cAMP theory, a recent study of *Drosophila*, mouse, and humans identified cAMP cascade alterations in the neural FX phenotype [5].

The FX neurophenotype has distinctive features which may be explained in part by cAMP alterations during neural differentiation. Developmentally, dysregulation of the cAMP cascade is known to impact growth cone motility and the structure of terminal varicosities in *Drosophila* [6]; to play an important role in AMPA trafficking [7], cell survival and neurogenesis [8], neural induction [9], and in neurite formation and synaptogenesis [10]; and to regulate spine density by gating brain derived neurotrophic factor (BDNF) signaling, a neurotrophin implicated in dendrite formation [11].

Prior studies examining development of the cAMP cascade in normal mice indicated that the inhibitory influence on adenylate cyclase (AC) early in development is followed by stimulatory AC responsiveness later in development [12,13]. We used this developmental responsiveness to adenylate cyclase stimulation as a biochemical marker to monitor neural maturation [14]. With the intent of guiding prenatal therapy, this work extends prior cellular *in vitro* fragile X studies [15] into the brain and provides a window into FX development through examination of the neuronal cAMP cascade. If FMRP deprivation limits production of cAMP in developing fragile X brain *in vivo*, pharmacologic agents known to act through the cAMP cascade may be therapeutic to prenatal neurodevelopment in FX.

Methods

All studies were conducted in accordance with the University of Wisconsin Institutional Review Board. The neural progenitor cell assay was conducted following the differentiated neural precursor cell protocol which was previously reported [5]. Briefly, cyclic AMP levels in FX and two control human neural progenitor cell lines were quantified using a fluorescence assay (Mediomics, LLC; St. Louis, MO). Cells were quantified in the presence of IBMX, a nonselective inhibitor of phosphodiesterase which metabolizes cAMP, and in the presence (5 μ M) or absence of forskolin, a potent activator of adenylate cyclase. We compared raw fluorescence (RF; increased RF corresponds to cAMP decreases), which measures cAMP levels, and fractional decreases in raw fluorescence (FDRF; increased FDRF corresponds to cAMP increases), a measure of changes in cAMP levels. Statistical analyses were conducted in SPSS 14.0 (SPSS, Inc.). All fluorescence trials were replicated in triplicate and are reported using two-tailed significance unless otherwise noted. Human neural progenitor cell studies were conducted in a blinded fashion with respect to FX status and unblinded for analysis. Neural progenitor cell data was acquired in parallel with and compared to differentiated neural precursor cell results which were previously reported [5].

A correlation matrix and dendrogram of FDRF neural progenitor and differentiated cell measures were generated in R [16]. The correlation matrix shows the Pearson correlation values in the upper triangle and scatter plots with smoothed LOWESS fit in the lower triangle. A dendrogram is the end result of agglomerative hierarchical cluster analysis, an exploratory multivariate statistical method of analysis to group predefined elements.

Given our interest in observing the unsupervised grouping of cyclic AMP across the parameter space of cell type [Control 1, Control 2, FX] and development [neural progenitor, differentiated cell], we examined two dendrograms in which the elements of interest were the vector of FDRF measures for a given cell type across developmental stage trials and for a given developmental stage trial across cell types, respectively. AHCA requires two specifications including a definition of distance and a clustering algorithm. We chose Euclidean distance as the grouping statistic:

$$D(A, B) = \sqrt{\sum_{i=1}^n (a_i - b_i)^2}$$

where, for example, n is the number of cell types and A and B are vectors of cell FDRF measures from a different developmental stage trial. Euclidean distance was chosen because it preserves the magnitude of FDRF differences and is a grouping statistic based on the individual differences of FDRF measures which are consistent across trials. The Lance-Williams algorithm[17] was used to implement a complete linkage clustering in which distance is determined as the largest similarity between a point in the first cluster and a point in the second cluster (furthest neighbor method). Dendrograms were then sorted by mean FDRF values in a given trial and are displayed at the edges of a FDRF heatmap.

Results

We compared the responsiveness of neural progenitor and differentiated cells for the FX and two control cell lines. Neural progenitor cell RF was comparable between groups in the presence [t(6)=0.85;p=0.43] or absence [t(6)=0.72; p=0.50] of forskolin (Figure 1a).

Neural progenitor cell FDRF was comparable across cell lines [$F(2,6)=2.84;p=0.14$] and the FX FDRF was comparable to control cells [$t(6) = -0.661;p=0.53$] (Figure 1b).

When the FDRFs from differentiated cells were compared to neural progenitor cells, the main effect for cell stage (neural progenitor cell vs. differentiated) was significant [$F(1,12)=82.84;p=0.00$]; the main effect for cell line was significant [$F(2,12)=3.845;p=0.05$]; and, the cell line by stage interaction was significant [$F(2,12)=5.48;p=0.02$]. The control cell lines showed a stage dependent difference in cAMP induction in that there was a marked increase in the levels of FDRF based cAMP production in differentiated cells compared to undifferentiated neural progenitor cells [$F(1,8)=126.8;p = 0.00$]. Among human control cell lines, the FDRF based cAMP induction stage difference was marginally significant between control cell lines [$F(1,8)=4.15;p=0.076$; DC Control 1 = 0.74 ± 0.026 (Mean $\pm$ SD); DC Control 2 = 0.76 ± 0.041 ; NS Control 1 = 0.135 ± 0.14 ; NS Control 2 = 0.30 ± 0.069] and the interaction between cell stage and control cell lines was not significant [$F(1,8)=2.38;p=0.16$]. In FX cells, however, FDRF based stimulated cAMP levels for differentiated cells and neural progenitor cells were comparable [$t(4)=2.135;p=0.10$; DC = 0.485 ± 0.181 ; NS = 0.261 ± 0.0125] (Figure 2).

Next, we generated a dendrogram and correlation matrix of FDRF measures to determine the uniqueness of the FX cAMP response in development. The unsupervised dendrogram of FDRF measures produced clusters in which control cell lines were agglomerated into a separate cluster from FX cells and both the neural progenitor and differentiated cells were

agglomerated into distinct clusters (Figure 3). A correlation matrix was generated using FDRF measures across all developmental trials (Figure 4). The control cell lines were more strongly correlated to each other [$r(4)=0.983$; $t(4)=10.8$; $p=0.000$] compared to the correlations of the FX cell line with either control cell line [Control 1: $r(4)=0.718$; $t(4)=2.065$; $p=0.108$; Control 2: $r(4)=0.642$; $t(4)=1.68$; $p=0.169$].

Conclusions

Cyclic AMP signal transduction mediates neurotransmission [18] and is a molecular mechanism [19] by which FMRP can affect protein expression [20]. We investigated the role of FMRP in development and neural maturation by assaying cyclic AMP responsiveness and levels in neural progenitor cells and differentiated human neural precursor cells. Induced FX cAMP production was comparable to controls in neural progenitor cells (Figure 1). When compared to results from a prior study [5], neural progenitor cell cAMP production was lower than controls in differentiated cells but FX differentiated cells and neural progenitor cells had comparable cAMP production. Using human FX neural cells, we showed that the cAMP cascade is altered in FX cells only when the cells have differentiated into neurons and glia. Stimulated cAMP production in FX neural progenitor cells is not different from unaffected progenitors (Figure 1).

The positive association between cAMP production and cell stage is lacking in FX neural development (Figure 2). The cAMP cascade is known to be developmentally regulated in mouse and rat brain [12,13,21] and results from human neural control cells in our study follow this pattern. The lack of a cAMP production defect in undifferentiated human

neural progenitors is therefore likely due to the fact that FMRP, and potentially specific cAMP generation factors under FMRP control, are not necessary at this stage in development. The lack of increased cAMP production as FX cells differentiate from progenitors suggests that FMRP operates in the maturation of AC responsiveness [14] and that differentiated human FX neural cells maintain an immature cAMP signaling phenotype comparable to that detected in neural progenitor cells.

Based on the dendrogram and correlation matrix, FX cells have a unique developmental cAMP trajectory distinct from the other two control cell lines. Neural progenitor and differentiated cells were distinguishable in the dendrogram as distinct clusters. Furthermore, the pattern of unsupervised clustering indicates that FX and control neural progenitor and differentiated cells were distinguishable on the basis of FDRF cAMP responsiveness (Figure 3). Cyclic AMP responsiveness may be a useful temporal biomarker to monitor the functional consequence of FMRP loss at the molecular level. In addition, the vertical height of the dendrogram indicates that control cells had a higher level of similarity based on the Euclidean distance of FDRF measures. This was confirmed by the significant correlation [$r(4)=0.983$; $t(4)=10.8$; $p=0.000$] among the FDRFs of the control cell line and non-significant associations between the FX and either control cell line (Figure 4). The mechanism for the distinctive development of the FX cAMP response remains unknown. EphB2 is a likely candidate because it is involved in the development of cAMP responsiveness to forskolin [22] and is a key component in dendritic spine formation that may contribute to abnormal pruning in FX [23-25]. Although the developmental time scale of cAMP responsiveness is coarse, our findings

indicate that FMRP loss alters the trajectory of cAMP responsiveness during development in a pattern that distinguishes FX from controls.

A potential target for prenatal therapy involves recalibration of the cyclic AMP production system. Although cAMP does not influence FMRP production through CREB [26], our results give insight into the influence of FMRP on cAMP and help determine the potential benefit of pharmaceuticals that increase cAMP levels to improve FX neuronal biochemical function [27]. With pharmaceutical calibration of FX cAMP levels, the intracellular cAMP signaling cascade may be able to properly regulate the cAMP second messenger in response to extracellular signals to ultimately improve FX development and behavior. For example, the mood stabilizer lithium increases cAMP production and is known to improve FX *Drosophila* memory behavior, as have mGluR2 antagonists which elevate cAMP levels [28]. However, prenatal pharmacotherapy to stimulate cAMP generation may be least effective very early in development and most effective during the temporal window during which the cAMP responsiveness of FX and control cells can be distinguished. Further studies will be necessary to improve the temporal window and resolution of this finding and to determine whether FMRP impedes maturation of the cAMP production system in series or in parallel with mGluR alterations.

Acknowledgements

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Figures

Figure 1: Fluorescence Assay of Cyclic AMP Production in Human Neural

Progenitor Cells. (a) Neural progenitor cell (control M045, M046 and FX : M049) raw fluorescence was comparable between groups ($n=3$ for each group) in the presence [$t(6)=0.85$; $p=0.43$] or absence [$t(6)=0.72$; $p=0.50$] of forskolin. (b) Neural progenitor cell FDRF was comparable across cell lines [$F(2,6)=2.84$; $p=0.14$] and the FX FDRF was comparable to control cells [$t(6) = -0.661$; $p = 0.53$]. Error bars are pooled SEM.

Figure 2: Development of cAMP Responsiveness is Reduced in Fragile X.

Control cells showed a marked increase in the levels of cAMP responsiveness (FDRF) in differentiated cells compared to undifferentiated neural progenitor cells [$F(1,8)=126.8$; $p=0.00$]. However, FX FDRF for differentiated cells and neural progenitor cells were comparable [$t(4)=2.135$; $p=0.10$; $DC = 0.485 \pm 0.181$; $NS = 0.261 \pm 0.0125$]. The rate of neural development indicated by the linear slope is significant in controls [$t(10)=9.34$; $p=0.00$; $r=0.947$] but not significant in FX [$t(4)=2.13$; $p=0.10$; $r=0.730$]. Error bars are SEM.

Figure 3: Two Dimensional Dendrogram of Neural Progenitor and Differentiated

Cell FDRF. The dendrogram of Euclidean FDRF distances successfully agglomerated neural progenitor (P) and differentiated cells (D) as distinct clusters across three trials while at the same time agglomerated control (black) and FX cells (red) as distinct clusters. The pattern of unsupervised clustering indicates that FX and control neural

progenitor and differentiated cells were distinguishable on the basis of cAMP responsiveness (FDRF).

Figure 4: Correlation Matrix of FDRF Neural Progenitor and Differentiated Cell

Trials. The correlation matrix plots correlation values in the upper triangle and scatter plots with a smoothed LOWESS fit (red) in the lower triangle. A significant correlation is denoted with red stars. The control cell lines were more strongly correlated to each other [$r(4)=0.983$; $t(4)=10.8$; $p=0.000$] compared to the FX cell line [Control 1: $r(4)=0.718$; $t(4)=2.065$; $p=0.108$; Control 2: $r(4)=0.642$; $t(4)=1.68$; $p=0.169$]. FX cells have a unique developmental cAMP trajectory compared to the two control cell lines.

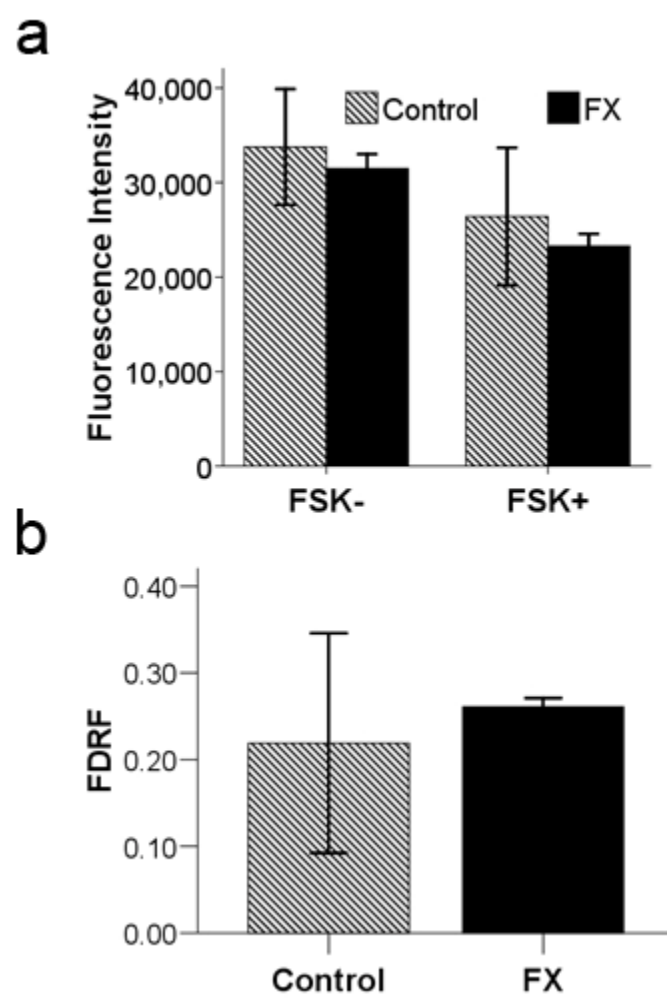


Figure 1

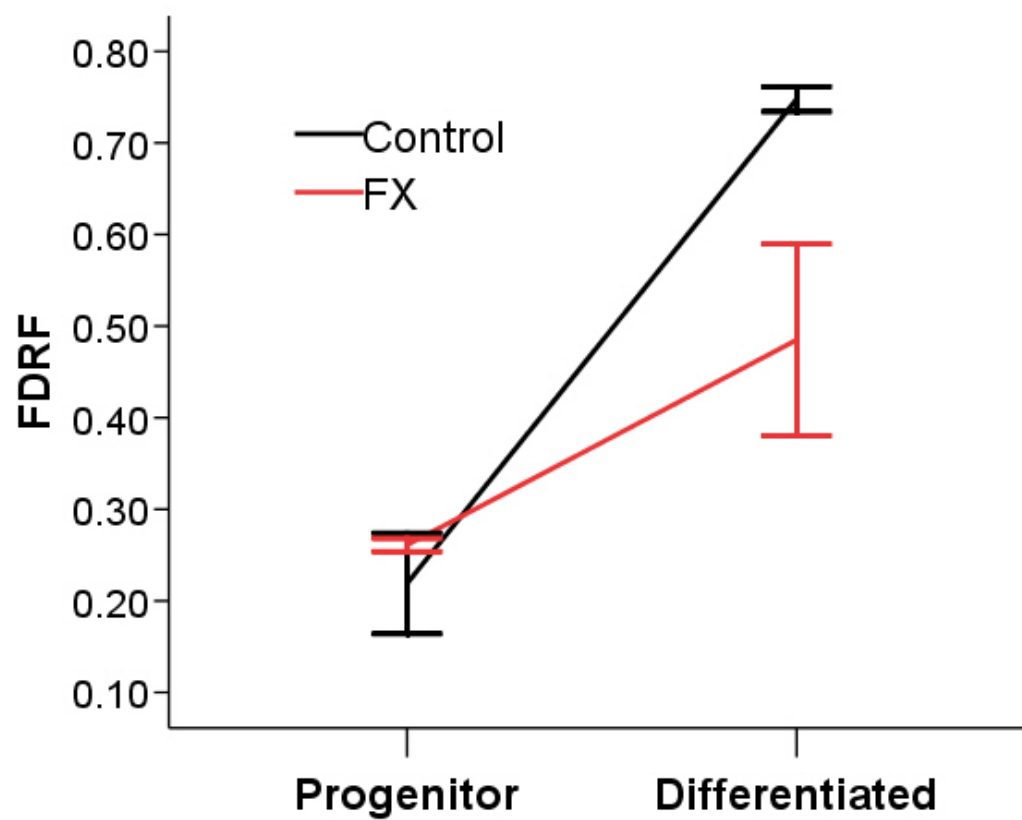


Figure 2

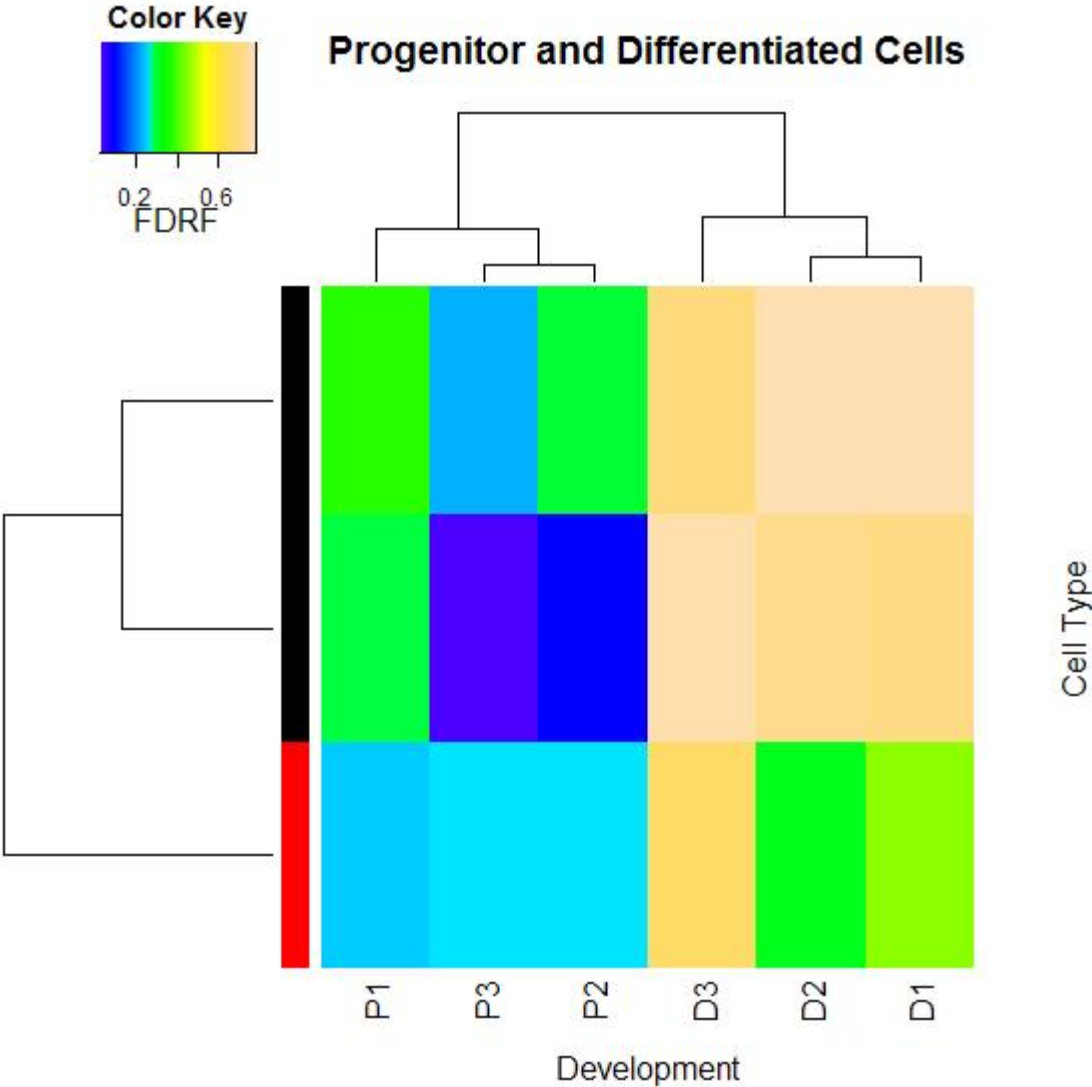


Figure 3

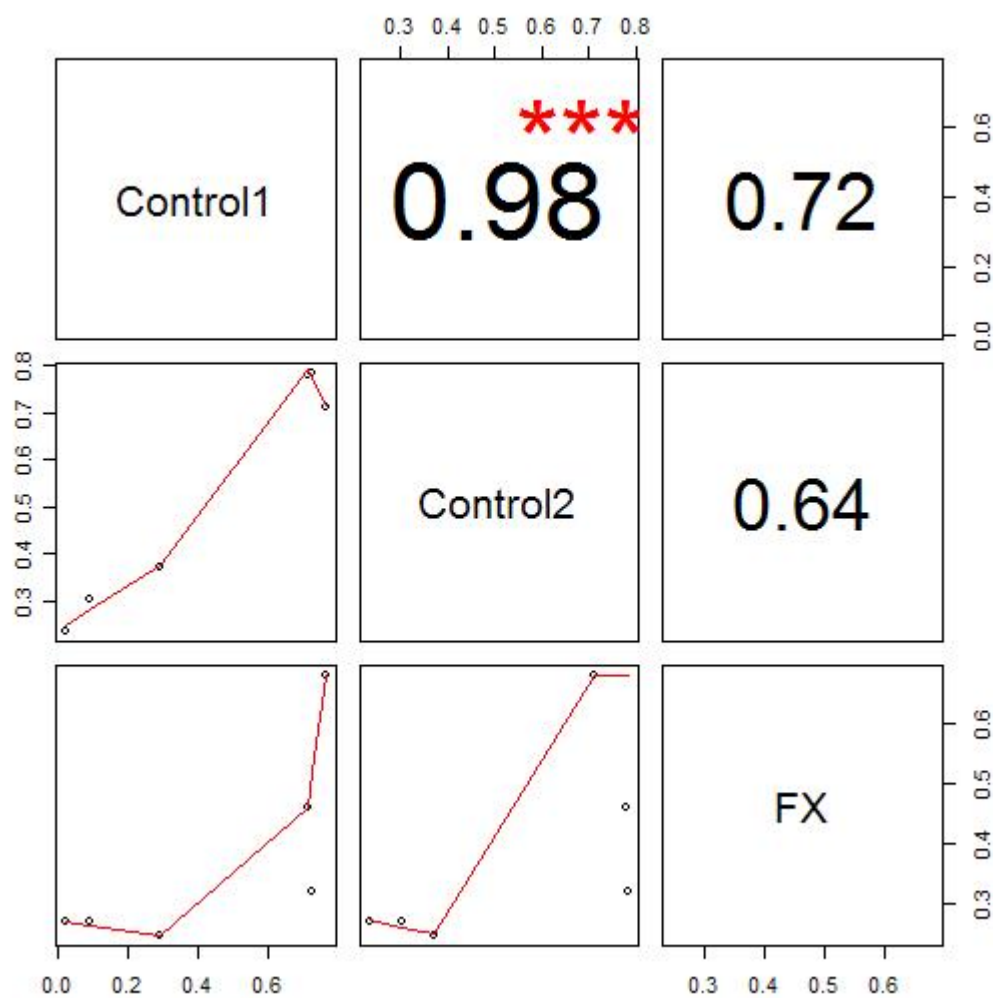


Figure 4

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Title: **Diffusion Tensor Imaging of Cortical Surface Development**

Running Title: DTI of cortical maturation

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Diffusion tensor imaging (DTI) is a robust MRI method that expands the capabilities of structural MRI beyond volumetric and morphometric investigations toward the characterization of tissue microstructure. The three-dimensional diffusion of water in brain tissue can be imaged using DTI and quantified by DTI indices. Fractional anisotropy (FA) is a commonly reported DTI index that quantifies the magnitude of asymmetric diffusion, or anisotropy, in tissue. This value is high in tissues with directionally oriented structural characteristics such as the myelinated axons in white matter. The magnitude of diffusion perpendicular to and along the axis of anisotropic tissue can be further characterized using DTI indices of parallel and perpendicular diffusivity, respectively. When used in combination with surface visualization, DTI measures provide a powerful tool to map structural characteristics across the entire cortex. Although DTI has gained substantial traction in human research, the application of DTI to animal studies is less common, but growing.

In a recent publication of the *Journal of Neuroscience*, Huang et al. reported that FA could be used to quantitatively map spatio-temporal patterns of cortical development in the rat brain (Huang et al., 2008). Although well investigated at the cellular level, cortical development during the early postnatal period remains largely unmapped across all cortical regions in the whole brain due to the practical limitations of histological methods. To characterize whole brain structural development, high-resolution DTI images of *ex-vivo* rat brains were acquired at five time points during postnatal development (P0, P3, P7, P11 and P19). Using FA to index the degree of cortical columnar organization at single measurement points, the authors identified an age-dependent decrease in FA with regional specificity in the cortex (Huang et al., [Figure 4](#)). In addition to single point analysis, maps of FA values across the entire brain surface were generated at each developmental time point to visualize cortical maturation dynamics (Huang et al., [Figure 3](#)). The observed patterns indicated that FA reduced during early cortical maturation in an anterior to posterior fashion.

Although the results of this paper were generally sound, the methods used to determine measurement location could have led to inaccuracies. The authors recognized the importance of using a region-selection method that reduced point misplacement and aligned the data from different developmental time points by selecting FA map slices from the same anatomical locations. This slice selection was accomplished by proportional distance scaling along the anterior-posterior axis (Huang et al., [Figure 1](#)). Cortical measurement points were then defined for these slices by conserving the angle from midline of an initial point set created using an atlas overlay on the P19 brain (Huang et al., [Figure 2](#)). For each measurement point, the inner, middle and outer layers of cortex were also defined. This alignment method greatly reduced the potential for user bias associated with region placement in developing brains but may not have accounted for measurement point misplacement due to anatomical changes in brain shape and local growth during development. A different or complementary registration approach, such as surface based methods (Kroenke et al., 2007) or the use of a developmental rat brain atlas, may have provided more accurate measurement point placement and validation of the current technique. Measurement location for the FA map relied on the inward shrinkage of a cortical surface mesh by a constant number of two voxels. Given the variability in cortical thickness across different regions, perhaps mesh placement based on proportion of cortical thickness would have been more appropriate. The findings were nonetheless robust in describing a spreading reduction in FA across the outer layers of the cortex.

Another potential problem for interpretation was the choice of statistical measure used to characterize regional patterns of cortical maturation. Huang et al. tested the null hypothesis that the FA developmental time courses were comparable among cortices based on their subregion averages and found significant differences based on the Kruskal-Wallis statistic. Since sixteen cortical measures were taken from a given rat, these six mean cortical FA

measures were likely interdependent and therefore in violation of the independence assumption of the Kruskal-Wallis test. A preferable statistical approach may be to block on the rat factor and use the Friedman test, a non-parametric repeated measures ANOVA of ranks (Friedman, 1937). Further, inter-regional differences in FA maturation were identified by thresholding with a Bonferroni correction for seven comparisons (Huang et al., [Table 2](#)). However, if the authors would have used a more conservative Bonferroni correction for all fifteen reported comparisons ($0.05/15=0.003$) rather than for the seven cortical regions compared, the cortical FA development would not have been significantly different among brain regions. Further studies with a larger sample size ($n > 2$) at each time point will be necessary to determine the significance of regional differences in cortical FA maturation trajectories.

The results of this study indicate a general trend of FA reduction during postnatal maturation in outer cortical layers and further suggest the existence of four distinct regional maturation patterns (prelimbic and cingulate, insula, somatosensory and motor, auditory and visual). Based on previous histological literature, the authors proposed that this change in FA is reflective of dendritic and axonal growth, interneuron infiltration, and the disappearance of radial glia. It is also possible that oligodendrocyte myelinogenesis and myelination of cortical axons contribute to the observed FA loss and that the variation in cytoarchitecture across cortical regions contributed to the heterogeneous FA profiles. The reported parallel and perpendicular diffusivity (Huang et al., [Figure 5](#)) may reflect different cellular correlates (Budde et al., 2007). Therefore, it would be interesting to know the structural substrates for selective loss of parallel diffusivity in this study.

The DTI analysis of postnatal cortical maturation presented by Huang et al. advances both the understanding of spatio-temporal dynamics in developing cortex and the contribution of DTI methods to basic neuroscience research. Despite the caveats in anatomical

localization, statistics, and developmental interpretation we identified in this paper, the future possibilities for work of this nature are promising and may include investigations of different regions such as the hippocampus or cerebellum, which is known to undergo both radial and tangential development (Goldowitz et al., 1998). The development of DTI quantification and visualization tools, such as described by Huang et al., and the extension of DTI to *in-vivo* animal imaging have the potential to advance numerous areas of neuroscience research including whole brain development, disease models, and network plasticity.

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Statistical Approaches to Functional Connectivity of Brain Networks

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Abstract

The human brain connectome is intrinsically organized into small world networks. The network topology of brain can be investigated using anatomical, functional, and effective connectivity. Here we review modern functional connectivity statistics for functional MRI (fMRI) analyses of brain integration in subjects at rest and during task performance. Modern functional connectivity techniques are not limited to temporal correlations because spectral decomposition of time series, information theory measures, and both univariate and multivariate statistical approaches in the frequency, wavelet, and fractal domain are available to identify human chronoarchitecture in whole networks and specific functional circuits.

Introduction

The human brain connectome exhibits intrinsic anatomical and functional organization [1-3]. The network topography of human brain can be studied using anatomical, functional, and effective statistical analyses of images collected using a variety of in vivo imaging techniques. Networks of brain function can be understood using functional MRI (fMRI) by examining the segregation of localized activity or the integration of brain regions that form a network [4]. Statistical mapping of brain function using fMRI traditionally focused on the magnitude of segregated activity during task-specific paradigms. Localized activation within the brain was first detected with fMRI using product moment correlation [5, 6] which was used as an index of association between the recorded fMRI signal at each voxel and a task design vector. As an alternative to mapping segregated patterns of localized brain activation using correlation, information transfer within the brain can be investigated by examining the integration of BOLD responses among brain regions using functional connectivity imaging analysis. The integrity of functional connectivity statistics between nodes determines the extent of integration of circuits in the functional network.

Functional connectivity was defined by Friston [7] as the “temporal correlations between remote physiological events” and is a means by which information integrated between and within brain regions can be detected. Functional connectivity using fMRI was first applied to the motor cortex of resting human brain using the product moment correlation of BOLD time courses [8, 9]. Since then, methods which vary with experimental design have been developed to examine functional connectivity during a task [10]. Block design

studies are able to utilize the variance at the beginning and end of a block for connectivity analysis and condition specific block connectivity is possible using simple subtraction to separate blocks. As the stimulus presentation shortens to that of an event-related design, measuring connectivity for specific trial conditions becomes more difficult since the individual trial signal variance is masked by the overlapping hemodynamic response functions of adjacent trials [11].

Functional connectivity is distinct from effective connectivity, “the influence one neuronal system exerts over another” [12]. Effective connectivity requires an *a priori* model for causal relationships, unlike functional connectivity which is considered to be more exploratory in nature [13]. Functional connectivity, unlike effective connectivity, neither presumes a causal connection between regions nor explains the driving force of interregional correlations. In the context of connectivity analysis, correlation and regression are not comparable because regression has a causal interpretation. In fact, regression can be considered the simplest structural equation model, an effective connectivity technique.

In this report, we review functional connectivity approaches that fit within the formal temporal definition of functional connectivity and those that have expanded the functional connectivity definition. These analyses use univariate and multivariate [14] techniques that take the form of correlations among time series using temporal, fractal, wavelet, and frequency based approaches [15], the spectral decomposition of time series into their spatial modes [16], and correlations of segregated activations (beta weights)

across event types [17] or across subjects [18]. In particular we will focus on the Pearson product moment correlation and its application in intraregional connectivity analysis (COSLOF), beta series correlation, real-time connectivity, and asymmetric functional connectivity analysis; rank, partial, semipartial, multiple semipartial, multiple, partial, and canonical correlations; time dependent regression and psychophysiological interaction analyses; wavelet correlation; coherence analysis; spectral decomposition methods, and information theory approaches.

Functional Connectivity Statistical Approaches

Pearson Product Moment Correlation

The **Pearson product moment correlation** in fMRI analysis can be defined as:

$$r = r_{xy} = \frac{s_{xy}}{s_x s_y} = \frac{\sum_{i=1}^n x_i y_i}{\sqrt{\sum_{i=1}^n x_i^2 \sum_{i=1}^n y_i^2}}; s_{xy} = \frac{\sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})}{(n-1)}$$

where s_x and s_y are the sample standard deviations, $\bar{x}$ and $\bar{y}$ are the sample means based on n time series measurements of random variables X and Y [19]. Rather than using this index as a measure of association between the recorded fMRI signal at each voxel and a task design vector [5, 6, 20], this statistic can also be used for connectivity analysis [8, 9] and to determine the number of edges and distances between nodes in a graph theoretic framework [21]. The correlation coefficient, used as a functional connectivity measure, can be derived from LSE regression, an effective connectivity measure, via the equation [22]:

$$r^2 = \beta_{xy} \beta_{yx}$$

where β_{xy} and β_{yx} are the coefficients for regressing x on y and y on x respectively. This equation identifies the squared correlation of functional connectivity as the interaction between effective connectivity weights with exchanged dependent and independent variables. A disadvantage of product-moment correlation is that the sampling distribution of non-zero correlations is skewed. This can be corrected using the nonlinear Fisher r to z' transformation [23]:

$$z' = \frac{\ln(1+r) - \ln(1-r)}{2} \quad \text{or} \quad z' = \tanh^{-1}(r) ; \quad sd_{z'} = \frac{1}{\sqrt{n-3}}$$

The linear correlation can be tested using [24, 25]:

$$t = r \frac{\sqrt{n-2}}{\sqrt{1-r^2}} ; \quad df = n - 2$$

For seed correlation analyses, t-transformed correlations of Gaussian smoothed time series form a t-field and can be tested for significance on a t-distribution; however, cross-correlation, autocorrelation, and homologous correlation fields do not form a t-field when t-transformed so tests of significance need to be based on random field theory [16, 26].

Thresholded correlations are a useful statistic to detect functional connectivity in local networks but may be less useful localizing long range networks compared to PCA and SVD discussed below [27]. Another disadvantage is that the product moment correlation only describes linear association. In contrast, the **generalized correlation** coefficient is a correntropy statistic based on mutual information that captures both linear and non-linear contributions, unlike Pearson's correlation, and does so preserving the sign of the correlation measure unlike canonical correlation [28]. Curvilinear relations can be assessed using the **index of correlation**, r_{yx} [29]:

$$r_{yx} = \frac{\sigma_{\hat{y}}}{\sigma_y}$$

where $\sigma_{\hat{y}}$ is the standard deviation of the estimates for $y, \hat{y}$, when y is regressed on x , and σ_y is the standard deviation of the actual y values.

Intraregional Connectivity Analysis: COSLOF Index

The cross-correlation coefficients of spontaneous low frequency synchrony (COSLOF index) was originally developed to identify alterations in hippocampal functional synchrony due to neurodegeneration in Alzheimer disease [30]. As an estimate of intraregional connectivity, the COSLOF index serves as a metric for functional synchrony within a region of interest. The COSLOF index is generated by:

$$COSLOF = \frac{2}{K(K-1)} \sum_{i,j=1, i>j}^K cc_{ij}$$

where K is the number of voxels and cc_{ij} is the cross-correlation coefficient between the i th and j th voxel time course in the ROI. Reductions or increases in the COSLOF index would be interpreted as dysfunctional connectivity. While originally developed for resting state connectivity, a COSLOF-like index representing the average cross correlation coefficient within a region of interest may serve equally well as a functional connectivity index during an event-related task. The disadvantage of this technique is that it is restricted to linear associations since it is based on Pearson's correlation.

Beta Series Correlation Analysis

Beta series correlation analysis (BSCA) operates within the general linear model to estimate beta values for individual events as an estimate of BOLD response magnitude. These parameter estimates for each trial can be categorized to produce a beta series for each trial type. The BSCA multivariate approach is distinct from more typical univariate approaches in that variability of individual trials is taken into account with the former, but not the latter, method [17]. The collection of beta series within an ROI can then be used as seeds in a voxelwise correlation analysis. This technique has been validated with coherence measures of connectivity on a previously published dataset and applied in a working memory study investigating functional connectivity with the fusiform face area during a delayed face recognition task [31]. The disadvantage of this technique is that it is essentially a correlation of regression estimates and is less robust than a structural equation modeling approach.

Real-time Connectivity Analysis

Real-time functional connectivity analyses have applications in behavioral therapy as a biofeedback mechanism. Predictive models are able to forecast future connectivity estimates and would give subjects feedback closer to real-time. Examples of forecasting estimates include mean absolute deviation, Theil's U-statistic, and state-space models [32]. Dynamic linear models have applications in real-time analyses, are state space models using Bayesian inference, and have the form:

$$Y = X \Theta + v$$

where Y is the dependent variable vector, X is the independent variable vector, Θ is an unknown parameter vector, and v is an error vector. Online correlation, principal component (PCA), independent component (ICA), and entropy analyses have been applied to evaluate connectivity features of the brain in real-time [33]. Online correlation analysis in particular is easily implemented (See Kasabov [33], page 22) and is useful for observing the evolution of the correlation coefficient over time, but does not take into account non-linear associations.

Asymmetric Functional Connectivity Analysis

Although no anatomical constraints are required in functional connectivity analysis, anatomical constraints may be useful in identifying asymmetric chronoarchitecture. One measure of asymmetric functional connectivity is the **laterality index** which has the form:

$$\text{Laterality Index} = \frac{L - R}{L + R}$$

where L is left hemisphere activation and R is activation in the corresponding region of the right hemisphere [34, 35]. A disadvantage of the laterality index is that its application is limited to functional systems whose anatomical connections are asymmetric. An alternative statistic is the **spatial coincidence coefficient** (SCC) for contralateral connectivity [36]:

$$SCC_{LR} = SCC_{RL} = \frac{2n_{LR}}{2n_L n_R + n_{LO} + n_{RO}}$$

This statistic is useful for determining the extent of asymmetric functional connectivity using Pearson's correlation based on the mean number of voxels above threshold in the

right ROI (R), in the left ROI (L), or outside either ROI (O). A disadvantage of this technique is that it is threshold dependent, although it was originally developed as a means to estimate an appropriate threshold in resting state networks by penalizing undesirable connectivities.

Rank Correlations

Rank correlation coefficients are non-parametric measures of similarity that can be implemented by converting the fMRI time series to ranks. The most well known non-parametric correlation statistic is the **quadrant correlation**, also known as Blomqvist's q statistic or the medial correlation statistic [37]. The quadrant correlation is the sample correlation between the signs of deviations from the median and has the form [22, 38]:

$$r_Q = \frac{1}{n} \sum \text{sign}[(x_i - \text{median}(x))(y_i - \text{median}(y))]$$

Spearman rank correlation, r_s , can be rewritten using the difference in ranks $d_i = x_i - y_i$ using the equation:

$$r_s = 1 - \frac{6 \sum_{i=1}^n d_i^2}{N(N^2 - 1)}$$

However, when the proportion of tied ranks is large, a correction factor, T , is required:

$$r_s = 1 - \frac{(N^3 - N) - 6 \sum_{i=1}^n d_i^2 - (T_x + T_y)/2}{\sqrt{(N^3 - N)^2 - (T_x + T_y)(N^3 - N) + T_x T_y}}; \quad T = \sum_{i=1}^g (t_i^3 - t_i)$$

where r_s is the Spearman rank correlation with correction for tied ranks, g is the number of different tied rank groupings, and t_i is the number of tied ranks in the i th grouping [19].

A disadvantage of the Spearman correlation is that its significance testing can only be

approximated [39]. The Spearman correlation is closely related to Pearson's correlation and can be converted to a product moment correlation using Pearson's approximation [40]:

$$\rho_s = \frac{6}{\pi} \sin^{-1} \left(\frac{1}{2} \rho \right)$$

Kendall's rank order correlation or Kendall's tau, τ , is an alternative statistic that quantifies the disagreement of rankings in X and Y and has the form:

$$\tau = \frac{4P}{n(n-1)} - 1$$

where P is the total number of agreements [41]. With tied ranks, the equation becomes:

$$\tau = \frac{P - Q}{\sqrt{[n(n-1)/2] - K_x} \sqrt{[n(n-1)/2] - K_y}}; K_x = \frac{1}{2} \sum f_i(f_i - 1)$$

where f_i is the number of tied observations in each group of ties on X and

$$K_y = \frac{1}{2} \sum f_i(f_i - 1)$$

where f_i is the number of tied observations in each group of ties on Y. In practice, a general rule for non-parametric tests is for $n < 30$ use Spearman's correlation if there are no expected differences in ranks or outliers; otherwise, Kendall's correlation is preferred. For $n > 30$, either Spearman's or Kendall's correlation is acceptable [39], however, Spearman's coefficient may perform better than Kendall for a large n and is easier to calculate [42]. The Spearman or Kendall coefficients can be z-tested for significance after a Fisher r to z conversion [24].

Partial Correlation

Partial correlation is used to measure the association between two time series, A and B, after the effects of other time series are partialled out from both A and B [24, 43]. If three time series are involved and a multivariate normal distribution is assumed, the sample estimate for the Pearson partial correlation coefficient $\rho_{12.3}$ can be defined as:

$$r_{12.3} = \frac{r_{12} - r_{13}r_{23}}{\sqrt{(1 - r_{13}^2)(1 - r_{23}^2)}}$$

Yule generalized the partial correlation to n time series as follows [44]:

$$r_{12.34\dots n} = \frac{r_{12.34\dots(n-1)} - r_{1n.34\dots(n-1)}r_{2n.34\dots(n-1)}}{(1 - r_{1n.34\dots(n-1)}^2)^{1/2}(1 - r_{2n.34\dots(n-1)}^2)^{1/2}}$$

The Pearson partial correlation is usually smaller than the product-moment correlation unless a covariate of no interest is present that is highly correlated with the predictors but uncorrelated with the dependent variable [45]. **Kendall's partial tau correlation** has the form:

$$\tau_{12.3} = \frac{(\tau_{12} - \tau_{13}\tau_{23})}{[(1 - \tau_{13}^2)(1 - \tau_{23}^2)]^{1/2}}$$

where τ_{12} , τ_{13} , and τ_{23} are Kendall's rank correlations[46]. Kendall also described a partial correlation for **Spearman's partial correlation** and suggested this approach could only be defended by analogy with Kendall's partial correlation:

$$\rho_{23.1} = \frac{\rho_{23} - \rho_{12}\rho_{13}}{(1 - \rho_{12}^2)^{1/2}(1 - \rho_{13}^2)^{1/2}}$$

where $\rho_{23.1}$ is the partial correlation between variables 2 and 3 when variable 1 is constant. The partial correlation can be tested on a t-distribution:

$$t = r_{xy.1\dots q} \frac{\sqrt{n-q-2}}{\sqrt{1-r_{xy.1\dots q}^2}}; df = n - q - 2$$

where q is the number of variable partialled out [47].

Semipartial and Multiple Semipartial Correlation

A **semipartial correlation**, also known as a part correlation, quantifies the relationship between time series A and B after partialling out other time series from A but not B. The partial correlation is greater than or equal to the semipartial correlation and can be determined from the multiple correlation discussed below [43]. The **multiple semipartial correlation** quantifies the correlation of A with B and C and the effects of other variables are removed from B and C. Both the squared semipartial and squared multiple semipartial correlation can be determined from the difference of squared multiple correlations[43]. The advantage of these correlations is that specific network interactions can be assessed. The disadvantage is that only linear effects are modeled.

Multiple Correlation

The **multiple correlation** coefficient, R, is the product moment correlation between a time series, A, and the estimated time series from multiple regression when regressing A on the linear combination of remaining time series [41]. In the multiple regression framework, the squared multiple correlation $R_{Y.1,\dots,J}^2$ can be interpreted as the

proportion of variance in the dependent variable explained by the independent variables [48]:

$$R_{Y.1,\dots,J}^2 = \frac{SS_{regression}}{SS_{total}}$$

Multiple correlation is the maximum correlation possible between the dependent variable and the linear combination of independent variables [43]. There is discrepancy whether the multiple correlation statistic should be reported as squared, i.e. as the coefficient of multiple determination, [48] or not [49]. The sample multiple correlation can be adjusted to account for its overestimation of the population multiple correlation [48] and can be tested on the F-statistic [47]:

$$F(J, n - J - 1) = \frac{R_{Y.1,\dots,J}^2}{1 - R_{Y.1,\dots,J}^2} * \frac{n - J - 1}{J}$$

Partial Multiple Correlation

The partial multiple correlation, also known as multiple partial correlation, quantifies the linear relationship between a dependent variable and a group of independent variables after partialling out another group of variables from both the independent and dependent variables [43, 50, 51]. The squared **partial multiple correlation** has the form:

$$R_{a.b(c)}^2 = \frac{R_{a.bc}^2 - R_{a.c}^2}{1 - R_{a.c}^2}$$

where $R_{a.b(c)}^2$ is the squared partial multiple correlation between variable a and dependent variables in b $[x_1, x_2, x_3 \dots x_n]$ while partialling out the control variables in c $[z_1, z_2, \dots, z_n]$ from both a and b, $R_{a.bc}^2$ is the squared multiple correlation between the dependent variable a and all other variables in b and c, i.e. $[x_1, \dots, x_n, z_1, \dots, z_n]$, and $R_{a.c}^2$ is the

multiple correlation between the independent variable a and control variables in c [43].

The basic difference between a partial and partial multiple correlation is the number of independent variables. The partial correlation has one independent variable but the partial multiple correlation has more than one independent variable [43].

Comparison of Part, Partial, and Multiple Correlations

In the context of time series, multiple and partial correlation work best when the form of multiple regression is linear [52]. As seen in Figure 1, multiple and partial correlation are distinct [53]. Multiple correlation is used to determine the relation between a given time series and all other time series; whereas, partial correlation characterizes the relation between two time series while controlling for all additional time series [44]. In partial correlation, each variable in the correlation is residualized on the variables of no interest. This is in contrast to the semipartial correlation statistic in which the correlation is between an unmodified variable and a variable residualized on one or more other variables. The squared semipartial correlation statistic can be calculated by subtracting the squared multiple correlation between the dependent variable and variables of no interest from the squared multiple correlation between the dependent variable and all independent variables [43]. The advantage of these techniques is that multiple connectivity patterns can be modeled; however, these patterns are only modeled in a linear fashion.

Canonical Correlation

The canonical correlation[54] is a multivariate statistic that measures the overall strength of interrelationships between a set of multiple dependent variables and a set of multiple independent variables [55]. Calculating the canonical correlation involves the maximization of the correlation between set X and set Y, each of which contains variables representing time series from separate voxels [56]. The canonical correlation can be derived by analogy with multiple regression and multiple correlation [57] and has the form:

$$| R_{yy}^{-1} R_{yx} R_{xx}^{-1} R_{xy} - \lambda I | = 0$$

where $||$ denotes the determinant, R_{yy} is the correlation matrix of the variables in set Y, R_{xx} is the correlation matrix of variables in set X, R_{xy} is the correlation matrix of set X variables with set Y variables, R_{yx} is the transpose of R_{xy} , λ are the eigenvalues, and I is the identity matrix. The canonical correlation, R_c , varies between 0 and 1 and is the correlation between the two canonical variates, the weighted linear combination of each set that maximizes R_c and has the form [43]:

$$R_c = \sqrt{\lambda}$$

The canonical variates are the results from a canonical variates analysis [58]. As indicated by this equation, canonical correlation does not provide directionality of the correlation unlike the Pearson correlation [28]. The maximum number of canonical correlations corresponds to the lowest number of variables in either set X or Y. Subsequent pairs of canonical variates are orthogonal to all prior pairs and produce canonical correlations with decreasing magnitude. The square of the canonical correlation represents the variance shared by each pair of canonical variates in X and Y. Multiple

regression is a type of canonical analysis in which only one dependent variable is present [43]. The canonical correlation statistic is useful in functional connectivity because, unlike multiple regression, the canonical correlation is symmetric in the sense that either set could be the dependent or independent set and still produce the same result [59]. Canonical variates analysis can be used in a multivariate framework to identify patterns of task dependent The canonical correlation can be used to formulate Wilk's lambda [43]:

$$\Lambda = (1 - R_{c_1}^2)(1 - R_{c_2}^2) \dots (1 - R_{c_j}^2)$$

where Λ is Wilk's lambda, $R_{c_1}^2$ is the square of the first canonical correlation, $R_{c_2}^2$ is the square of the second canonical correlation, and $R_{c_j}^2$ is the square of the jth canonical correlation. The significance of the canonical correlation can be tested using Wilk's lambda directly using random field theory [60] or using Bartlett's significance test of lambda using the chi-squared distribution:

$$\chi^2 = -[N - 1 - 0.5(p + q + 1)]\ln(\Lambda); df = pq$$

where N is the number of subjects; p is the number of variables on the left; q is the number of variables on the right; and df is the degrees of freedom [43]. Correction of the maximum or principal canonical correlation for multiple comparisons can be determined using random field theory [61]. A difficulty with canonical correlation is that it is sensitive to multicollinearity.

Time Dependent Regression Estimation

Dynamic changes in connectivity are not accounted for by the stationary and constant network estimates [62, 63]. Variable parameter regression models beta variation and has the form [64]:

$$y_t = x_t \beta_t + u_t$$

$$u_t \sim N(0, \sigma^2); t = 1, \dots, T$$

Variable parameter regression with Kalman filtering models the time-varying parameter estimate using a random walk [65]. A disadvantage of the Kalman filter is that it is a one sided filtering technique that requires estimation of the initial state. A preferred approach in the economics literature is Schlicht's method for variable parameter regression which uses a two-sided filter that makes no assumptions about the initial state and uses all the time points to estimate each coefficient [66]. Alternatively, partly conditional time varying coefficient modeling estimates regression parameters as a function of predicted and predictor variable timing using an extension of marginal regression analysis [67]. These methods may be useful to observe the task-dependent changes in fMRI connectivity studies and are comparable to the psychophysiological interaction analyses discussed below.

Psychophysiological Interaction (PPI)

Rather than estimating the beta weight as a random walk over time, the beta coefficient could be estimated as a function of time-dependent explanatory variables [65]. A **psychophysiological interaction** refers to a change in the slope, when regressing activity from two brain regions, brought about by a psychological variable [68]. PPI enables the determination of the contribution that activity in a given brain region is influenced by the

interaction between a psychological variable and the activity of another brain region [69]. Activity in PPI analysis is modeled using three explanatory regressors including a voxel time course as the physiological, brain response variable; a psychological variable based on the context of the stimuli presented [70]; and the psychophysiological product of the former two variables [13, 71]. The first two regressors are treated as regressors of no interest and the third is used to generate statistical maps. In other words, this analysis can identify a region, A, of brain that is active in response to a stimulus only when another region, B, of brain is active. The interpretation would be that region B's activity modulates the activity of region A indicating they are effectively connected [68]. However large number of inputs to a region are required to interpret results in the context of effective connectivity; and, signal from one ROI can only be interpreted in the context of functional connectivity [68, 71]. This may be one reason why PPI analysis has been categorized as a functional connectivity technique [13]. Unlike functional connectivity analysis, PPI provides insight into the directionality of the “contextual modulation of connectivity,” because the statistical maps generated depend upon which physiological node in the functional circuit was used to generate the psychophysiological interaction regressor [72].

Fractal Connectivity

Fractals are self-similar patterns that follow a power law of the form:

$$N = s^d$$

where d is the fractal dimension. The term fractal is derived from Latin meaning broken. The term fractal in fractal dimension describes the “broken” non-integer nature of d and dimension refers to the power law relationship that defines the self-similarity property [73]. Fractal analysis methods to determine the fractal dimension include the Hausdorff, Minkowski-Bougliand, calliper, box counting, and mass-radius methods [74]. The fractal dimension of a given time series can be assessed using a dispersion method based on the standard deviation [75]:

$$SD(m=1) = \frac{1}{N} \sqrt{N \sum_{i=1}^N x^2(i) - [\sum_{i=1}^N x(i)]^2}$$

This calculation is then repeated for a new time series that has adjacent points averaged and $N/2$ points to determine $SD(m=2)$, and with 4 points averaged to produce $SD(m=4)$, until the number of total points is less than 4. The slope of a $\log SD(m)$ vs $\log m$ plot can then be subtracted from 1 to quantify the fractal dimension[75], which can be interpreted as a measure of internal or self-similar connectivity. Fractal networks of brain connectivity exhibit small world properties that can be assessed using measures of degree, the clustering coefficient, and the minimum path length [76]. Fractals have the advantage of characterizing networks across several levels of complexity; however, fractal analysis of real data, unlike mathematical constructs, may not have adequate sensitivity to characterize subtle differences in complexity which rarely has more self-similarity than two orders of magnitude [74].

Wavelet Correlation

Wavelets are fractal functions that take into account the $1/f$, long memory properties of the BOLD response and, for this reason, wavelet analysis is preferred to simple

correlation analysis as an estimator of functional connectivity [77]. The wavelet coefficients produced from decomposition of each time series can be used to calculate the **wavelet correlation coefficient** which is derived from the ratio of wavelet covariances and variances [78, 79] and has the form [23]:

$$\rho_x(\lambda_j) = \frac{\gamma_x(\lambda_j)}{\sigma_1(\lambda_j)\sigma_2(\lambda_j)}; \gamma_x(\lambda_j) = \frac{1}{2\lambda_j} \text{Cov}(w_{1,j,t}, w_{2,j,t})$$

where $\gamma_x(\lambda_j)$ is the covariance of $(x_{1,t}, x_{2,t})$ for scale λ_j ; $w_{1,j,t}$ and $w_{2,j,t}$ are the scale λ_j wavelet coefficients for $x_{1,t}$ and $x_{2,t}$; and $\sigma_1(\lambda_j)$ and $\sigma_2(\lambda_j)$ are the wavelet variances for $x_{1,t}$ and $x_{2,t}$. Wavelet correlation has an advantage over other correlation procedures because this statistic integrates both phase and amplitude information and overcomes the difficulties of window width selection [80]. Further development of the wavelet correlation using the MODWT, maximal overlap discrete wavelet transform, can be found in its application to small world properties of low frequency neural fluctuations [81]. A Matlab toolbox is available at: <http://www.atmos.washington.edu/~wmtsa/>.

Coherence Analysis

Event-related fMRI integration can be examined using coherence, a frequency based correlation measure [82, 83]. More specifically, **coherence** is defined as:

$$\text{Coherence}_{xy}(g) = |R_{xy}(g)|^2 = \frac{|f_{xy}(g)|^2}{f_{xx}(g)f_{yy}(g)}$$

where $R_{xy}(g)$ is the complex valued coherency of time series x and y , $f_{xy}(g)$ is the cross-spectrum of x and y , and $f_{yy}(g)$ is the power spectrum of y . An advantage of coherence analysis is that functional connectivity can be measured independent of HRF form and independent of hemodynamic spatial heterogeneity. A disadvantage of this technique is that the temporal waveforms of condition specific trial types are unable to be separated due to overlapping hemodynamics of adjacent event-related trials. Task dependent changes in coherence over time may be detectable using time-varying transfer functions[84].

Spectral Decomposition Methods

For further elaboration of the techniques discussed below and significance testing, the reader is referred to a recent review[85, 86].

Eigenimage or spatial mode analysis

Functional connectivity can be measured with eigenimage analysis using singular value decomposition [87, 88]. **Singular value decomposition** (Figure 2) is a matrix factorization technique that is applicable to canonical correlation and principal component analysis [89] and decomposes a matrix into orthonormal bases using [90];

$$A = UDV^T$$

where A is a scan time by brain voxel matrix containing BOLD signal intensities; U is a time by eigenvoxel matrix whose orthonormal columns contain the left singular vectors, temporal eigenvectors, or eigenvariates; D is an eigenvoxel by eigentime diagonal matrix of singular values in A which, when squared, are the eigenvalues that indicate

eigenintensity; and V is a voxel by eigentime matrix whose orthonormal columns are the right singular vectors, spatial eigenvectors, or eigenimages [91, 92]. Each eigenimage, or spatial mode, in V is a voxel-dependent profile of each eigenvariate and defines a functionally connected, but distributed, brain system; and, each eigenvariate in U is a time-dependent profile of each eigenimage [87]. **Generalized eigenimage analysis** can be used to identify group differences in eigensystems:

$$C_j d = C_i d \lambda$$

where C is the correlation matrix of groups i and j and d is the generalized eigenimage [87]. A disadvantage of these techniques is that they are sensitive to preprocessing and significant results can only be characterized descriptively [86].

Multidimensional Scaling

Metric **multidimensional scaling**, or principal coordinates analysis, is a multivariate technique to visualize functional connectivity by mapping anatomy into functional space [93] and takes the form:

$$AA^T = U\lambda U^T; X = A^T U$$

Using MDS, singular value decomposition of the correlation matrix, AA^T , produces eigenvectors in U used to determine the location of voxels in functional space, X . The distance between nodes derived with MDS can be related to the correlation coefficient [94]:

$$d_{ij} = \sqrt{2} \sqrt{(1 - \rho_{ij})}$$

where d is the distance between nodes i and j , and ρ_{ij} is the correlation of time series x_i and y_j . A disadvantage of this technique is that the graphical display restricts the number of data points that can be plotted and too many points make interpretation difficult.

Principle and Independent Component Analysis

PCA and ICA are exploratory methods in functional connectivity [86] that can be implemented using SVD [92]. The key difference between PCA and ICA analysis is that PCA identifies uncorrelated, Gaussian source signals but ICA identifies sources that are non-Gaussian and independent [92]. Principle component analysis (PCA) can give insight into functionally connected spatial modes by identifying the orthogonal axes producing maximal signal variance [88]. In principal components analysis, the covariance matrix of the data, is used to produce the eigenimages in V unlike eigenimage analysis which uses the data matrix itself or MDS which uses the correlation matrix [88, 95]. ICA approaches are considered data driven and do not require an *a priori* model which makes them especially useful for identifying resting state networks [10]. The application of either spatial or temporal independent component analyses to functional connectivity depends on the task and hypothesis [96]. In functional connectivity analysis, an advantage of ICA is that it is not dependent on voxel seed, unlike simple correlation, and is robust to structured noise; but, the bases may not fully characterize the networks and the resulting maps are difficult to threshold [97]. Nonlinear ICA and PCA techniques attempt to overcome the orthogonal and linear drawbacks of eigenimage analysis [87].

Partial Least Squares

Partial least squares is a multivariate regression technique that predicts the dependent variable set from the independent variable set by maximizing the covariance between the decomposed sets of dependent and independent variables [98]. Partial least squares was implemented in fMRI analysis using the single value decomposition of a covariance matrix between brain images and a behavioral or task design [99]. Rather than applying the SVD to a correlation or covariance matrix of identical brain images as with eigenimage analysis and MDS [99], PLS uses the covariance matrix of two different sets of images to investigate connectivity between brain systems [87]. In the context of interhemispheric connectivity, PLS can identify systems of connectivity within a hemisphere that have the greatest connectivity between hemispheres:

$$A_i^T A_j = U D V^T ; D = U^T M_i^T M_j V$$

where the columns of U and V contain the singular images and the diagonal matrix, D, contains the singular values which represent the extent of functional connectivity between the two hemispheres, i and j [94]. Partial least squares is fundamentally different from canonical variates analysis because PLS optimizes covariance patterns but CVA optimizes canonical variate correlations [99]. The main disadvantages for PLS are the potential for inconsistent parameter estimation [100], difficulty interpreting independent latent variable loadings, and the need to bootstrap for significance testing [101].

Information Theory Approaches

Mutual information and entropy are statistics in information theory that can be applied to functional connectivity analysis. Mutual information, MI, measures the information about X contained in Y or vice versa since mutual information is a symmetric function [102]:

$$MI(X, Y) = H(X) + H(Y) - H(X, Y)$$

where differential entropy is:

$$H(X) = -\int_X p(x) \log_2(p(X)) dX$$

Entropy is a measure of information gain over time [103, 104] and cross-entropy is a non-linear method to assess the complexity of interaction between time series [75].

Friston and colleagues [94] showed that mutual information between two time series from voxels p and q can be written in terms of the correlation coefficient

$$MI_{pq} = -\log(1 - \rho^2) / 2$$

An advantage of mutual information over simple correlation is that mutual information can be extended to multivariate assessment. For example, when determining the relationship between multiple time series from region p and q, mutual information can be written as [94]:

$$MI_{pq} = \log \left(\left[\frac{|A_p^T A_p| * |A_q^T A_q|}{[A^T A]} \right]^{\frac{1}{2}} \right)$$

A disadvantage of mutual information is that it does not differentiate between positive and negative associations like Pearson's correlation[105] but has the advantage of being able to identify relations between p and q that lack dependence, unlike Pearson's correlation[106].

Conclusion

Functional networks can be mapped in subjects at rest or performing tasks using fMRI functional connectivity analyses. Both univariate and multivariate statistical approaches are available to identify human chronoarchitecture in specific functional circuits or whole networks respectively. Functional connectivity analysis is centered on the spectral decomposition of time series or temporal associations in the time and frequency domain. Time series analysis in the time, frequency, wavelet, or fractal domains may be conducted across voxels, clusters, or groups of individuals. Functional connectivity analysis techniques are no longer just “temporal correlations” and may require a revision of Friston’s original definition of functional connectivity that adequately characterizes newer approaches such as fractal, coherence, and beta series correlation approaches.

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Figures

Figure 1: Correlations for Functional Connectivity

Figure 2: Singular Value Decomposition (SVD).

Adaptation of SVD matrix visualization [90] where A is a scan time by brain voxel matrix containing BOLD signal intensities; U is a time by eigenvoxel matrix whose orthonormal columns contain the left singular vectors, temporal eigenvectors, or eigenvariates; D is an eigenvoxel by eigentime diagonal matrix of singular values in A which, when squared, are the eigenvalues that indicate eigenintensity; and V is a voxel by eigentime matrix whose orthonormal columns are the right singular vectors, spatial eigenvectors, or eigenimages [91, 92]. Each eigenimage, or spatial mode, in V is a voxel-dependent profile of each eigenvariate and defines a functionally connected, but distributed, brain system; and, each eigenvariate in U is a time-dependent profile of each eigenimage [87].

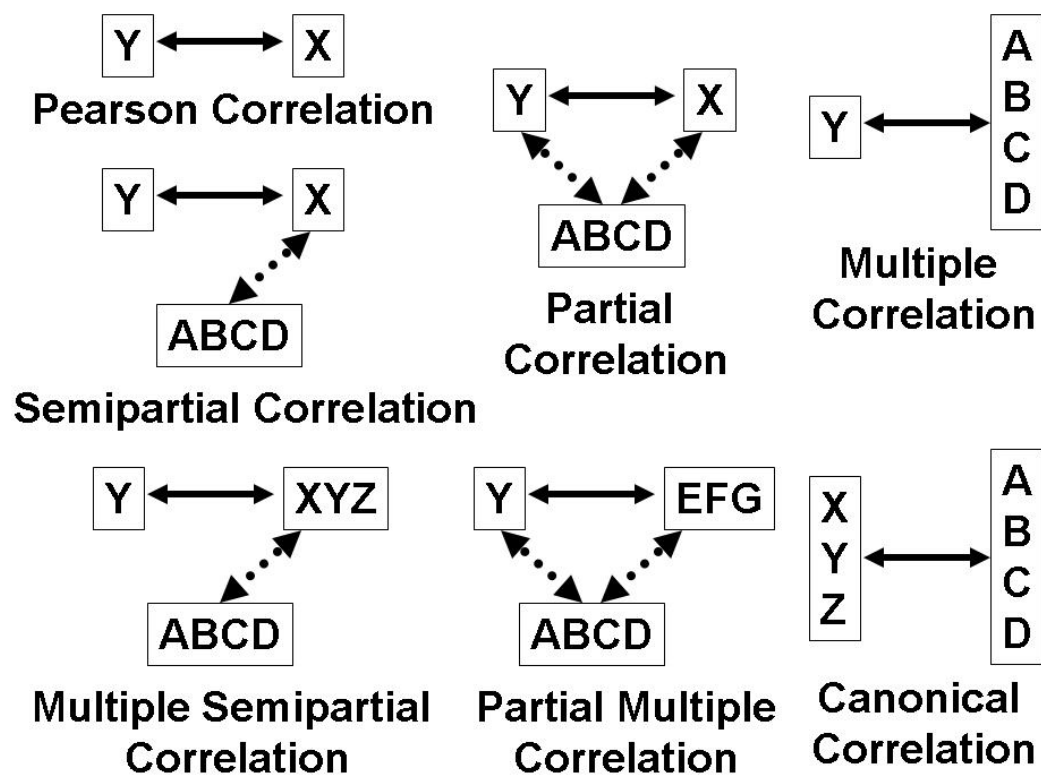


Figure 1

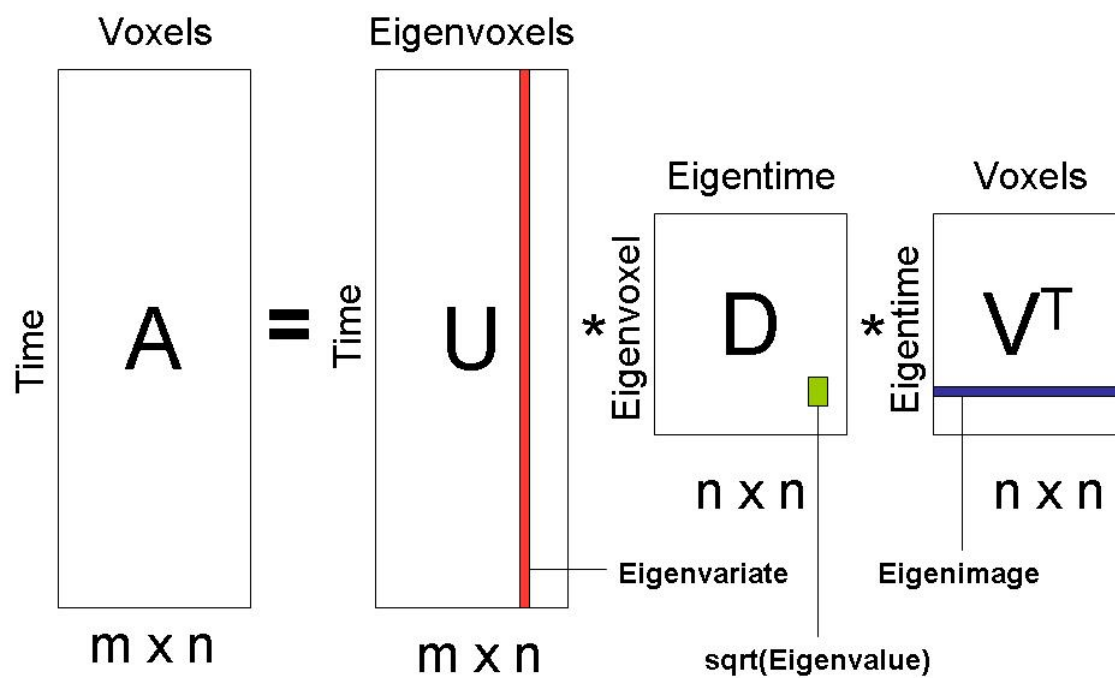


Figure 2

Citation:

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Creating Physical 3D Stereolithograph Models of Brain and Skull

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Abstract

The human brain and skull are three dimensional (3D) anatomical structures with complex surfaces. However, medical images are often two dimensional (2D) and provide incomplete visualization of structural morphology. To overcome this loss in dimension, we developed and validated a freely available, semi-automated pathway to build 3D virtual reality (VR) and hand-held, stereolithograph models. To evaluate whether surface visualization in 3D was more informative than in 2D, undergraduate students (n=50) used the Gillespie scale to rate 3D VR and physical models of both a living patient-volunteer's brain and the skull of Phineas Gage, a historically famous railroad worker whose misfortune with a projectile tamping iron provided the first evidence of a structure-function relationship in brain. Using our processing pathway, we successfully fabricated human brain and skull replicas and validated that the stereolithograph model preserved the scale of the VR model. Based on the Gillespie ratings, students indicated that the biological utility and quality of visual information at the surface of VR and stereolithograph models were greater than the 2D images from which they were derived. The method we developed is useful to create VR and stereolithograph 3D models from medical images and can be used to model hard or soft tissue in living or preserved specimens. Compared to 2D images, VR and stereolithograph models provide an extra dimension that enhances both the quality of visual information and utility of surface visualization in neuroscience and medicine.

Introduction

Leonardo da Vinci was the first to model brain structure by injecting molten wax into the ventricle of an oxen brain [1]. Since then, neuroanatomical models have shown utility in neuroscience and medicine in areas such as education, diagnosis, and surgical planning [2,3]. Currently, classical modelling techniques are being supplanted by modern methods that emphasize three-dimensional anatomical relationships using imaging techniques [4,5]. In this report, we describe and evaluate a modern method to replicate an individual's anatomy as a physical, hand-held model by using reverse engineering and rapid prototyping stereolithography [6].

Reverse engineering is a process in which a 3D physical object is scanned using an MRI or CT, for example, and the images are used to render a three-dimensional virtual model. Rapid prototyping is a new technology that uses this virtual model to print or fabricate a physical model. Stereolithography is a rapid prototyping technique with several variants; and, in our application, layers of plaster can form a solid, physical model after a binding agent is applied to each printed layer.

Although other hand-made and computerized modelling approaches have been used to model neuroanatomy [7,8], rapidly prototyped models preserve anatomical scale and anatomical relationships, are three-dimensional, are understood by visual and tactile learners, do not require software training, and can be produced to the anatomical specifications of the individual patient. Compared to computerized virtual models which have the capability to virtually dissect an object in an infinite number of ways, physical

models can be intuitively held and rotated, can be interactively manipulated regardless of complexity, and are accessible without the need for computers or advanced training.

In this report, we developed a streamlined procedure to replicate human brain and skull morphology using freely available software to produce CT/MRI-assisted reverse engineered VR and stereolithograph models; validated the scaling precision of our modelling pathway for quality control; and evaluated 2D images, virtual models (VM), and stereolithograph models (SM) of brain and skull for the quality of visual information and utility of these models.

Analysis

Algorithm

We developed an open-source processing pathway to visualize VR and stereolithograph models (Figure 1). With University of Wisconsin-Madison IRB approval, reverse engineering of brain began with 3D SPGR MRI acquisition of living human brain images on a 3.0 Tesla GE Signa (General Electric Medical Systems; Waukesha, WI) scanner (114 axial slices 1.3 mm thick; 256 x 256 resolution; pixel size 0.9375 x 0.9375). DICOM images were loaded into the NIH Analysis of Functional Neuroimaging (AFNI) software package [9] for automated inhomogeneity correction and brain isolation through skull stripping. Although a mesh is commonly used to model the brain's surface (as in Figure 2b), this method failed to print because the mesh lacks information about the thickness of the surface and is too thin to print. For this reason, we preserved the volume dimension of this dataset by generating an isosurface which preserves surface

morphology and has enough thickness to be printed. After exporting these files in an ANALYZE format, these images were reconstructed in three dimensions and saved in the visualization tool kit (.vtk) format using 3dSlicer [10]. The reconstructed images were converted to the virtual reality markup language (VRML) for three dimensional printing using isosurface visualization without scalar coloring in MayaVi [11]. We viewed and navigated the VRML brain model with VRMLView (Systems in Motion; Norway). The virtual whole brain isosurface (Figure 2a) and cortical mesh clearly delineated sulci from gyri (Figure 2b).

Validation

We fabricated an intact, half scale, whole brain 3D stereolithograph model (SM) which preserved cortical morphology using the VRML model to drive the 3D Printing (3DP) [6] process on a Zcorp Z406 3D printer (Z Corporation; MA, USA) at the UW-Madison New Media Center. The whole brain 3D stereolithograph model (Figure 2c) preserved the cortical morphology (Figure 2d; compare to Figure 2b) of the virtual reality markup language (VRML) model.

Maximum length (mm) measurements along the primary right-left (RL), anterior-posterior (AP), and superior-inferior (SI) axes were respectively taken for the VM and SM models with AFNI using DICOM images and with ImageJ [12] using images collected with an HP (Hewlett Packard, USA) PSC 1210 scanner. The primary axes length measures for the scaled stereolithograph model (RL,AP,SI: 67.2, 90.1, 63.2 mm) were as expected based on the virtual brain model (RL,AP,SI: 134.1, 179.1, 133.9 mm).

The SM brain was reduced 3.75 mm (5.9%) in the SI dimension during model construction because the model was printed in layers from the inferior to superior direction and the weight of the model caused compression along this dimension. Temperature, humidity, and seasonal calibration also contributed to error in the SI dimension. The printer operator can correct this printing artifact by adjusting the length of the model in the dimension of model growth.

For validation, we tested whether the average virtual to stereolithograph ratios for the

primary axes lengths $\left[mean \left(\frac{RL_{VM}}{RL_{SM}} + \frac{AP_{VM}}{AP_{SM}} + \frac{SI_{VM}}{SI_{SM}} \right) \right]$ preserved the 2:1 VM:SM axis

scaling factor and the stereolithograph model average axis proportions

$\left[mean \left(\frac{AP}{RL_{VM}} + \frac{AP}{SI_{VM}} + \frac{RL}{SI_{VM}} \right) \right]$ preserved the 1:1 VM:SM ratio. These measures

indicated that our half-scale stereolithograph brain model preserved the dimensions of the virtual model (Figure 3).

We then applied our method to produce a VRML (Figure 4a) and physical 3D model (Figure 4b) of Phineas Gage's skull. As the raw CT scans of Phineas Gage's skull were not available because scan requests from prior authors failed, we produced the 3D model from a video clip of multi-plane, computed tomography (CT) images taken of Phineas Gage's skull in 2004 [13]. The accurate physical reproduction of Phineas' skull required a virtual model with the correct dimensions; however, voxel size was not reported with the

video clip [13] and a prior publication did not report measurements taken of the authentic Phineas skull [14]. Accurate replication of the virtual model was possible using a voxel size (RL,AP,SI: 0.48,0.8,0.8 mm) in which the VR skull dimensions matched the anatomical dimension measures of the authentic Phineas Gage skull (Table 1) provided by Dominic Hall, Curator of the Warren Anatomical Museum at Harvard Medical School which houses the authentic skull of Phineas Gage. The mpeg video clip was brought into AFNI, cropped to isolate sagittal images (RL,AP,SI: 458,352,342 voxels), exported in ANALYZE format, entered into our processing pathway (Figure 1), and volume rendered in Video S1. Caliper measurements (12" digital caliper; Neiko Tools, USA) indicated that the half-scaled dimensions of the stereolithograph skull model were as expected (RL,AP,SI: 66.0,90.6,56.6 mm) and the printer operator successfully compensated for compression in the RL print out dimension. The stereolithograph skull model preserved the VM:SM average axes scaling factor of 2:1 [Mean $\pm$ SEM=1.99 $\pm$ 0.00; $t(2)$ = -1.73; p =0.23, 2-tailed] and the 1:1 average axes proportion [Mean $\pm$ SEM=1.0 $\pm$ 0.00; $t(2)$ = -0.32; p =0.78, 2-tailed].

Evaluation

Students enrolled in the University of Wisconsin-Madison course entitled “Ways of Knowing Biology,” voluntarily and anonymously evaluated the quality of visual information contained in the 2D, VRML, and stereolithograph models of brain and Phineas Gage’s skull. We used the Gillespie rating scale (Table S1) to assess students’ perception of biological image models relative to a baseline model [3]. In our assessment, 2D images were used as the baseline for assessing VRML and

stereolithograph models for the quality of visual information and biological utility.

Gillespie ratings were coded with 1=Inferior, 2=Similar/Equivalent, 3=Superior (similar information more rapidly assimilated), 4=Superior (additional information provided).

Since all models were derived from the same data, differences in perceived visual information were a function of model type.

The Gillespie rating survey indicated that the 3D models were superior in surface visualization quality (Table 2) compared to 2D images (Gillespie rating = 2) for the brain VRML [$t(49)=37.5$; $p=0.00$], brain stereolithograph [$t(49)=12.7$; $p=0.00$], Phineas VRML [$t(49)=20.8$; $p=0.00$], and the Phineas stereolithograph models [$t(49)=14.1$; $p=0.00$]. The Wilcoxon signed ranks test indicated that the quality of visualization using VRML is superior to stereolithograph models for brain [$Z=-4.88$; $p=0.00$; $n=50$] and skull [$Z=-2.29$; $p=0.022$; $n=50$] and that biological image utility was greater for VRML than 2D models [$Z=-6.13$; $p=0.00$; $n=50$], was greater for stereolithograph than 2D models [$Z=-4.97$; $p=0.00$; $n=50$], and was greater for VRML than stereolithograph models [$Z=-3.20$; $p=0.001$; $n=50$].

Discussion

The reverse engineering and rapid prototyping pathway we developed has medical applications in biomodel guided stereotactic surgery [15], cranioplasty [16], aneurysm research and repair [17], and craniofacial reconstruction [18]. In neuroscience, this pathway has applications in producing phantoms of living or preserved specimens and in both basic science and imaging education. Even though the 3D models were derived from the same data as 2D images, 3D models display anatomical relationships in an extra

dimension to enhance the quality of visual information and model utility compared to 2D images.

Efficient replication of the intact whole brain with high resolution was previously unattainable using rapid prototyping techniques due to limitations that have largely been overcome through advances in neuroimaging software, computer hardware, and higher field magnets. Disadvantages that remain for rapid prototyping include monetary cost (approximately 50 cents / cc, excluding the cost of MR imaging and data post-processing) and time to print (approximately 2.5 vertical cm / hour). Our half-scaled 219 cc physical brain model cost approximately \$110 and took 2.5 hours to print. The 1756 cc full brain would have cost about \$880 and taken 5.5 hours to print. The cost would have been significantly more using other rapid prototyping technologies.

We developed and validated a freely available method to model brain and skull anatomy in three dimensions using images collected with multiple modalities. Virtual reality and stereolithograph models are advantageous for observing surface morphology compared to two-dimensional planar images and can be manipulated using a data glove or bare hand, respectively. A disadvantage of the stereolithography approach compared to the VR approach is the lack of subcortical visualization and the inability to visualize the interior skull regions. Future adaptations of this technique should take advantage of 3D tissue printers which are currently being developed and the current color printing capability of 3D printers to delineate brain and skull regions.

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Figures

Figure 1: MRI-based Reverse Engineering Processing Pathway

Freely available software and file formats used to produce virtual and stereolithograph brain models are diagrammed. AFNI is available from [<http://afni.nimh.nih.gov/>]; 3dSlicer is available from [<http://www.slicer.org/>]; MayaVi is available from [<http://mayavi.sourceforge.net/>].

Figure 2: VR and Rapidly Prototyped Stereolithographic Human Brain Model

The processing pathway we developed can replicate living specimens. (a) Cortical isosurface of virtual whole human brain. (b) Right hemisphere cortical isosurface of pre- and postcentral (left) gyrus with wireframe mesh (black) and vertices (red). (c) Physical whole human brain stereolithograph replica. (d) Right hemisphere pre- and postcentral (left) gyrus for comparison with Figure 2b.

Figure 3: Validation of Processing Pathway

The stereolithographic model preserved the VM:SM average axes scaling factor of 2:1 [Mean $\pm$ SEM=2.03 $\pm$ 0.04; $t(2)=0.79$; $p=0.51$, 2-tailed] and the 1:1 average axes proportion [Mean $\pm$ SEM=0.96 $\pm$ 0.02; $t(2)=-2.2$; $p=0.16$, 2-tailed]. Error bars are SEM.

Figure 4: Stereolithograph of Phineas Gage's Skull

The processing pathway we developed can replicate preserved specimens. (a) Virtual model of Phineas Gage's skull. (b) With this physical 3D model of Phineas Gage's skull,

we illustrate the approximate path of the tamping iron that produced Phineas Gage's famous injury.

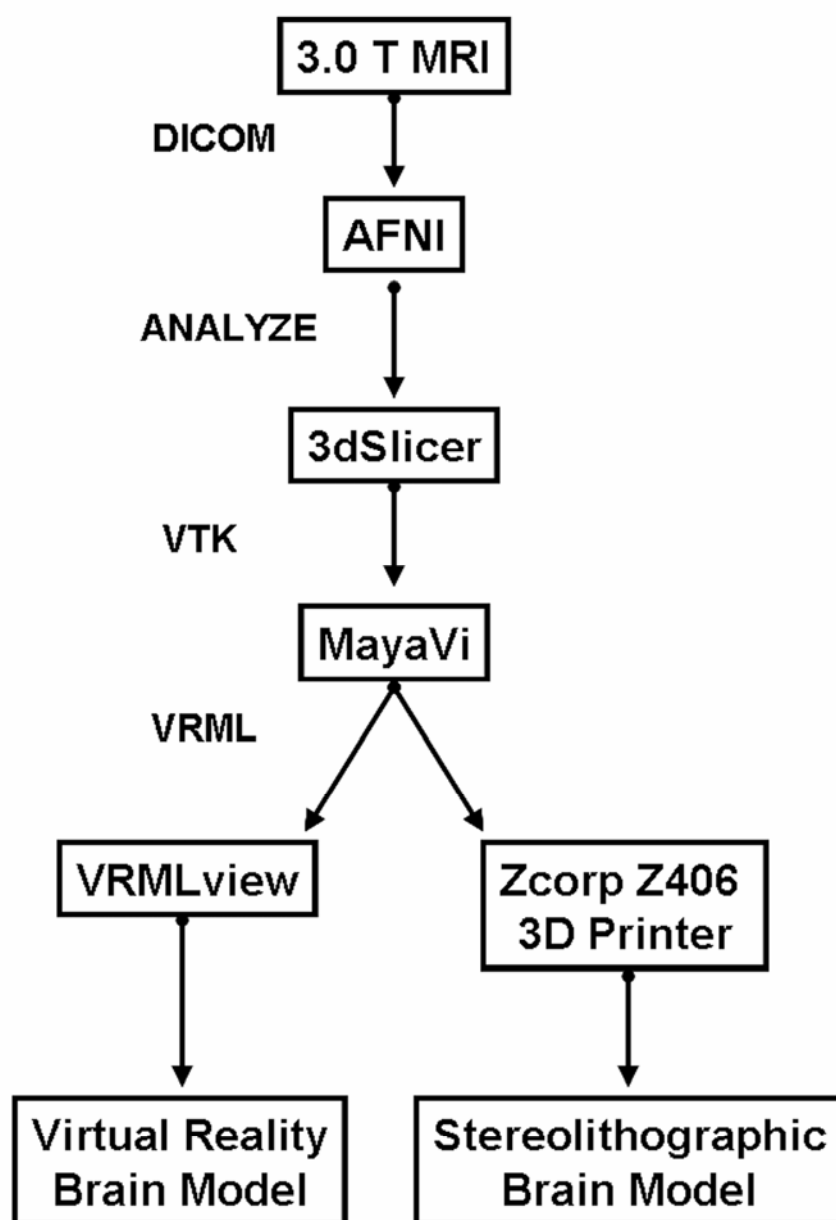


Figure 1

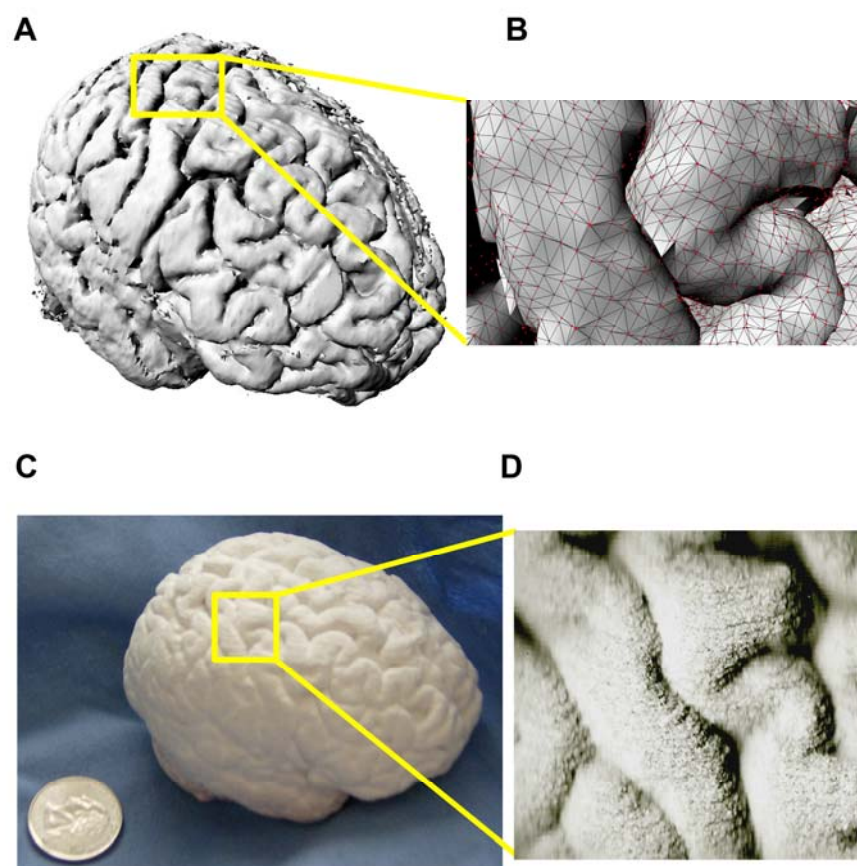


Figure 2

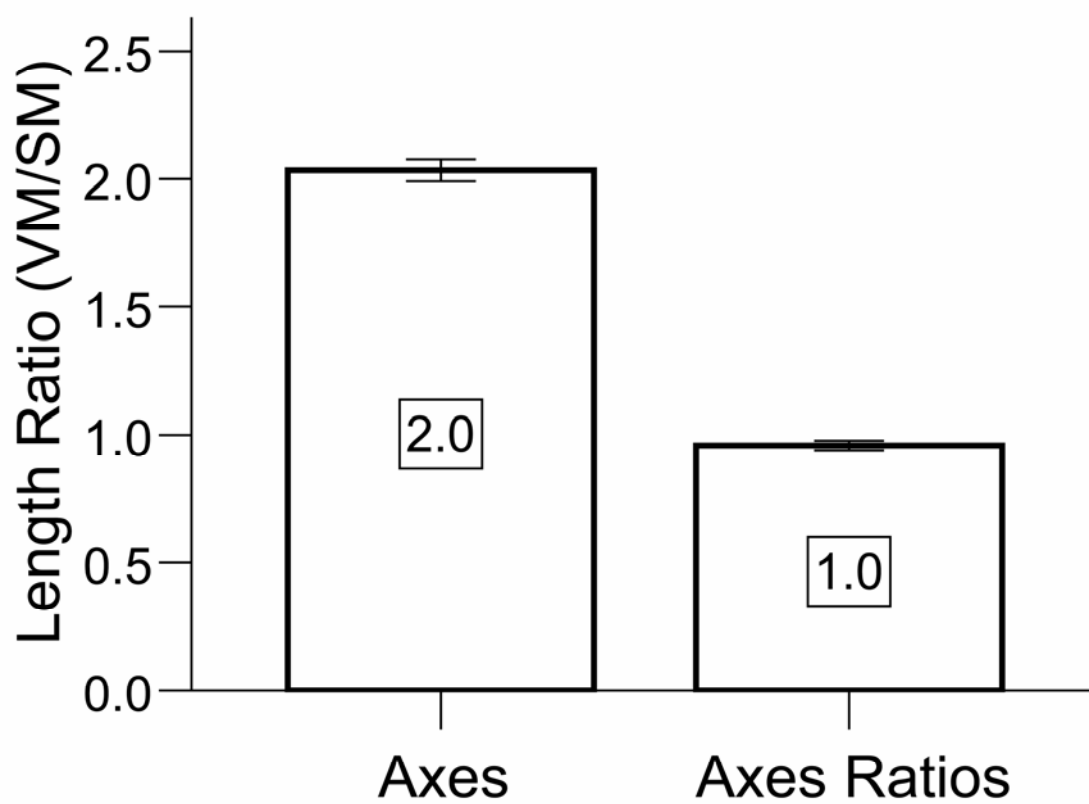


Figure 3

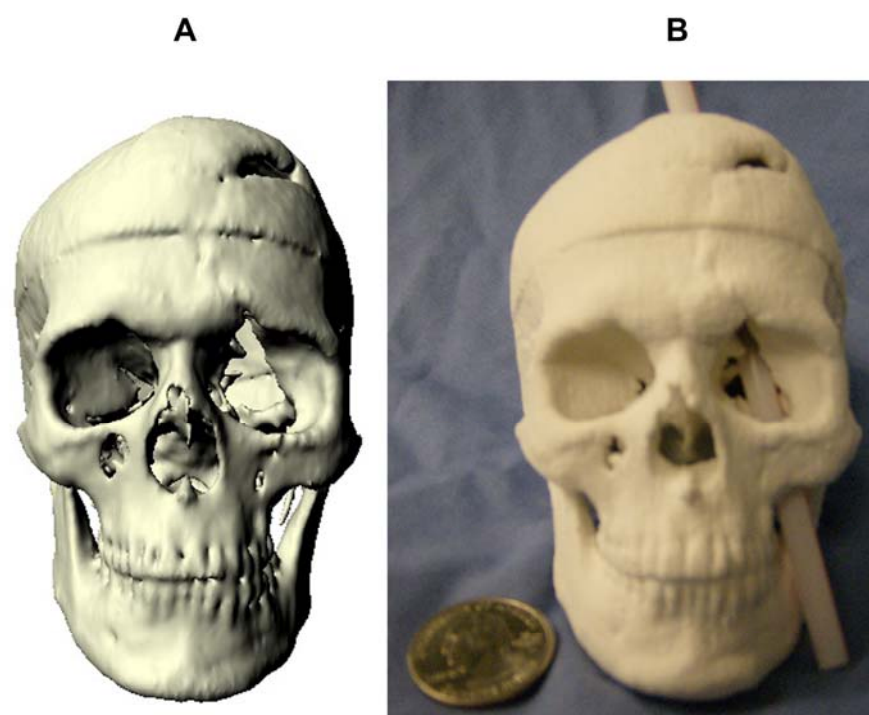


Figure 4

Tables:

Table 1: Dimension Estimates of Authentic Phineas Gage Skull.

Primary Axis Dimension	Anatomical Landmarks	Length Estimates (mm)
Right:Left (RL)	Right Extrema to Left Extrema	131
Anterior:Posterior (AP)	Glabella to Posterior Extrema	180
Superior:Inferior (SI)	Nasion to Gnathion	113

Table 2: Gillespie Ratings of Visual Information and Biological Utility.

Questionnaire results reporting mean, standard deviation, and distribution of responses in each Likert-scaled category.

Question	Topic	Mean	SD	1	2	3	4
VI	Quality of Visual Information, VI						
1	Brain VRML	3.86	0.35	0	0	7	43
2	Brain Stereolithograph	3.10	0.61	1	4	34	11
3	Phineas VRML	3.58	0.54	0	1	19	30
4	Phineas Stereolithograph	3.28	0.64	0	5	26	19
BU	Biological Utility, BU						
1	2D	2.20	0.70	6	30	12	2
2	VRML	3.74	0.56	0	3	7	40
3	Stereolithograph	3.18	0.80	2	6	23	19

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Brain mapping in cognitive disorders: a multidisciplinary approach to learning the tools and applications of functional neuroimaging.

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Abstract

Background: With rapid advances in functional imaging methods, human studies that feature functional neuroimaging techniques are increasing exponentially and have opened a vast arena of new possibilities for understanding brain function and improving the care of patients with cognitive disorders in the clinical setting. There is a growing need for medical centers to offer clinically relevant functional neuroimaging courses that emphasize the multifaceted and multidisciplinary nature of this field. In this paper, we describe the implementation of a functional neuroimaging course focusing on cognitive disorders that might serve as a model for other medical centers. We identify key components of an active learning course design that impact student learning gains in methods and issues pertaining to functional neuroimaging that deserve consideration when optimizing the medical neuroimaging curriculum.

Methods: Learning gains associated with the course were assessed using polychoric correlation analysis of responses to the SALG (Student Assessment of Learning Gains) instrument.

Results: Student gains in the functional neuroimaging of cognition as assessed by the SALG instrument were strongly associated with several aspects of the course design.

Conclusions: Our implementation of a multidisciplinary and active learning functional neuroimaging course produced positive learning outcomes. Inquiry-based learning activities and an online learning environment contributed positively to reported gains. This functional neuroimaging course design may serve as a useful model for other medical centers.

Background

A need in medical education is to inform students about the application of a continuum of multidisciplinary imaging techniques to understand disease and to encourage physicians to take part in clinical imaging trials [1]. Higher level brain functions, such as memory, metacognition, executive abilities, language, and emotions, form the basis of adaptive and rich social interactions and their dysfunction can now be better understood through experimentation and application of advanced functional imaging techniques. With statistical analyses of images collected using functional imaging modalities, clinicians and researchers have the capability to spatially localize significant brain activation in the form of functional brain maps that convey information about the neural systems subserving aspects of cognition. Functional brain mapping topics have yet to be incorporated into the curriculum at most medical centers even though these techniques have demonstrated great clinical potential for advancing medical practice by informing physicians about the function of scanned brain structures [2-5]. This is in contrast to radiological neuroimaging techniques like CT and MRI that are incorporated into the medical curriculum [6] and are readily used to provide information about gross structural anatomy and pathology in clinical diagnosis. In order to understand the distinctive features of structural and functional imaging methodologies and to catalyze the translation of functional neuroimaging techniques from the research laboratory into clinical applications for patient care, physicians require a practical understanding of the entire functional imaging process from experimental design to the interpretation of statistical brain activation maps [7].

Brain mapping has great potential to influence medicine by serving as a biomarker for cognitive disorders that has utility in predicting or detecting disease and

in quantifying the effect of therapeutic intervention [8]. For example, longitudinal studies of people at risk for Alzheimer's Disease may identify patterns of functional brain networks that are predictive of disease susceptibility. In addition, brain mapping studies may influence medical education by providing a rapidly growing knowledge-base of functional neuroanatomical relationships. However, to actualize the potential of brain mapping in medicine, practitioners must be educated in both the benefits and limitations of brain mapping in order to adequately interpret prior studies, take part in ongoing studies, and implement new studies.

The teaching of functional neuroimaging requires a multidisciplinary approach based on (1) the variety of functional imaging modalities used in basic and clinical neuroscientific investigations and (2) the multidisciplinary nature of the imaging sciences which requires expertise in neuroscience, neuropsychology, clinical neurology, neuroradiology, statistics, medical physics, computer science, and neuroanatomy, to name a few [9]. In the Fall 2006 course at the University of Wisconsin-Madison entitled, "Functional Imaging of Cognitive Disorders," we implemented a multidisciplinary, active learning course design which focused on cutting edge brain mapping methods that are used to measure structure and function in normal and previously normal brains and that, in particular, have demonstrated relevance to the assessment of brain function and localization in clinically defined cognitive disorders. Course design reflected the multidisciplinary approach used to brain map cognitive disorders and was weighted toward formal lectures on a variety of key topics regarding methodology and clinical applications, as well as student presentations and group discussions of seminal and current relevant scientific papers. The topics for neuroimaging techniques and cognitive disorders reflected the multidisciplinary nature of brain mapping in clinical research and patient case studies.

A complementary view to the idea that neuroimaging research holds great promise for improving classroom education [10-14] is that inquiries into the educational process of students may improve the teaching of functional neuroimaging. To assess the impact that our course design had on student gains in learning functional neuroimaging, henceforth referred to as neuroimaging cognition, we implemented the SALG [15] instrument. The SALG instrument was developed by Elaine Seymour at the University of Wisconsin Center for Educational Research to assess student perception of learning gains as a function of course design and delivery [16] and has been successfully used to this end [17, 18]. We converted the online SALG instrument to a paper format and assessed student learning gains as part of our end of semester course assessment.

Methods

Course Design

The inquiry-based, active learning methods [19, 20] used to engage students depended on regular attendance and active student participation. Activities included oral presentations, focused group discussions, and reading assessments which were developed by students. In addition, we assigned readings from textbooks [21, 22] to provide an overview of the techniques and their applications. Learn@UW [23], an online learning environment, was used to deliver topics that included all research papers, case studies, reading assessments, solutions, lecture notes, and grades to students. Students were required to prepare for each class by reading the assigned papers and by completing reading assessments which their classmates constructed.

Each student was asked to present one paper in detail including the theory supporting it, the methods, results, and interpretation. To account for the diversity of

student interests, students presented papers on topics which they selected and were approved by the instructor. Students were expected to prepare a 15 minute presentation and critique of the paper. The presentation was followed by an interactive period of commentary and discussion from the faculty and students. The presentations were assessed on clarity of content, scientific accuracy, and presentation quality. The presenting student was expected to consult several other sources beyond the paper (such as reviews and other empirical studies with similar or different methods) in order to develop a broader perspective on the topic, place the current paper in the context of what is known and not known about the topic, and to provide ideas for further study. In addition, one week prior to their presentation, students were asked to submit two questions (and answers) about the key concepts in the paper they were going to present which, upon approval, were forwarded to the class and posted on Learn@UW as reading assessments. Reading assessments consisted of responses to two cluster-style questions per week and were graded for content based on the assigned readings for that week. Course assessment was based on active class participation, individual student presentations, and weekly reading assessments

A vital component of the course was faculty lectures. Each week a presentation was given to students by UW faculty who are experts in their field. This was followed by a group discussion period in which students could ask questions and instructors engaged students through decision making questions.

We based topic selection on the advanced neuroimaging techniques currently in use and the expertise of faculty on the University of Wisconsin campus [see Additional file 1]. The neuroimaging techniques we selected for this course included: Magnetic Resonance Imaging (MRI), functional MRI (fMRI), Positron Emission Tomography, Single Photon Emission Computed Tomography, Transcranial

Magnetic Stimulation, Electroencephalography, Diffusion Tensor Imaging, Diffusion Weighted Imaging, Perfusion Imaging, and Connectivity Techniques. The focus of clinical neuroimaging applications included: Alzheimer's Disease, Parkinson's Disease, Stroke, fMRI Presurgical Mapping, Anxiety, Depression, Pain, Traumatic Brain Injury, Social Cognition, and Cerebral Recovery. Guest lecturers were recruited from the University of Wisconsin Departments of Medicine, Radiology, Neurology, Medical Physics, Psychiatry, Psychology, Biomedical Engineering, Kinesiology, the Medical Scientist Training Program, and the Neuroscience Training Program.

Survey and Statistical Analysis

Students voluntarily completed our implementation of the Student Assessment of Learning Gains (SALG), which was administered to assess student learning and gain feedback as part of the initial course evaluation. The questionnaire was voluntary and anonymous with no bearing whatsoever on the students' grades. Because the survey was part of the academic evaluation of the course, IRB approval was initially not required and not obtained. However, prior to publishing the material in this report, we received IRB approval for this secondary use of the data.

Online course access was determined using the number of topics accessed feature in Learn@UW. Responses to the SALG were anonymous and quantified by someone not affiliated with the course in the Neuroscience Training Program. Categorical responses were coded on a 5 point (0 = Not applicable; 1 = Not At All, 2 = A Little, 3 = Somewhat, 4 = A Lot, 5 = A Great Deal) or 4 point (0 = Not Applicable; 1 = Strongly Agree, 2 = Disagree, 3 = Agree, 4 = Strongly Agree) Likert scale. Questions in the neuroimaging cognition category focused on the gains in learning goals for this course and the course design questions were centered around

engagement activities and course content. The polychoric correlation, ρ , is a useful statistic to understand associations in categorical data and is preferred to the Spearman correlation because the discretizing latent variable thresholds are estimated [24]. The association of course contributions to neuroimaging cognition learning gains using the SALG instrument were determined using polychoric [25] correlation software in R [26]. Two-tailed significance was assessed after a ρ to t conversion on $N-2$ degrees of freedom. A corrected p -value ≤ 0.05 was considered significant.

Results

Of all the students ($N = 20$) attending the course, ten were advanced undergraduates and the rest were professional students in graduate or medical school. All students completed the survey. Students accessed 94 $\pm$ 3 percent of the course topics listed in the Learn@UW course website. Student responses to the SALG questions focusing on student neuroimaging cognition and course design are reported in Table 1 as means ($\pm$ SD) and percentage frequency of Likert scaled responses. The polychoric correlation matrix (Figure 1) displays the association of student gains in medical neuroimaging cognition with course design. Significant association ($p \leq 0.05$, 2-tailed) are plotted in the p -value matrix of Figure 2 and listed in Table 2. A marginally significant association was also found in learning gains between guest lecturers and future neuroimaging courses using the same format ($\rho = 0.73$, $df = 18$, $p = 0.07$).

Discussion

In this paper, we describe our implementation of a functional neuroimaging course intended to meet the need in medical education to inform students about the distinctive features of functional brain mapping and to foster an enthusiasm for

functional imaging that has the potential to translate into clinical trial involvement [1]. Based on a prior student perception study of medical imaging education [6], we would recommend incorporating this course, or portions of it, early in the medical school curriculum.

Student gains in brain mapping methodology and gains attributed to specific aspects of course design were positive overall (Table 1). The largest average gains in the functional imaging of cognition (mean ≥ 4 out of 5) were made in increasing students' understanding that functional imaging of patients with cognitive disorders can improve our understanding of basic neuroscience/psychology (i.e., normal brain function), that future patients may benefit from ongoing functional neuroimaging research, that functional neuroimaging may improve the diagnosis and care of cognitive disorders, and in understanding that new brain mapping techniques can impact medicine. The learning gains due to course design were maximally attributed (mean ≥ 4 out of 5) to the multidisciplinary panel of lecturers, the topics covered, the course overall, the treatment of neuroimaging techniques overall, the functional MRI topic, the importance of the neuroimaging field to human disorders, the critical review of papers, and the summarization of articles. In addition, students strongly agreed (mean ≥ 3 out of 4) that functional neuroimaging should be an integral part of medical education, that the overall content of the course was appropriate, that the online resource Learn@UW was easy to use and contributed positively to this course, that designing reading assessments required key concepts in the paper to be identified, and that future neuroimaging courses should use the same or similar format as this course.

Students exhibited both positive and negative associations in learning gains between neuroimaging cognition and course design (Figure 1). Among the thirty-three

questions that comprised the SALG instrument, sixteen unique learning gains pertaining to neuroimaging cognition and course design were significantly associated at some level after multiple comparison correction (Figure 2). Given that all of the significant associations were positive, the gains from course design contributed positively to student gains in neuroimaging cognition (Table 2). In addition, this indicates a positive alignment between perceived learning gains in neuroimaging cognition and specific engagement activities used in our course design. The gains in student enthusiasm for brain mapping were strongly associated with the overall course design, the topics covered, the multidisciplinary panel of lecturers, and the importance of applied functional neuroimaging. Lecturers with expertise contributed positively to students' perception that functional imaging can improve our understanding of normal brain function. Students also gained an association between fMRI and the ability of neuroimaging to benefit patients with cognitive disorders. Aspects of neuroimaging cognition (Q3,6,7,9,10), which were not significantly associated with any aspect of course design, included instilling student confidence (Q6) and the notion of improving medicine through current imaging practices (Q3,7,9,10). This result may be related to the introductory nature of our course or insufficient multidisciplinary prerequisites for students to build confidence and, respectively, the course emphasis on laboratory research methods for imaging rather than clinical practice methods for individualized medicine which are largely in development. Aspects of course design which were not significantly associated with any aspect of neuroimaging cognition included subcategories of the online system (Q11,Q14), subcategories of active learning engagement activities (Q10,16,19,20,21), subcategories of course materials (Q8,22), and subcategories of overall course content (Q4,Q12). Nevertheless, our course design was assessed favorably overall by students (Table 1) and both the significant

and non-significant associations between course design and neuroimaging cognition (Table 2) may be useful metrics to modify subcategories in this course design to further align instructor learning goals with student outcomes.

Future clinical neuroimaging courses should consider using an online learning environment and an active learning approach in their course design. Students' perception of the online learning environment, Learn@UW, was strongly associated with instilling an enthusiasm for applied neuroimaging. Through active learning engagement activities including reading assessments and critical review of articles, students gained an understanding that new imaging techniques can impact medicine and that future patients will benefit from ongoing functional neuroimaging research. The key aspects of the course that were significantly associated with gains in neuroimaging cognition and that are important for future neuroimaging courses included the topics covered, summarizing and critically reviewing papers, the importance of neuroimaging applications to human disease, and the inquiry-based, active learning engagement activity in which students could design reading assessments.

Based on our experience, future introductory neuroimaging courses should incorporate more active learning exercises into their multidisciplinary course design. For example, an introductory laboratory unit using neuroimaging software may be a useful alternative to oral presentations by students. Incorporating the design of a neuroimaging experiment as an active learning activity may engage students and foster gains in neuroimaging cognition and confidence. Future studies should consider investigating the efficacy of course design on neuroimaging cognition using alternative measures. Although the SALG instrument is a useful tool to examine the association between course design and student learning gains, these self-report

measures are limited by the qualitative subjective experience of each individual that we are attempting to quantify. As an alternative, results from laboratory analysis exercises could provide objective student outcome measures.

Conclusions

Using the SALG instrument, we conclude that the learning gains from this multidisciplinary neuroimaging course contributed positively to student gains in functional neuroimaging of cognitive disorders as evidenced by the significant, positive polychoric correlations. An active, inquiry-based learning approach in which students designed and answered reading assessments contributed positively to learning outcomes. Future functional neuroimaging courses would benefit from incorporating an active learning approach and an online learning environment into the medical neuroimaging curriculum.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DJK participated in the survey design and carried out the statistical analysis and drafted the manuscript. SCJ participated in the design and coordination of the survey and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1: Polychoric Correlation Matrix of SALG Associations between Course Design and Neuroimaging Cognition.

Figure 2: P-value Matrix of Significant ($p \leq 0.05$) Polychoric Correlations in Figure 1, Bonferroni corrected for multiple comparisons.

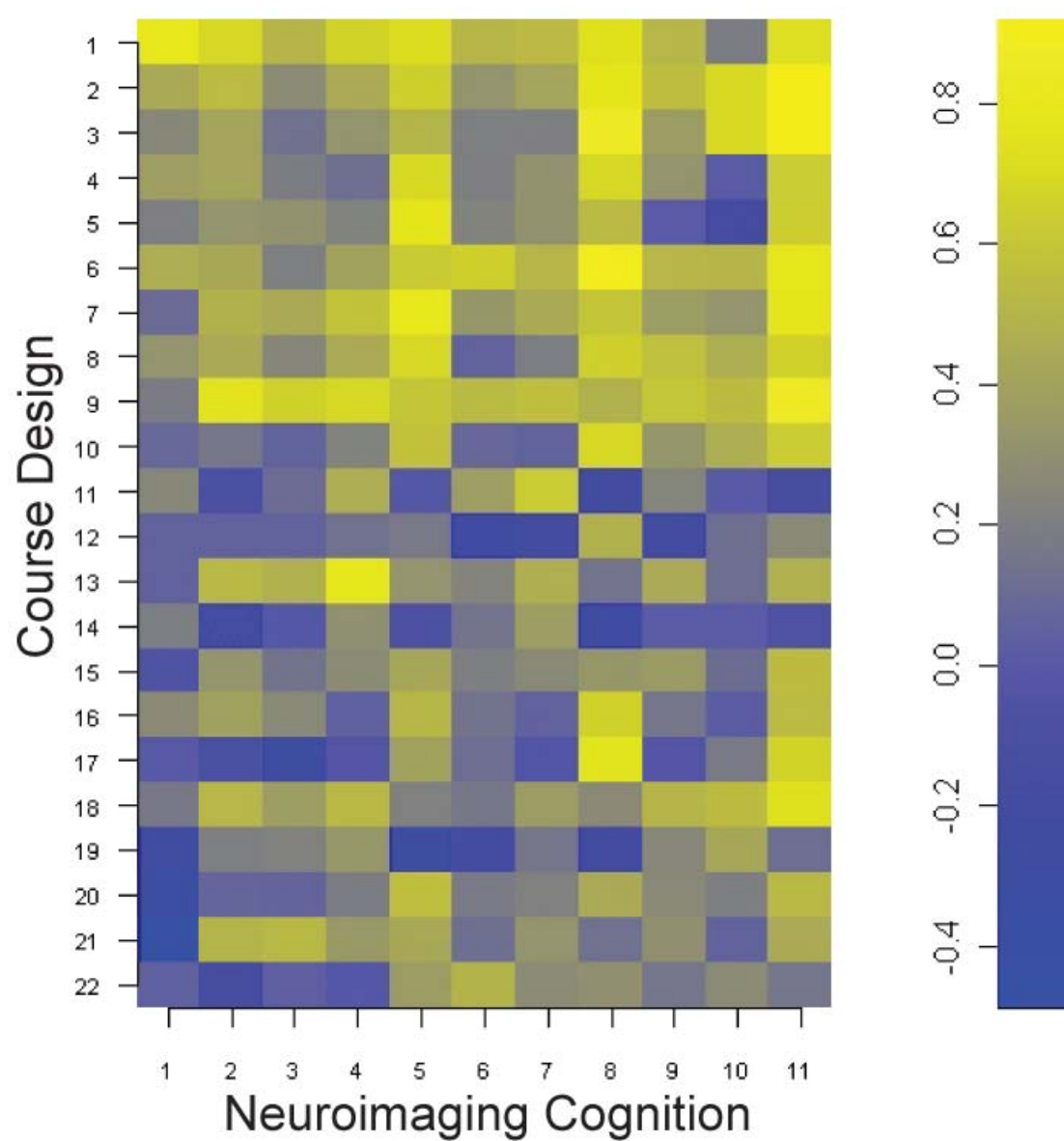


Figure 1

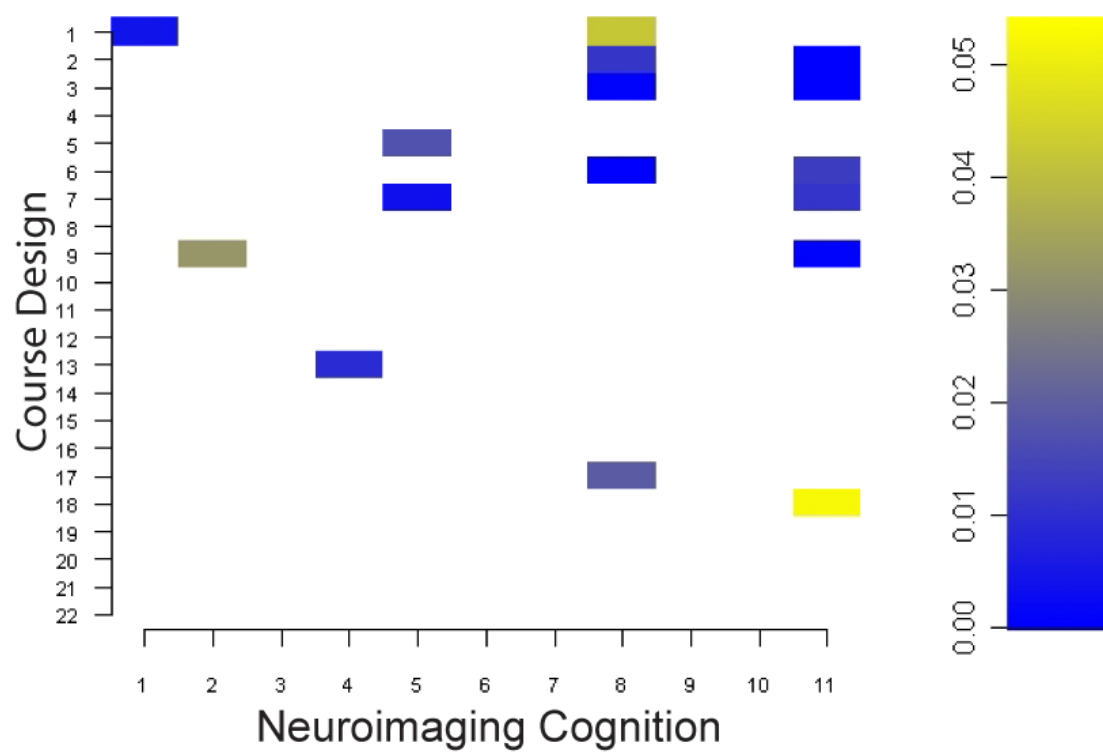


Figure 2

Tables

Table 1: SALG Questionnaire Results Reporting Mean, Standard Deviation, and Percentage of Responses in each Likert-scaled Category (N = 20).

<u>Neuroimaging Cognition</u>		Mean	SD	0	1	2	3	4	5
Q1	Functional imaging patients with cognitive disorders can improve our understanding of basic neuroscience/psychology (i.e., normal brain function)	4.45	0.51	0%	0%	0%	0%	55%	45%
Q2	Understanding that future patients will benefit from ongoing neuroimaging research	4.15	0.81	0%	0%	5%	10%	50%	35%
Q3	Understanding that neuroimaging can improve the <u>diagnosis</u> of cognitive disorders	4.15	0.88	0%	0%	5%	15%	40%	40%
Q4	Understanding how neuroimaging techniques can impact medicine	4.05	1.00	0%	5%	0%	15%	45%	35%
Q5	Functional imaging can benefit patients with cognitive disorders	3.85	0.93	0%	0%	10%	20%	45%	25%
Q6	Confidence in your ability to take part in this field	3.85	0.93	0%	0%	5%	35%	30%	30%
Q7	Understanding that neuroimaging can improve the <u>treatment</u> of cognitive disorders	3.80	0.95	0%	0%	10%	25%	40%	25%
Q8	Enthusiasm for this field	3.80	1.06	0%	0%	10%	35%	20%	35%
Q9	Understanding that neuroimaging can improve the <u>prognosis</u> of cognitive disorders	3.75	1.07	0%	5%	5%	25%	40%	25%
Q10	Neuroimaging should be an integral part of medical education	3.50	0.61	0%	0%	5%	40%	55%	-
Q11	Future neuroimaging courses should use the same format as this course	3.05	0.69	0%	5%	5%	70%	20%	-
<u>Course Design</u>									
Q1	Guest Lectures	4.40	0.60	0%	0%	0%	5%	50%	45%
Q2	The Topics Covered	4.35	0.81	0%	0%	5%	5%	40%	50%
Q3	Overall Course	4.35	0.81	0%	0%	5%	5%	40%	50%
Q4	Neuroimaging Techniques Overall	4.20	0.89	0%	0%	0%	30%	20%	50%
Q5	Functional MRI	4.15	0.81	0%	0%	5%	10%	50%	35%
Q6	Importance of this field	4.15	0.81	0%	0%	0%	25%	35%	40%
Q7	Critically Reviewing Articles	4.10	0.79	0%	0%	5%	10%	55%	30%
Q8	Selected Papers	4.10	0.91	0%	0%	5%	20%	35%	40%
Q9	Summarizing Articles	3.95	0.94	0%	0%	5%	30%	30%	35%
Q10	Giving an Oral Presentation (neuroimaging skills)	3.80	0.95	0%	5%	0%	25%	50%	20%
Q11	Learn@UW was easy to use	3.70	0.47	0%	0%	0%	30%	70%	-
Q12	The overall content of the course was appropriate	3.65	0.49	0%	0%	0%	35%	65%	-
Q13	Answering Reading Assessments	3.65	1.23	5%	5%	0%	15%	60%	15%
Q14	Learn@UW contributed positively to this course	3.60	0.50	0%	0%	0%	40%	60%	-
Q15	Cognitive Disorders Overall	3.60	0.88	0%	0%	10%	35%	40%	15%
Q16	Classroom Discussions	3.60	0.88	0%	0%	5%	50%	25%	20%
Q17	Learn@UW	3.55	0.89	0%	0%	10%	40%	35%	15%
Q18	Designing Reading Assessments	3.45	1.00	0%	5%	5%	45%	30%	15%
Q19	Designing reading assessments required you to identify key concepts in the paper	3.35	0.67	0%	0%	10%	45%	45%	-
Q20	Listening to Student Presentations	3.15	0.88	0%	0%	25%	40%	30%	5%
Q21	Giving an Oral Presentation (learning neuroimaging)	3.10	1.07	0%	10%	10%	50%	20%	10%

Table 2: Significant Associations Between Neuroimaging Cognition and Course Design from Figure 2.

Question Numbers	Neuroimaging Cognition	Course Design	Polychoric Correlation	df	p-value (corrected)
1,1	Functional imaging patients with cognitive disorders can improve our understanding of basic neuroscience/psychology (i.e., normal brain function)	Guest Lectures	0.81	18	0.00
2,9	Understanding that future patients will benefit from ongoing neuroimaging research	Summarizing Articles	0.75	18	0.03
4,13	Understanding how neuroimaging techniques can impact medicine	Answering Reading Assessments	0.79	18	0.01
5,7	Functional imaging can benefit patients with cognitive disorders	Critically Reviewing Articles	0.81	18	0.00
5,5	Functional imaging can benefit patients with cognitive disorders	Functional MRI	0.77	18	0.02
8,6	Enthusiasm for this field	Importance of this field	0.89	18	0.00
8,3	Enthusiasm for this field	Overall Course	0.84	18	0.00
8,2	Enthusiasm for this field	The Topics Covered	0.78	18	0.01
8,17	Enthusiasm for this field	Learn@UW	0.77	18	0.02
8,1	Enthusiasm for this field	Guest Lectures	0.74	18	0.04
11,3	Future neuroimaging courses should use the same format as this course	Overall Course	0.92	18	0.00
11,2	Future neuroimaging courses should use the same format as this course	The Topics Covered	0.92	18	0.00
11,9	Future neuroimaging courses should use the same format as this course	Summarizing Articles	0.84	18	0.00
11,7	Future neuroimaging courses should use the same format as this course	Critically Reviewing Articles	0.78	18	0.01
11,6	Future neuroimaging courses should use the same format as this course	Importance of this field	0.78	18	0.01
11,18	Future neuroimaging courses should use the same format as this course	Designing Reading Assessments	0.74	18	0.05

Supplemental Material

D. J. Kelley and S. C. Johnson: Brain mapping in cognitive disorders: a multidisciplinary approach to learning the tools and applications of functional neuroimaging.

Syllabus excerpts: topics and sources of educational material.

Textbooks:

Huettel SA, Song AW, McCarthy G: *Functional magnetic resonance imaging*. Sunderland, Mass.: Sinauer Associates Publishers; 2004.

Optional

Jezzard P, Matthews PM, Smith SM, NetLibrary Inc.: *Functional MRI an introduction to methods*. Oxford ; New York: Oxford University Press; 2001.

D'esposito, M., *Functional MRI: Applications in Clinical Neurology and Psychiatry*, United Kingdom: Informa Healthcare; 2006.

Timeline

Week 1

Lecture: Course overview: brain mapping in clinical populations

Week 2

Lecture: MRI and fMRI Fundamentals

Huettel SA, Song AW, McCarthy G: **Chapter 6: From Neuronal to Hemodynamic**. In: *Functional magnetic resonance imaging*. pp. xviii, 492. Sunderland, Mass.: Sinauer Associates Publishers; 2004: 127-157.

Huettel SA, Song AW, McCarthy G: **Chapter 7: BOLD fMRI**. In: *Functional magnetic resonance imaging*. pp. xviii, 492. Sunderland, Mass.: Sinauer Associates Publishers; 2004: 159-184.

Lecture: Is fMRI feasible in clinical populations?

Huettel SA, Song AW, McCarthy G: **Chapter 11: Experimental Design**. In: *Functional magnetic resonance imaging*. pp. 284-319. Sunderland, Mass.: Sinauer Associates Publishers; 2004: 284-319.

Price CJ, Crinion J, Friston KJ: **Design and analysis of fMRI studies with neurologically impaired patients**. *J Magn Reson Imaging* 2006, 23:816-826.

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D'Esposito M, Deouell LY, Gazzaley A: **Alterations in the BOLD fMRI signal with ageing and disease: a challenge for neuroimaging.** *Nat Rev Neurosci* 2003, **4**:863-872.

Week 3

Lecture: Brain mapping with PET

Toga AW, Mazziotta JC: **Chapter 18: Imaging Brain Function with Positron Emission Tomography.** In: *Brain mapping : the methods*, 2nd ed. pp. xvii, 877. Amsterdam ; Boston: Academic Press; 2002: xvii, 877.

Buckner RL, Snyder AZ, Shannon BJ, LaRossa G, Sachs R, Fotenos AF, Sheline YI, Klunk WE, Mathis CA, Morris JC, Mintun MA: **Molecular, structural, and functional characterization of Alzheimer's disease: evidence for a relationship between default activity, amyloid, and memory.** *J Neurosci* 2005, **25**:7709-7717.

Lecture: fMRI, TMS and Working memory

Postle BR, Ferrarelli F, Hamidi M, Feredoes E, Massimini M, Peterson M, Alexander A, Tononi G: **Repetitive transcranial magnetic stimulation dissociates working memory manipulation from retention functions in the prefrontal, but not posterior parietal, cortex.** *J Cogn Neurosci* 2006, **18**:1712-1722.

Week 4

Lecture: Parkinson's Disease and dopamine PET

Brooks DJ, Piccini P: **Imaging in Parkinson's disease: the role of monoamines in behavior.** *Biol Psychiatry* 2006, **59**:908-918.

Lecture: Autism

Ch. 12 Huettel Statistical Analysis

Just MA, Cherkassky VL, Keller TA, Minshew NJ: **Cortical activation and synchronization during sentence comprehension in high-functioning autism: evidence of underconnectivity.** *Brain* 2004, **127**:1811-1821.

Week 5

Lecture: Alzheimer Disease: clinical perspective and early intervention

Nestor PJ, Scheltens P, Hodges JR: **Advances in the early detection of Alzheimer's disease.** *Nat Med* 2004, **10 Suppl**:S34-41.

de Leon MJ, DeSanti S, Zinkowski R, Mehta PD, Pratico D, Segal S, Clark C, Kerkman D, DeBernardis J, Li J, Lair L, Reisberg B, Tsui W, Rusinek H: **MRI and CSF studies in the early diagnosis of Alzheimer's disease.** *J Intern Med* 2004, **256**:205-223.

Student Presentations: fMRI of hippocampal function AD/ amnesic disorders

Bassett SS, Yousem DM, Cristinzio C, Kusevic I, Yassa MA, Caffo BS, Zeger SL: **Familial risk for Alzheimer's disease alters fMRI activation patterns.** *Brain* 2006, **129**:1229-1239.

Johnson SC, Schmitz TW, Trivedi MA, Ries ML, Torgerson BM, Carlsson CM, Asthana S, Hermann BP, Sager MA: **The influence of Alzheimer disease family history and apolipoprotein E epsilon4 on mesial temporal lobe activation.** *J Neurosci* 2006, **26**:6069-6076.

Week 6

Lecture: Diffusion Tensor Imaging

Le Bihan D, Mangin JF, Poupon C, Clark CA, Pappata S, Molko N, Chabriat H: **Diffusion tensor imaging: concepts and applications.** *J Magn Reson Imaging* 2001, **13**:534-546.

Claudia A.M. Wheeler-Kingshott GJB, Stefan C. A. Steens, Mark A. van Buchem,: **D: The Diffusion of Water.** In: *Quantitative MRI of the Brain* Edited by Tofts P. pp. 203-256; 2004: 203-256.

Lecture: Stroke perfusion-diffusion

Rowley HA: **Extending the time window for thrombolysis: evidence from acute stroke trials.** *Neuroimaging Clin N Am* 2005, **15**:575-587.

Week 7

Lecture: White Matter imaging, DTI

Dong Q, Welsh RC, Chenevert TL, Carlos RC, Maly-Sundgren P, Gomez-Hassan DM, Mukherji SK: **Clinical applications of diffusion tensor imaging.** *J Magn Reson Imaging* 2004, **19**:6-18.

Student Presentations: Is there a default mode?

Greicius MD, Srivastava G, Reiss AL, Menon V: **Default-mode network activity distinguishes Alzheimer's disease from healthy aging: evidence from functional MRI.** *Proc Natl Acad Sci U S A* 2004, **101**:4637-4642.

Greicius MD, Krasnow B, Reiss AL, Menon V: **Functional connectivity in the resting brain: a network analysis of the default mode hypothesis.** *Proc Natl Acad Sci U S A* 2003, **100**:253-258.

Week 8

Student Presentations: Memory: What to encode?

Kao YC, Davis ES, Gabrieli JD: **Neural correlates of actual and predicted memory formation.** *Nat Neurosci* 2005, **8**:1776-1783.

Wheeler ME, Buckner RL: **Functional-anatomic correlates of remembering and knowing.** *Neuroimage* 2004, **21**:1337-1349.

Student Presentations: fMRI and presurgical mapping

Liegeois F, Cross JH, Gadian DG, Connelly A: **Role of fMRI in the decision-making process: epilepsy surgery for children.** *J Magn Reson Imaging* 2006, **23**:933-940.

Baciu MV, Watson JM, Maccotta L, McDermott KB, Buckner RL, Gilliam FG, Ojemann JG: **Evaluating functional MRI procedures for assessing**

hemispheric language dominance in neurosurgical patients.
Neuroradiology 2005, **47**:835-844.

Week 9

Lecture: Brain Injury and cerebral recovery

Weiller C, May A, Sach M, Buhmann C, Rijntjes M: **Role of functional imaging in neurological disorders.** *J Magn Reson Imaging* 2006, **23**:840-850.

Student presentations: Mapping cerebral recovery

Nyffeler T, Muri R, Pflugshaupt T, Wartburg R, Hess CW: **Cortical reorganization after brain damage: the oculomotor model.** *Eur J Neurosci* 2006, **23**:1397-1402.

Saur D, Lange R, Baumgaertner A, Schraknepper V, Willmes K, Rijntjes M, Weiller C: **Dynamics of language reorganization after stroke.** *Brain* 2006, **129**:1371-1384.

Week 10

Lecture: Functional Connectivity

Ramnani N, Behrens TE, Penny W, Matthews PM: **New approaches for exploring anatomical and functional connectivity in the human brain.** *Biol Psychiatry* 2004, **56**:613-619.

Student Presentations: Emotion and task dependent connectivity

Schmitz TW, Johnson SC: **Self-appraisal decisions evoke dissociated dorsal-ventral aMPFC networks.** *Neuroimage* 2006, **30**:1050-1058.

Week 11

Lecture: Depression and Anxiety

Cannistraro PA, Rauch SL: **Neural circuitry of anxiety: evidence from structural and functional neuroimaging studies.** *Psychopharmacol Bull* 2003, **37**:8-25.

Davidson RJ, Pizzagalli D, Nitschke JB, Putnam K: **Depression: perspectives from affective neuroscience.** *Annu Rev Psychol* 2002, **53**:545-574.

Student Presentation: fMRI of appraisal: depression and anxiety

Rosenkranz MA, Busse WW, Johnstone T, Swenson CA, Crisafi GM, Jackson MM, Bosch JA, Sheridan JF, Davidson RJ: **Neural circuitry underlying the interaction between emotion and asthma symptom exacerbation.** *Proc Natl Acad Sci U S A* 2005, **102**:13319-13324.

Dalton KM, Kalin NH, Grist TM, Davidson RJ: **Neural-cardiac coupling in threat-evoked anxiety.** *J Cogn Neurosci* 2005, **17**:969-980.

Week 12

Lecture: Pain

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An Imaging Roadmap for Biology Education: From Nanoparticles to Whole Organisms

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Abstract

Imaging techniques provide ways of knowing structure and function in biology at different scales. The multidisciplinary nature and rapid advancement of imaging sciences requires imaging education to begin early in the biology curriculum. Guided by the NIH Roadmap initiatives, we incorporated a nanoimaging, molecular imaging, and medical imaging teaching unit into three one-hour class periods of an introductory course on ways of knowing biology. Activities were derived from NIH Roadmap initiatives in nanomedicine, regenerative medicine, and nuclear medicine. The course materials we describe contributed positively to student learning gains in quantifying and interpreting images, in characterizing imaging methods that provide ways of knowing biological structure and function, and in understanding scale in biology and imaging. The NIH Roadmap provides a useful context to educate students about the multidisciplinary imaging continuum.

Introduction

Currently, undergraduate science courses are not keeping pace with research advances and are lacking an intellectual continuum that fosters quantitative, multidisciplinary studies¹. Curriculum reform at the predoctoral level is especially needed in biology and its integration with the imaging sciences² because of the rapidity of imaging advances and multidisciplinary expansion of imaging applications³. With educational opportunities being created by neuroinformatic endeavors like the Human Brain Project⁴, there is a need to integrate imaging into the undergraduate biology curriculum in order to develop information technology skills for all students and provide the necessary foundation for those biology students interested in taking advanced imaging courses⁵. The demand for multidisciplinary imaging skills is high in the marketplace⁶ and the imaging infrastructure has been consolidated by formation of the National Institute of Biomedical Imaging and Bioengineering⁷ and the NIH Roadmap initiative^{8,9}. The NIH Roadmap [<http://nihroadmap.nih.gov/>] was designed to accelerate research and medicine based on three major themes: New Pathways to Discovery, focused on improving our understanding of biological systems; Research Teams of the Future, which explores alternative models to organize research teams; and Reengineering the Clinical Research Enterprise, which aims to better integrate current research infrastructure and improve clinical outcomes assessment. This course was structured around the initiative within the New Pathways to Discovery theme: to advance nanomedicine, tissue engineering and regenerative medicine, and nuclear medicine using a variety of imaging techniques. The overarching learning goals of an imaging teaching unit early in the introductory biology curriculum are that students understand the integrated landscape of imaging

science requires preparation through multidisciplinary studies and that students are better prepared for this rapidly advancing field whether as consumers or future providers of molecular medicine ¹⁰.

Biological imaging is a technique that can bring quantification into biology education and offers new insights into biological structure and function through visualization of processes at the nano, molecular, cellular, and system-level scale. Innovations in biological imaging require translation from the laboratory bench to the undergraduate, graduate, and medical classroom. However, it is challenging to provide a context in the undergraduate curriculum that will highlight the multidisciplinary scope of imaging science and its integrated continuum with biology. Furthermore, instruction in biological imaging must be able to overcome student misconceptions (Table 1), to engage students with content that is interesting to all students but requires little background knowledge, and to encourage learning gains that are self-directed within an active learning framework. Here, we introduce a teaching unit on biological imaging developed for three one-hour class periods in an introductory undergraduate biology course, Ways of Knowing Biology (WOK). We show that our course design, which drew from NIH Roadmap initiatives, was significantly associated with student learning gains in understanding key concepts and developing core skills in imaging.

Method

Scientific Teaching

We used scientific teaching and backward design approaches to create this teaching unit^{11,12} for the WOK course. The main goal of WOK was to introduce seventy-five first-year college students to the breadth and methods of biology in a pass/fail environment. Supplemental materials for this imaging unit (including the syllabus, slides, pre/post questionnaires, Gillespie survey, SALG instrument, and imaging internet resources) are available through the Wisconsin Program for Scientific Teaching (WPST) Digital Library website [<http://scientificteaching.wisc.edu/materials/molecularbiology.htm>]. Specifically, we established learning goals in the context of the course and literature, we designed assessments that described student performances to indicate achievement of those goals, we developed activities to help students achieve the goals, and we evaluated the success of the materials by quantifying student learning gains.

Since imaging misconceptions develop where education does not keep up with technological advances¹³, we identified concepts in the imaging and biology literature that would help students integrate biology and modern imaging methods, that were reported as misconceptions, or that were recommended for incorporation into the biology curriculum^{2,13-16}. These concepts provided a basis for primary learning goals and specific learning outcomes (Table 1). We incorporated learning goals into student activities to delineate measurable criteria for assessment purposes. The activities to achieve these goals took the form of mini-lectures which progressed in scale from the nanoscale to system level scale, NIH Image J analysis, in-class NIH Roadmap activities, and the Gillespie, SALG, and Pre/Post assessments.

In-class activities and course content were developed and unified around the NIH Roadmap initiatives. More specifically, assessment activities in nanoimaging, molecular imaging, and medical imaging were drawn from nanomedicine, tissue engineering in regenerative medicine, and nuclear medicine. The course activities progressed in biological and imaging scale from nano through macroscale. As a nanoimaging activity, students quantified electron microscopy images of carbon nanofibers arranged into the shape of Bucky Badger (NanoBucky)¹⁷. The molecular imaging activities involved interpreting fluorescent microscopy images of fluorescently labeled bovine pulmonary artery endothelial cells (available in NIH Image J), enhanced green fluorescent protein (eGFP) expressing stem cells¹⁸, and macroscopic fluorescent images of the eGFP rabbit Alba¹⁹. System level activities required clinical evaluation of a NIH Positron Emission Tomography (PET) exercise²⁰ supplemented with a patient case study based on 18-FDG PET images collected at the UW-Madison Cyclotron and PET Research Center. In addition, students evaluated computed tomography (CT) images of Phineas Gage's skull²¹ and MRI images of human brain in two dimensional (2D) images, three dimensional (3D) virtual reality (VR), and physical 3D formats. Virtual reality models of human brain and Phineas Gage's skull were generated and then printed in 3D as physical stereolithograph models at the UW-Madison New Media Center²². We viewed the VR brain and skull models in the virtual reality markup language (VRML) format with VRMLView (Systems in Motion; Norway).

To provide a better understanding of the actual lessons and student activities, we will provide an in depth description and active learning approach for the NanoBucky exercise.

Students were asked to bring in their laptops for this in-class activity. Students organized themselves into groups and used the NIH image J applet [<http://rsb.info.nih.gov/ij/applets.html>] which did not require prior installation but did require an internet enabled classroom. Students were provided with NIH Image J instructions to use NIH image J applet software from the Image J Documentation Wiki [<http://imagejdocu.tudor.lu/imagej-documentation-wiki>]. Students actively learned how to initiate the software, load an image, and analyze the height of NanoBucky. This activity familiarized students with nanomaterials that are being developed as part of the NIH Roadmap initiative and enabled students to understand the nanoimaging scale by calculating the height of NanoBucky based on the scale legend provided in the image [<http://hamers.chem.wisc.edu/research/nanofibers/index2.htm>]; to understand how imaging provided scientists with ways of viewing and assessing nanomaterials and, in the future, nanodevices for quality control; and to recognize that imaging is a quantitative tool in biology by observing, measuring, and interpreting biological images. The NanoBucky activity was completed in about 30 minutes.

All the course activities aligned directly with the learning goals (Table 1) and exemplified how biological images advance the NIH Roadmap initiatives. The relationship between biological scale and imaging scale was exemplified by nanoimages of NanoBucky, molecular images of fluorescent stained endothelial cells available in Image J²³, eGFP labeled human embryonic stem cells, the eGFP rabbit Alba, MRI images of brain, and CT images of Phineas Gage's skull. Students could understand that imaging provides ways of knowing biological structure and function by interpreting the

intensity of eGFP labeled gene expression during human embryonic stem cell differentiation and images of brain function using 18-FDG PET and functional MRI. The use of MRI and CT images to create stereolithograph models of brain and skull, respectively, exemplified the broader application of images and novel visualization approaches. In addition, students could develop their evaluation skills by comparing 2D images, 3D VR, and stereolithograph models in terms of visual information quality and biological utility. Computer skills could be developed by using the NIH Image J software to quantify NanoBucky's height and intensity and to apply this knowledge when interpreting fluorescently labeled endothelial and stem cells. With NanoBucky, students could gain an understanding that nanoimaging of nanomaterials influences the development of nanodevices which have applications in nanomedicine; that molecular imaging of human embryonic stem cells can inform tissue engineering which can advance regenerative medicine; and that PET imaging with radioactive tracers can influence nuclear medicine.

Diversity

This course addressed diversity in learning styles, but it was much more challenging to simultaneously address gender, cultural, and socioeconomic diversity. Diversity in students' educational backgrounds was accounted for in this teaching unit by incorporating multiple modes of teaching and assessment activities. Mini-lectures provided background information that minimized discrepancies in educational background. Audiovisual aids were used to clarify concepts in an interesting and understandable fashion. The video, "Powers of Ten," introduced the concept of scale and

a movie clip of the “Hulk” illustrated fluorescence. Using NIH Image J, students quantified images and observed how images can be utilized using hand held models of a brain and of Phineas Gage’s skull. We accounted for different test taking styles by using a variety of formats to assess learning gains: oral discussion, written answers, online and in-class surveys, and online pre and post course assessments. To address gender and cultural diversity, the significant contributions of minority and female scientists from various countries were specifically incorporated into the mini-lectures. For example, the work of the Polish, female chemist and physicist Marie Curie and English biophysicist Rosalind Franklin were highlighted for the contributions to nuclear medicine and genetics, respectively. To address socioeconomic diversity, we built upon the common campus culture held by all UW-Madison students and incorporated the work of scientists who made contributions to imaging and who were either from the state of Wisconsin or were affiliated with UW-Madison. For example, we highlighted the work of Raymond Damadian, who attended UW-Madison on a Ford Foundation Scholarship as an undergraduate and went on to invent and develop the first MRI scanner, Indomitale.

Active Learning

Students were engaged in learning actively in multiple ways. For example, visualization of biological processes using images engaged students in scientific thinking. By viewing and analyzing nanoimages, molecular images, and medical images, students could gain an understanding of the relationship between biological and imaging scale and realize that biological science and imaging science are related by scale. By viewing and analyzing images with fluorescent probes and radionuclear markers, students were

encouraged to understand how images provided ways of knowing biological structure and function. When introducing different imaging topics, we selected interesting images that students could observe, quantify, and interpret. As examples, students used a freely available software program, NIH Image J²³, to quantify images and evaluated image visualization and utility by comparing 2D slices of brain and of Phineas Gage's skull with 3D VR and printed 3D stereolithograph models. Through introduction of NIH Image J analysis software, students could extend their conceptual understanding of imaging analysis into a technical skill using real data. By evaluating images, students understood how biological images advanced nanomedicine, regenerative medicine, and nuclear medicine and contributed to the NIH Roadmap initiatives.

Several varieties of images were used in this course module and are described in Table 1. These can be categorized into two dimensional (2D) and three-dimensional (3D) images. Two dimensional images included (1) nanoimages of NanoBucky; (2) molecular images of fluorescent probe intensity from (a) bovine pulmonary artery endothelial cells and (b) eGFP labeled human embryonic stem (ES) cells; (3) system-level images of (a) the eGFP rabbit Alba, (b) MRI images of human brain, and (c) CT image of Phineas Gage's skull. The 2D images of human brain and Phineas Gage's skull were rendered into a computerized, three-dimensional volume in the VRML format and displayed to students through a projector. In addition, the computerized VRML models were printed in three dimensions as physical stereolithograph models which students held in their hands.

Assessment

Through these activities, we were able to evaluate student learning gains summatively by comparing the results of a pre and post survey and formatively throughout the teaching unit by using assessments for active learning activities, the Gillespie scale²⁴ for rating visual information quality, and the Student Assessment of Learning Gains (SALG)²⁵ which is an instrument developed by Elaine Seymour at the University of Wisconsin Center for Educational Research to assess student perception of learning gains as a function of course design and delivery²⁶.

Summative pre and post assessments showed that the learning gains in the unit were achieved. Students were asked to define biological scale, list and explain three imaging modalities, and quantitatively interpret X-ray images of lung with and without pneumonia. To assess learning gains, a rubric was established to code responses with a three point scoring scale with 1=incorrect/unknown, 2=a basic understanding, 3=an advanced explanation using quantitative terminology.

In-class assessments included group discussions, activities, and evaluations that indicated to teachers, as well as students, how well concepts were understood. Here we report technical gains in the ability to use the NIH Image J program to quantify biological images in partial fulfillment of our learning goals. Responses from student groups were collected and compared to repeated measures by the instructor. Student proficiency with Image J software was assessed with a t-score. We used the Gillespie rating scale²⁴ to assess students' perception of biological image models relative to a baseline model to

examine whether students made an association between image visualization and utility in biology. In our assessment, 2D images were used as the baseline for assessing VRML and stereolithograph models for the quality of their visual information and for their biological utility. Gillespie ratings were coded on a 4-point Likert scale [1=Inferior, 2=Similar/Equivalent, 3=Superior (similar information more rapidly assimilated), 4=Superior (additional information provided)]. Students also completed our implementation of the SALG instrument which has been successfully administered to assess student learning^{27,28}. Responses to the SALG were anonymous and collected online using Zoomerang software (MarketTools, Inc.; San Francisco, CA). Categorical responses were coded on a 5-point Likert scale [0=Not applicable; 1=Not at all, 2=A Little, 3=Somewhat, 4=A Lot, 5=A Great Deal]. To clarify the choice of Likert scale coarseness, a three point scale was arbitrarily used for pre-post assessment, whereas the Gillespie and SALG ratings used the historically recommended four and five point scale, respectively. Questions in the student gains category focused on the gains in learning goals for this imaging course, and the course design questions were centered on engagement activities and course content. The association between student gains and course design was used to assess alignment between student learning gains and the activities which were developed to help students achieve the learning goals²⁹. The Gillespie associations and SALG associations were determined using polychoric³⁰ correlation software in R³¹. The polychoric correlation, ρ , is a useful statistic to understand associations in categorical data and is preferred to the Spearman correlation because the discretizing latent variable thresholds are estimated³². Two-tailed significance was assessed after a ρ to t conversion on $N-2$ degrees of freedom. A

corrected p-value ≤ 0.05 was considered significant. Analyses were conducted in R version 2.4.1 and SPSS version 14.0 (Chicago, IL).

Results

Summative Assessments

The distribution of the pre and post assessment learning scores for biological scale, imaging tools, and image interpretation are displayed in Figure 1. The Wilcoxon signed rank test on pre and post assessment learning scores indicate that students made significant learning gains in interpreting images [$Z=-2.81$; $p=0.005$; $n=34$], distinguishing imaging modalities [$Z=-3.70$; $p=0.00$; $n=36$], and understanding biological scale [$Z=-4.64$; $p=0.00$ (2-tailed); $n=35$].

In-class Assessments

Students quantified the height of Nanobucky using NIH Image J (Figure 2). Groups of students reported Nanobucky's height in micrometers (Mean $\pm$ SEM: $31.9 \mu\text{m} \pm 0.49$, $n = 3$) and pixels (Mean $\pm$ SEM: $342.9 \text{ pixels} \pm 2.0$). When compared to instructor estimates for Nanobucky's height in micrometers (Mean $\pm$ SEM: $32.2 \mu\text{m} \pm 0.25$, $n=10$) and pixels (Mean $\pm$ SEM: $337.7 \text{ pixels} \pm 2.7$, $n=10$), the height estimates in micrometers [$t(11)=-0.52$; $p=0.61$; unpaired, two-tailed] or pixels [$t(18)=1.56$; $p=0.14$; unpaired, two-tailed] were not significantly different between the students and instructor.

The Gillespie ratings indicate that students perceived a strong association between the quality of visual information contained in images and their biological utility (Figure 3a).

After multiple comparison correction (Figure 3b), significant associations were positive except for a negative association between the visual information contained in the stereolithograph of Phineas' skull and the general utility of VRML models in biology (Table 2). This indicates students made evaluations across the different model types and identified a tradeoff in utility between VRML and stereolithograph models for biological visualization. This may be due to the instructor demonstrating that the virtual reality model permitted navigation of structures internal to the skull as students took the perspective of the tamping iron that passed through the Phineas Gage skull when the instructor used the zooming tool to navigate within the cranial vault from superior to inferior and vice versa. Students could not physically navigate the stereolithograph model in the same way which may have produced the negative polychoric correlation.

The SALG instrument identified aspects of course design that contributed to student learning gains (Table 3). Significant associations between learning gains and course design (Figure 4a) after correcting for multiple comparisons (Figure 4b) were positive and involved the primary learning goals that were key features of our backward design (Table 1).

Discussion

Our teaching unit used the NIH Roadmap as the central feature of course context and spanned a wide spectrum of imaging scales from nanoimaging to whole organisms. We used a scientific teaching approach to align learning goals with assessments and activities (Table 1). In this unit, we measured student learning through (1) pre and post assessment

of gained knowledge in biological scale, in a variety of imaging tools, and in the interpretation of biological images; (2) assessment activities drawn from the NIH Roadmap initiative; (3) the Gillespie rating scale to quantify student associations of biological utility with the quality of visual information contained in images; and (4) the SALG instrument which allowed us to quantify associations between course design and student learning.

Summative measures of student learning gains indicated that our learning goals were achieved. Based on pre and post assessment, students made significant gains in understanding biological scale, distinguishing among a variety of imaging modalities, and interpreting images (Figure 1). These results respectively aligned with our learning goals which were for students to understand the continuity of biological and imaging scale, the quantitative utility of imaging across a wide range of biological scales, and the importance of images for interpretation of structure and function.

In-class metrics indicated that our activities contributed to student learning gains. Students demonstrated proficiency with quantifying images using NIH Image J during the NanoBucky exercise (Figure 2). Incorporation of Image J into the course design contributed positively to gains in using imaging software [$r=0.71$; $p=0.00$; $n=37$] and to various aspects of student learning (Figure 4). Using the Gillespie rating scale, students evaluated and perceived a strong association between the quality of visual information contained in biological images and the utility of these images within model types and an inverse association across model types (Figure 3, Table 2). The SALG instrument

identified a strong association between the overall course and student gains in our learning goals including recognizing biological scale [$r=0.84;p=0.00;n=37$] and understanding structure and function from images [$r=0.77;p=0.00;n=37$]. Furthermore, the NIH Roadmap context in our course design was associated with gains in nanoimaging [$r=0.61;p=0.01;n=37$], molecular imaging [$r=0.75;p=0.00;n=37$], system level imaging [$r=0.71;p=0.00;n=37$], biological imaging tools [$r=0.56;p=0.04;n=37$], and using imaging software [$r=0.61;p=0.01;n=37$]. Interestingly, student learning gains were not strongly associated with instructor evaluations (data not shown). This suggested that student learning was self-directed and took place within an active learning framework as intended by our course design.

The current module would be useful as an introductory imaging unit in an introductory biology course. In addition, the NIH Roadmap initiatives we used to design this module provide a useful framework to introduce imaging concepts into the undergraduate, graduate, or medical school curriculum. Faculty with an imaging background would be best suited to teach an imaging module; however, those without an imaging background may want to separate the nanoscale, molecular, and medical imaging subunits and build upon the subunit most applicable to their overall course. The module in its current form could be adapted to a classroom of any size provided that computers and internet access were made available. Students were provided with an overview of how the imaging equipment actually works and collects data. For example, with GFP, students were given a historical and practical overview of GFP discovery, the physics of fluorescence, and the meaning of excitation and emission spectra. Since this was an introductory course, we

provided an overview of imaging quantification using images previously acquired with, for example, scanning electron microscopy and fluorescent imaging microscopy. Future implementations of this module as a full length course should incorporate finer details of image quantification by focusing on capture methodology including the importance of different exposures and neutral density filters for image correction. Future assessments using this course design may want to compare the utility of in-class activities to a purely lecture based curriculum among two cohorts comprised of those who attended lecture and those who attended lecture and participated in the active learning activities to confirm the utility of the activities we developed based on the NIH Roadmap.

By using a scientific teaching approach and the NIH Roadmap to guide activities, this course made positive contributions to student gains in understanding that imaging is a useful way of knowing biology. This conclusion was based on summative pre-post assessments and in-class assessment using activities, Gillespie associations, and SALG associations. Early exposure to an imaging teaching unit addressed several misconceptions and may generate interest in advanced imaging education. Both the multidisciplinary nature of imaging and the NIH Roadmap context make this teaching unit accessible not only to students of biology but also physiology, engineering, mathematics, and physics. This teaching unit was limited by the number of class periods in the WOK course but may serve as a useful framework on which to build a longer, more in depth course.

Accessing Materials

Materials for this imaging unit (including the syllabus, slides, pre/post questionnaires, Gillespie survey, SALG instrument, and imaging internet resources) are freely available through the Wisconsin Program for Scientific Teaching (WPST) Digital Library website [<http://scientificteaching.wisc.edu/materials/molecularbiology.htm>].

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Tables:**Table 1: Alignment of Misconceptions, Learning Goals, Learning Outcomes, and Activities.****Misconceptions** *Students mistakenly think that:*

-Biological science and imaging science are unrelated	-Biological images can only reveal structure but not function	-Biological images are pretty pictures but not quantifiable	-The impact of imaging techniques on biology and medicine is limited

Learning Goals *Students will:*

-Conclude that the scale of biology studied must match the scale captured by an imaging device	-Appraise the utility of imaging tools to examine structure and function of biological processes	-Discover that imaging is a quantitative tool used to measure data across a wide range of biological scale	-Generalize that biological images hold prominent places in advancing biology and medicine and contribute to the NIH Roadmap initiative
-Develop interpretation skills by examining different imaging formats and multiple biological scales using real data	-Evaluate imaging methods that provide ways of knowing structure and function	-Develop computer and computation skills	-Understand that imaging is an integrated multidisciplinary field

Learning Outcome *Having achieved the learning goal, students will be able to:*

-Summarize the relationship between biological and imaging scale	-To predict the rabbit Alba's color from fluorescence emission of eGFP	-Proficiently use NIH Image J software to calculate Nanobucky's height and image intensity	-Value that (1) nanoimaging advances the development of nanodevices and nanomedicine
-Accurately interpret: (1) nanoimages of NanoBucky (2) molecular images of fluorescent probe intensity from (a) bovine pulmonary artery endothelial cells (b) eGFP labeled human embryonic stem (ES) cells (3) system-level images of (a) eGFP rabbit Alba (b) MRI image of brain (c) CT image	-Evaluate the intensity of eGFP labeled ES cells to understand ES function	-Apply ImageJ principles to: (1) Interpret the intensity of eGFP labeled cells (2) Quantify and localize brain	(2) molecular imaging advances tissue engineering and regenerative medicine (3) system-level imaging (eg, PET) advances nuclear medicine
	-Assess functional brain		-Identify disease using

of Phineas Gage's skull	activity using	function using	X-ray images of lung
-Compare 2D, 3D VR, and	18-FDG PET	PET images	with and without
stereolithograph models of	measures of		pneumonia and PET
brain and Phineas Gage's skull	glucose		images of brain with
and contrast them for visual	metabolism		and without a tumor
information quality and	-To explain and		
biological utility	give examples		
	of imaging tools		

Activities *Students engage in the following activities to achieve the goals:*

Mini-lectures
ImageJ Analysis
In-class NIH Roadmap Activities
Gillespie Assessment
SALG Assessment
Pre and Post Instruction Assessment

Table 2: Significant Gillespie Rating Associations Between Visual Information and Biological Utility ($p \leq 0.05$, corrected). Students gained a positive association between the visual information contained in images and their biological utility. The negative association indicates students evaluated these models and recognized a tradeoff between stereolithograph visual information (Phineas) and VRML utility.

Question (VI, BU)	Visual Information	Biological Utility	Polychoric Correlation	df	p-value (corrected)
1,2	Brain VRML	VRML	0.61	48	0.00
3,2	Phineas VRML	VRML	0.57	48	0.00
4,2	Phineas Stereolithograph	VRML	-0.44	48	0.02
2,3	Brain Stereolithograph	Stereolithograph	0.65	48	0.00
4,3	Phineas Stereolithograph	Stereolithograph	0.60	48	0.00

Table 3: Questionnaire results reporting mean, standard deviation, and distribution of responses in each Likert-scaled category of course design (CD) and student gains (SG). Selected CD and SG topics were respectively based on active learning activities and learning goals.

Question CD	Topic Course Design, CD	Mean	SD	1	2	3	4	5
1	Mini Lectures	3.76	0.86	0	2	13	14	8
2	Group Activities	3.05	0.88	1	7	21	5	3
3	Image J	3.08	0.92	0	11	15	8	3

4	Printed 3D Models	3.57	0.80	0	3	14	16	4
5	Virtual 3D Models	3.95	0.74	0	1	8	20	8
6	The Topics Covered	3.84	0.76	0	1	11	18	7
7	Overall Course	3.70	0.74	0	1	14	17	5
8	Course Emphasis that Imaging Provides a Way of Knowing Biology at Different Scales	4.19	0.70	0	0	6	8	13
9	NIH Roadmap	3.54	0.93	0	5	13	13	6
SG	Student Gains, SG	Mean	SD	1	2	3	4	5
1	Nanoimaging	3.70	0.94	0	4	11	14	8
2	Molecular Imaging	3.73	0.84	0	3	10	18	6
3	System Level Imaging	3.49	0.84	0	4	15	14	4
4	Imaging as a Quantitative Tool	3.76	0.86	0	2	13	14	8
5	Biological Scale	3.76	0.86	0	3	10	17	7
6	Biological Imaging Tools	3.73	0.77	0	0	17	13	7
7	Using imaging software	3.73	0.80	0	1	15	14	7
8	Interpreting Imaging Studies	3.59	0.69	0	2	13	20	2
9	Recognizing Biological Scale in Images	3.70	0.81	0	2	13	16	6
10	Quantifying Images	3.46	0.84	0	3	19	10	5
11	Importance of this Field	3.92	0.76	0	0	12	16	9
12	Understanding that Imaging offers New Insights into Biological Structure and Function	3.86	0.86	0	1	13	13	10
13	Understanding that Imaging Impacts Medicine	4.11	0.84	0	1	8	14	14
14	Understanding that Future Patients will Benefit from Ongoing Imaging Research	4.08	0.89	0	1	10	11	15
15	Imaging is an Integrated, Multidisciplinary Field	3.70	0.88	0	3	12	15	7

Figures:

Figure 1: Summative Assessment of Image Interpretation, Imaging Tools, and Biological Scale. Histogram of responses to pre and post course assessments focusing on learning goals in which students were asked (a) to interpret images of healthy and unhealthy lungs (n=34), (b) to explain and give examples of imaging tools (n=36), and (c) to define biological scale (n=35). The change in distributions between the pre and post course assessment indicated that students made positive learning gains toward our learning goals which were for students to understand the prominent place of quantitative imaging in medicine, to recognize the scope of imaging tools and their application to examine structure and function, and to understand the meaning of biological scale in imaging.

Figure 2: In-class Assessment of Image Quantification

Measurements of NanoBucky's height using ImageJ were reported as (a) micrometers (n=3 for students; n = 10 for instructor) or (b) pixels (n=10 for students; n = 10 for instructor). Students demonstrated proficiency in using imaging software because the height estimates in micrometers [$t(11)=-0.52$; $p=0.61$; unpaired, two-tailed] or pixels [$t(18)=1.56$; $p=0.14$; unpaired, two-tailed] were not significantly different between the students and instructor. This satisfied our learning goal for students to recognize imaging as a quantitative tool.

Figure 3: Gillespie Associations of Visual Information with Biological Utility

(a) Polychoric correlations of biological utility (1=2D, 2=VRML, and 3=Stereolithograph) with visual information (1=Brain VRML, 2=Brain Stereolithograph, 3=Phineas VRML, 4=Phineas Stereolithograph) were both positive and negative and indicated that students evaluated VRML and stereolithograph models. (b) P-values of significance ($p \leq 0.05$, corrected for multiple comparisons) for polychoric correlations in Figure 3a. Students made positive associations between the biological utility of these models with the quality of their visual information. Only the Phineas stereolithograph had a significant negative association with VRML utility which indicated that students made comparisons across VRML and stereolithograph model types and recognized a tradeoff between visual information and biological utility.

Figure 4: SALG Association of Course Design with Learning Gains

(a) Polychoric correlations of course design with student gains in Table 3 were positive and indicated a positive alignment between active learning activities and learning gains
(b) P-values of significance ($p \leq 0.05$, corrected for multiple comparisons) for polychoric correlations in Figure 4a. Among other positive associations, the NIH Roadmap context in our course design contributed to positive gains in understanding nanoimaging [$r=0.61$; $p=0.01$; $n=37$], molecular imaging [$r=0.75$; $p=0.00$; $n=37$], and system level imaging [$r=0.71$; $p=0.00$; $n=37$].

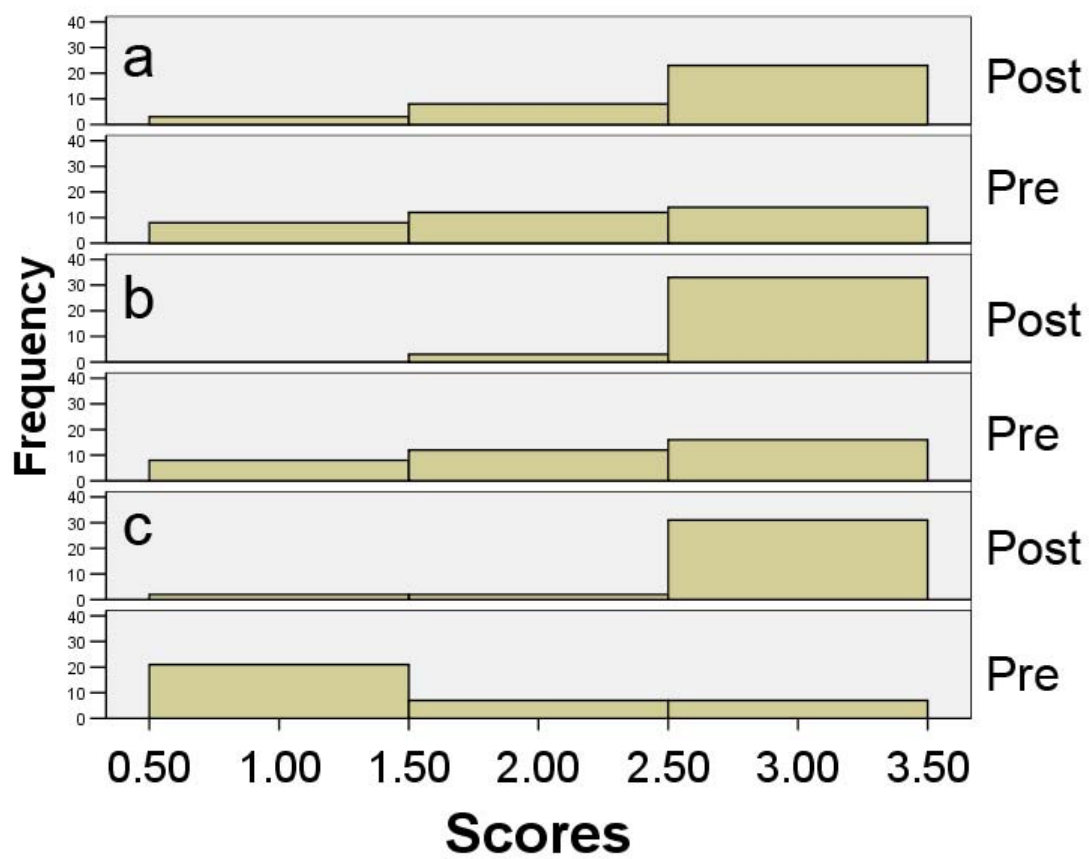


Figure 1

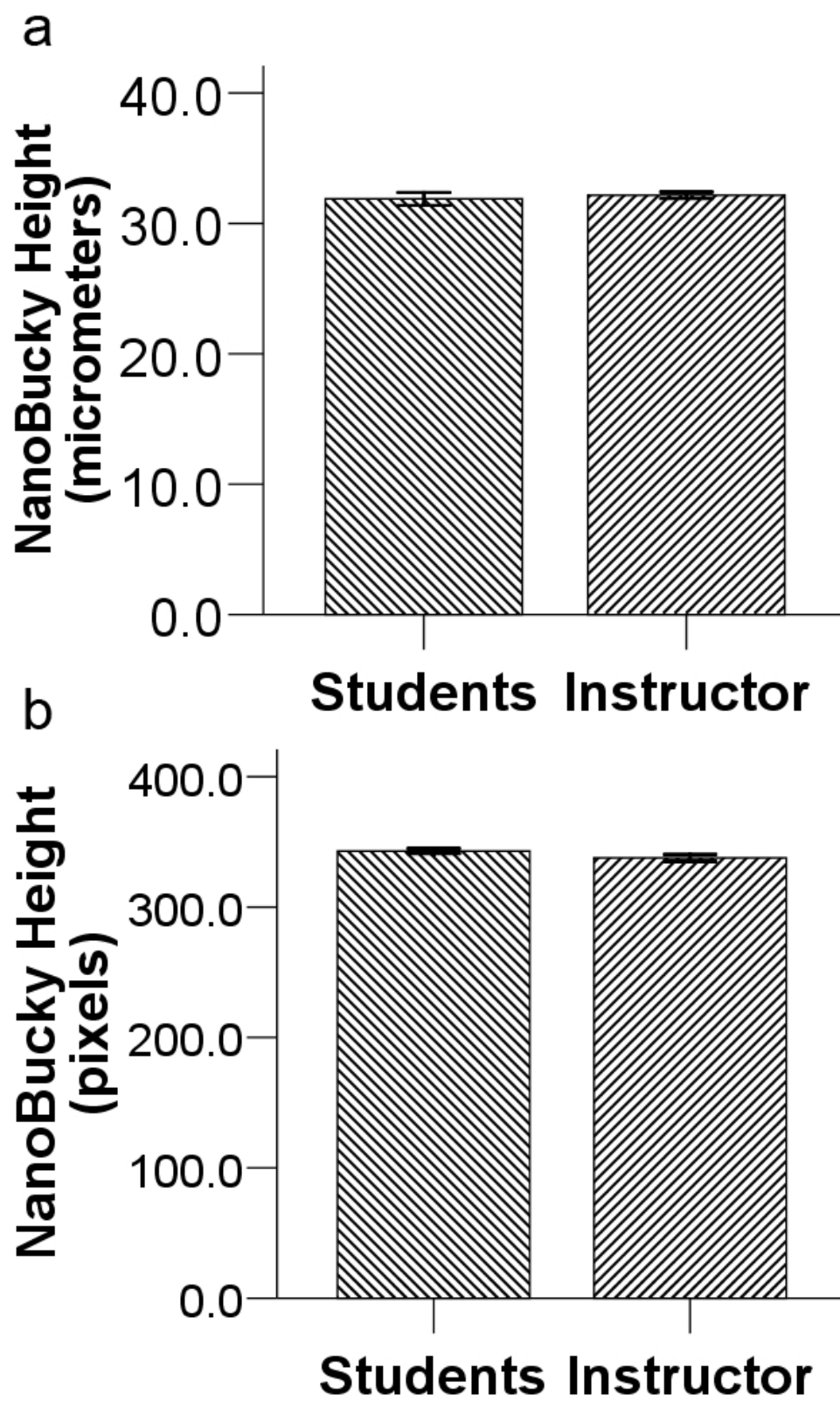


Figure 2

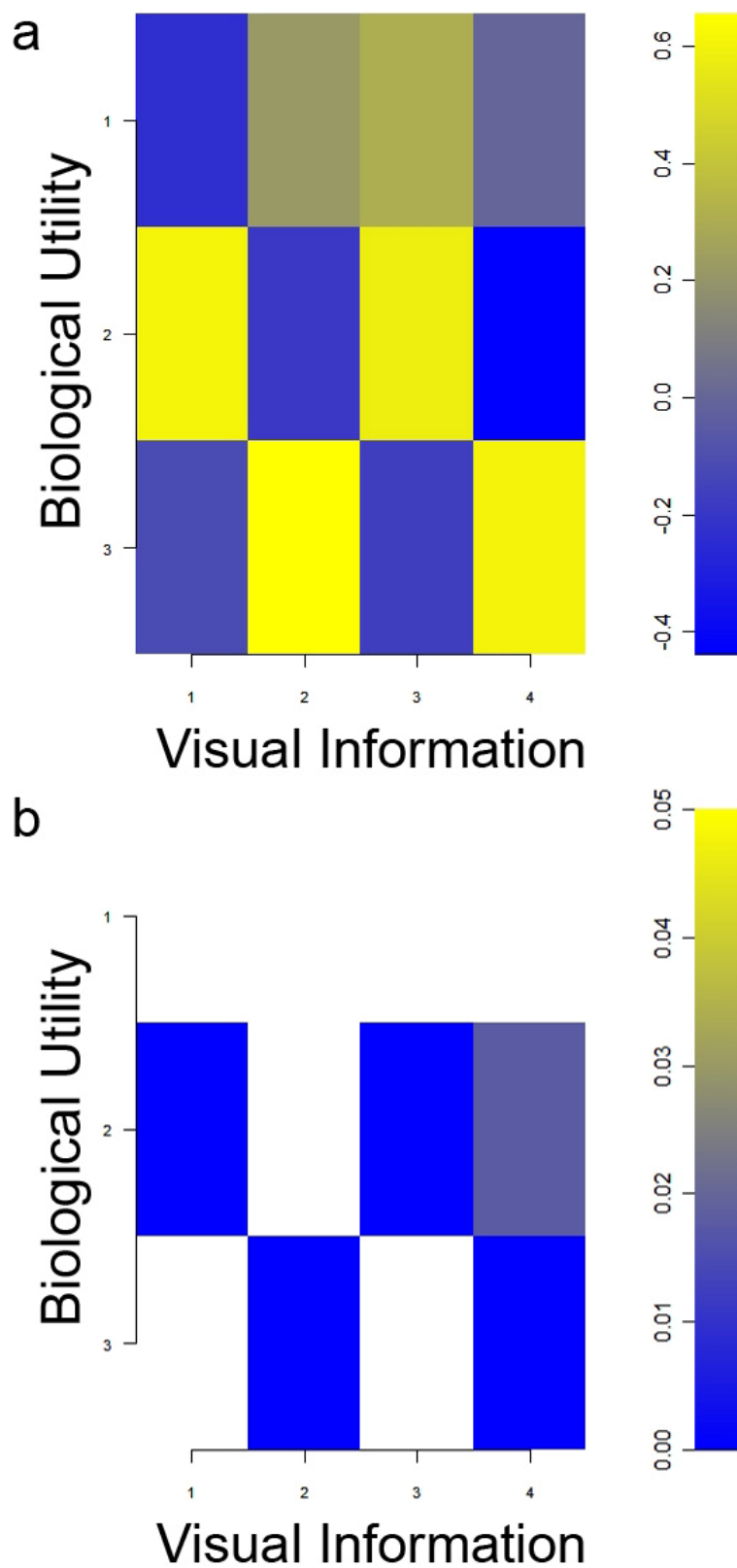


Figure 3

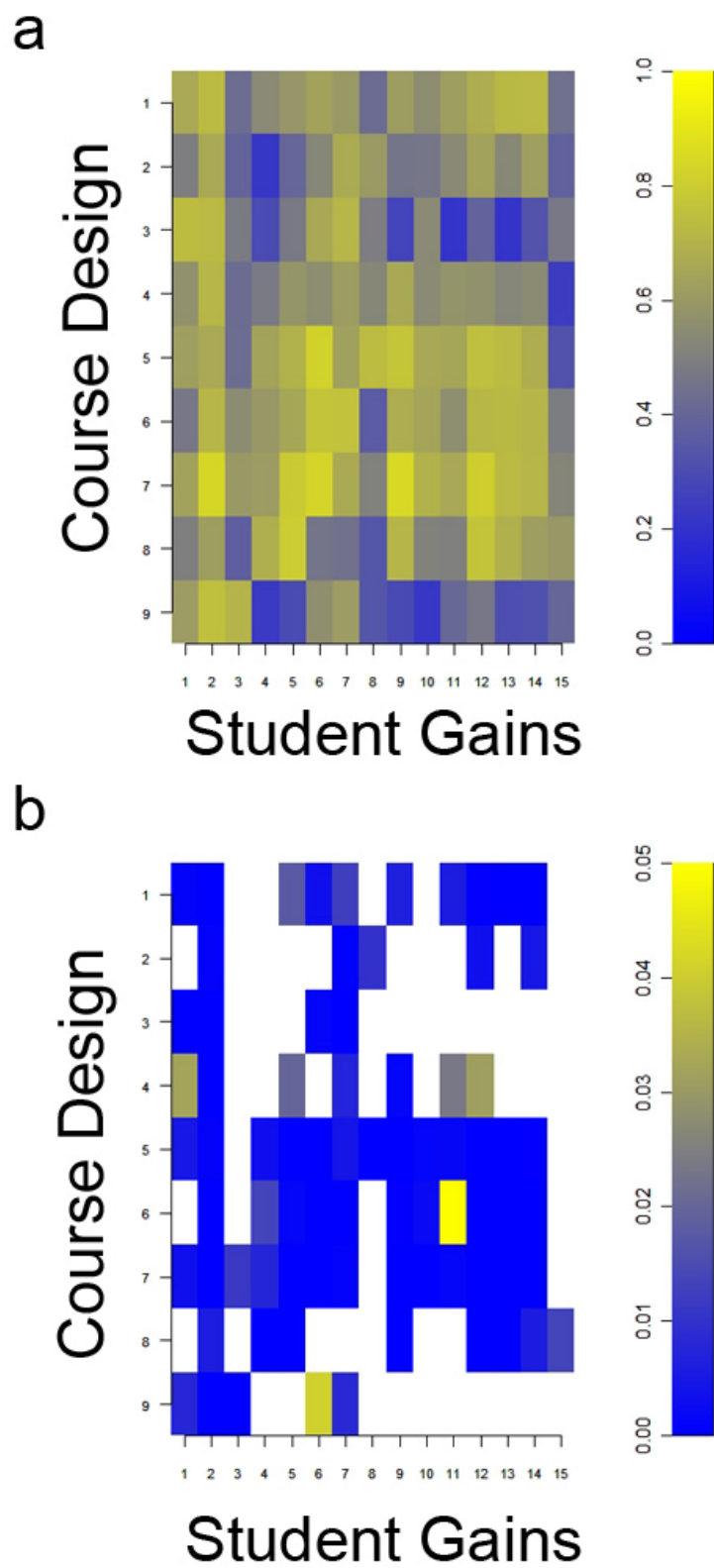


Figure 4