Int. J. Devl Neuroscience 29 (2011) 489–494

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Insular volume reduction in fragile X syndrome

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ARTICLE INFO

Article history:
Received 31 December 2010
Accepted 22 January 2011

Keywords:
Insula
Fragile X syndrome
Morphometry
Anxiety

ABSTRACT

Fragile X syndrome (FraX) is the most common form of inherited mental deficit and is caused by mutations of the *Fragile X Mental Retardation 1* (*FMR1*) gene on the X chromosome. While males and females with the full *FMR1* mutation are affected differently because the disorder is X-linked, both suffer from varying degrees of cognitive impairment, attention deficits and social anxiety. The insula is a sensory integrative region that has been increasingly suggested as a critical area involved in anxiety manifestation.

The current study was designed to examine possible changes in insular volume in FraX compared to age- and gender-matched typically developing healthy controls (HC) as well as age-, gender-, and intelligence-matched developmentally delayed controls (DD). An established native-space, manual morphometry method was utilized to quantify total and regional insular volumes using structural magnetic resonance imaging.

Total, anterior and posterior insular volumes were found to be reduced in FraX compared to both HC and DD. The current data add to a growing literature concerning brain abnormalities in FraX and suggests that significant volume reduction of the insula is a component of the FraX neuroanatomical phenotype. This finding also provides an intriguing potential neural correlate for hyperarousal and gaze aversion, which are prominent behavioral symptoms of FraX.

1. Introduction

Fragile X Syndrome (FraX) is a genetic disorder caused by mutations of the *FMR1* gene on the X chromosome, and is the most common form of inherited mental deficit, occurring in 1 of every 2000–6000 live births (Gustavson et al., 1986; de Vries et al., 1997). This single gene mutation alters the course of typical brain development and significantly impacts cognitive abilities as well as behavior. Cognitive dysfunction in fragile X syndrome includes deficiencies in working memory, executive function, mathematical, and visuospatial abilities (D'Hulst and Kooy, 2009; Bennetto and Pennington, 1996; Hagerman, 2002; Kemper et al., 1988). Speech and communication skills are also adversely affected in males with fragile X syndrome, who often exhibit autistic-like behavior including aversion to eye gaze, perseverative speech and behavior, shyness, social anxiety, and hand flapping (Merenstein et al., 1996; Tsiouris and Brown, 2004). Due to the fact that the syndrome is X-linked, females often display a milder phenotype of the disorder than do males. Heterozygous females typically exhibit not only mild intellectual disability to normal cognitive function, but also often manifest attention deficits, anxiety and difficulties with socialization (Eliez et al., 2001; Riddle et al., 1998).

Structural magnetic resonance imaging (MRI) studies of FraX neuroanatomy have noted a variety of anatomical variations in both males and females with the full *FMR1* mutation (i.e., ≥200 CGG repeats). Limbic structures, such as the hippocampus and amygdala, have been shown to have abnormal volume and/or morphology in human males and females with the full *FMR1* mutation compared to age- and gender-matched controls (Mazzocco et al., 1995; Hazlett et al., 2009; Gothelf et al., 2008; Jäkälä et al., 1997), and in the *FMR1* knockout mouse (Grossman et al., 2006). The hippocampus is a structure commonly associated not only with learning and memory, but also contributes to visuospatial processing, a cognitive function particularly affected in FraX (Freund and Reiss, 1991). Reduced amygdalar volumes may also play a role in

Abbreviations: FraX, Fragile X syndrome; *FMR1*, Fragile X Mental Retardation 1; MRI, Magnetic resonance imaging; HC, typically developing control subjects; DD, developmental delay; IQ, intelligence; WISC, Wechsler Intelligence Scale for Children; WAIS, Wechsler Adult Intelligence Scale; BIJ, *BrainImageJava*; FSL, FMRIB Software Library; FAST, FMRIB Automated Segmentation Tool; ROI, region-of-interest; ANOVA, analysis of variance; ANCOVA, analysis of covariance; MANCOVA, multivariate analysis of covariance.

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0736-5748/$36.00 © 2011 ISDN. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijdevneu.2011.01.003

FraX phenotype as *fmr1* knockout mice show an abnormal conditioned fear response (Paradee et al., 1999). Increased caudate nucleus volume, a region important in filtering and organizing information sent to the cortex has been detected repeatedly in both males and females with FraX (Eliez et al., 2001; Hoeft et al., 2008; Gothelf et al., 2008; Hazlett et al., 2009). The superior temporal gyrus, which includes primary and secondary auditory cortex, is decreased in size in FraX individuals (Reiss et al., 1994; Gothelf et al., 2008). Hypoplasia of the cerebellar vermis was also confirmed in several studies (Mostofsky et al., 1998; Reiss et al., 1991a,b). The cerebellar vermis has connections with both the hippocampus and amygdala, and anatomical variations in this region were hypothesized to be related to hyperactivity, attention deficits, tactile defensiveness and repetitive behaviors seen in FraX (Hessl et al., 2004).

The insula is a sensory integrative region (Craig, 2003) that has connections with amygdala (Mufson et al., 1981), hippocampus, superior temporal gyrus (Mesulam and Mufson, 1982a,b), and striatum (Chikama et al., 1997). Among its diverse functional roles, the insula appears to be important for the representation of aversive experiences, especially fear and anxiety (Paulus and Stein, 2006). One neurocircuitry model of anxiety features a central role for the anterior insula, and proposes that persons with high anxiety sensitivity perceive a heightened interoceptive prediction signal (Paulus and Stein, 2006). In this model, individuals who are prone to anxiety show an altered interoceptive prediction signal, i.e., they experience an augmented signaling of the difference between the observed and expected body state. Such individuals with high anxiety sensitivity would therefore be more prone to specific kinds of anxiety disorders. There is evidence of altered insular functioning in patients with anxiety disorders, including obsessive–compulsive disorder, posttraumatic disorder (Rauch et al., 1997), simple phobia (Rauch et al., 1997; Wright et al., 2003), panic disorder (Malizia et al., 1998), generalized anxiety disorder (Hoehn-Saric et al., 2004), and social anxiety (Lorberbaum et al., 2004).

The objective of the current study was to examine potential insular morphometric changes in FraX compared to age- and gender-matched typically developing controls as well as age-, gender-, and intelligence-matched developmentally delayed controls. To determine whether insular volume reductions in FraX can be attributed to specific elements of this genetic disorder rather than intellectual disability in general, it was important to compare findings from individuals with FraX to both control groups. The insula was targeted as a region of interest due to its role in anxiety and aversive behaviors, which are prominent behavioral features of FraX, as well as its connectivity to other brain regions already shown to be anomalous in FraX. Furthermore, previous studies using voxel-based morphometry suggest that insular gray matter volumes are reduced in individuals with FraX (Hoeft et al., 2008; Gothelf et al., 2008). Building on these previous findings, a native-space, manual morphometry method (Cohen et al., 2010) was utilized to quantify total and regional (i.e., anterior and posterior volumes) insular volumes in adolescents and young adults with FraX using structural MRI. Because of its proposed role in anxiety disorders (Paulus and Stein, 2006) and previous findings of insular volume reduction related to specific phobias (Cohen et al., 2010), it was expected that insular volumes would be reduced in FraX compared to both healthy and developmentally delayed controls. Furthermore, based on the model proposed by Paulus and Stein (2006), it was expected that the anterior insula would be more anomalous than posterior insula because of the specific role attributed to the anterior insula in anxiety manifestation. However, the methods used here were designed to be equally sensitive to possible volume changes in the anterior and posterior insula by direct measurement.

2. Methods

2.1. Subjects

Five female (15.3 ± 3.3 years) and six male (16.3 ± 2.8 years) participants previously diagnosed with FraX were recruited through advertisements in national and local FraX newsletters and referrals from physicians. Five female (16.5 ± 3.7 years) and three male (13.3 ± 2.6 years) typically developing healthy control subjects (HC) were recruited through advertisements in the local community. Six additional female (16.4 ± 2.3 years) and five male (16.0 ± 3.9 years) subjects with non-specific (non-FraX) developmental delay (DD) were also recruited as a developmental control group. The HC and DD subjects were recruited from the San Francisco/San Jose region. The groups were matched for gender and all groups were well matched for age. FraX subjects and DD subjects were further group matched for intelligence (IQ). Intelligence for each subject was measured by Wechsler Intelligence Scale for Children (WISC), except for two control females and three FraX females. These five participants were 17 years of age or older and were administered the Wechsler Adult Intelligence Scale (WAIS). The full *FMR1* mutation (i.e., ≥200 CGG repeats) was confirmed in all FraX subjects by standard DNA (Southern Blot) analysis. Written informed consent was obtained from all participants, and, when applicable, their parents or guardians. The human subjects review board at Stanford University School of Medicine, Stanford, CA, approved all protocols.

2.2. Image acquisition and processing

Volumetric MR images were acquired for each subject at Stanford University on a GE-Signa 1.5 T scanner (GE Imaging Systems, Milwaukee, WI, USA). High-resolution T1-weighted coronal brain images were acquired using the following 3D volumetric radio frequency spoiled gradient echo pulse sequence parameters: TR = 35, TE = 6 ms, flip angle = 45°, 24 cm field of view, 124 slices in the coronal plane, 256 × 192 matrix, acquired resolution = 1.5 × 0.9 × 0.9. Brain images were imported, aligned and skull stripped in *BrainImageJava* (BIJ) (Ng et al., 2001) (CIBSR.stanford.edu/tools), a freeware program developed in the Center for Interdisciplinary Brain Sciences Research. Total brain volume was calculated using BIJ and FMRIB Software Library (FSL) 4.1. The FMRIB Automated Segmentation Tool (FAST) was used to segment and bias correct the images. Total white matter, gray matter and CSF were computed from the FAST products; total brain volume was computed as the sum of total white matter and total gray matter. Insular regions-of interest (ROI) were drawn on the spatially aligned images in BIJ, and volumes were determined from the ROI drawings.

2.3. ROI analysis

The insula was manually delineated using the sagittal and coronal planes for each participant according to previously described methods (Cohen et al., 2010), using grayscale volume stacks derived from whole-brain analysis. These data sets were standardized in their orientation, parallel to the axial plane passing through the anterior and posterior commissures, using a six-parameter rigid-body transform. The resolution of the standardized coronal data sets was then increased from 256 × 256 to 512 × 512, using a bicubic interpolation algorithm to increase visualization accuracy. Insular ROIs were traced on the non-segmented grayscale images, and the resulting gray matter volumes were quantified by projecting the ROIs onto the segmented gray matter fraction stack for each subject. The inter-rater reliability of the insular ROIs was established by achieving intraclass correlation coefficient equal to 0.93.

The superior and inferior circular sulci separate the insula from the frontoparietal and temporal opercula, respectively. Since the inferior circular sulcus does not extend rostral to the limen of the insula, there is no well-defined boundary between the anterior insula and the orbital frontal cortex. The orbitoinsular sulcus is considered the topographic boundary between the anterior insula and adjacent orbitofrontal cortex. The superior and inferior circular sulci fuse to form the posterior pole of the insula. The medial boundary of the insula is a band of white matter called the extreme capsule. The anterior and posterior boundaries of the insula were viewed best from the sagittal plane. The coronal plane was utilized to define the superior, inferior and medial boundaries of the superior sulcus, inferior sulcus, and extreme capsule respectively.

2.4. Statistical analysis

2.4.1. Subjects

A two-way analysis of variance (ANOVA) was used to demonstrate that the groups were well-matched for gender and age. A two-way ANOVA was used to test for group differences in IQ, in which gender (male, female) and group (FraX, DD, HC) were the independent variables and IQ was the dependent variable. A one-way analysis of variance, in which group (FraX, DD, HC) was the independent variable and total brain volume (in cm^3) was the dependent variable, was used to test for group differences in total brain volume.

2.4.2. Insular ROIs

A one-way repeated measures analysis of covariance (ANCOVA) was applied with hemisphere (Left, Right) as the repeated measure, group as the independent

Table 1
Subject demographics.

		Group			Significance
		HC	DD	FraX	
Subjects	Males	3	5	6	
	Females	5	6	5	
	Total	8	11	11	$p = 0.759$
Age (years)	Males	13.3 (2.6)	16.0 (3.9)	16.3 (2.8)	
	Females	16.5 (3.7)	16.4 (2.3)	15.3 (3.3)	
	Total	15.3 (3.5)	16.2 (3.0)	15.9 (2.9)	$p = 0.666$
Intelligence (IQ)	Males	101.0 (15.6)	61.8 (4.7)	58.8 (12.3)	
	Females	115.6 (12.4)	60.8 (15.6)	84.0 (27.5)	
	Total	111.4 (13.9)	61.3 (10.9)	70.3 (23.5)	$p < 0.001$[a]
Total brain volume (cm^3)	Males	1310.27 (136.55)	1410.56 (283.51)	1342.43 (120.19)	
	Females	1216.04 (71.88)	1115.00 (107.04)	1218.29 (89.30)	
	Total	1251.38 (103.24)	1249.34 (248.40)	1286.00 (120.89)	$p = 0.955$

Table lists means (standard deviations) for participants' age, intelligence (IQ), and total brain volume. Data organized by total group means and gender by group means. *p*-Values refer to overall group difference significance levels.

[a] DD vs. FraX $p = 0.283$; HC > DD $p < 0.001$; HC > FraX $p < 0.001$.

variable and total insular volume as the dependent variable. In order to control for inter-individual differences in brain volume associated with gender or other factors, total brain volume was entered as a covariate. This is statistically analogous to normalizing brain volumes in automated methodologies. To test anterior and posterior insular differences, a multivariate analysis of covariance (MANCOVA) with repeated measures was used in which hemisphere was the repeated measure, group was the independent variable, anterior and posterior volumes were the dependent variables, and total brain volume was the covariate.

2.4.3. Asymmetry

Asymmetry for each ROI was examined by assessing the group by hemisphere (Left, Right) interaction that was used in the repeated measures ANCOVA and MANCOVA analyses described above.

3. Results

3.1. Subjects

There were no significant effects of group ($p = 0.666$), gender ($p = 0.493$), or group-by-gender interaction ($p = 0.377$) for age (Table 1). There was a significant group difference in IQ ($p < 0.001$) that was not related to gender ($p = 0.200$). Two-group ANOVAs confirmed that HC had significantly higher IQ than both FraX subjects ($p < 0.001$) and DD subjects ($p < 0.001$). Importantly, there was no significant group difference in IQ between FraX and DD ($p = 0.283$). There was no significant between-group difference in total brain volume ($p = 0.955$). However, to control for individual variability, total brain volume was included as a covariate for all insular volume comparisons.

3.2. Insular ROIs

3.2.1. Main effect of group

Insular volumes, adjusted for total brain volume, organized by region, hemisphere and group are given in Table 2. A repeated measures ANCOVA showed a significant group difference in total insular volume across groups ($p = 0.006$) that was unrelated to hemisphere (Table 2). Two-group comparisons showed that HC and DD total insular volumes did not significantly differ from one another ($p = 0.545$), but FraX total insular volumes were significantly reduced compared to both DD ($p = 0.008$) and HC ($p = 0.036$) (Fig. 1). The effect size for both significant results were large (d's > 0.81).

Table 2
Adjusted mean insular volumes.

		Group		
Hemisphere	Region	HC	DD	FraX
Left	Anterior	3.89 (0.55)	3.98 (1.04)	3.33 (0.75)
	Posterior	3.11 (0.75)	3.11 (0.81)	2.38 (0.87)
	Total	7.00 (0.85)	7.09 (1.61)	5.71 (1.52)
Right	Anterior	4.00 (0.58)	4.09 (1.03)	3.42 (0.92)
	Posterior	2.73 (0.66)	2.99 (0.65)	2.36 (0.72)
	Total	6.74 (0.93)	7.08 (1.65)	5.78 (1.50)

Means (standard deviations) in cm^3 for insular volumes by hemisphere, region and group, which are corrected for total brain volume.

A MANCOVA with repeated measures analysis of insular subregions yielded a significant effect of group at the multivariate level ($p = 0.040$). Both the anterior ($p = 0.026$) and posterior ($p = 0.016$) insular volumes were significantly different across groups (Fig. 1). FraX anterior insular volumes were significantly reduced compared to DD ($p = 0.019$, $d = 0.70$), and showed a trend difference with HC ($p = 0.087$, $d = 0.76$). Posterior insular volumes were significantly reduced in FraX compared to both DD ($p = 0.012$, $d = 0.87$) and HC ($p = 0.042$, $d = 0.74$). HC and DD did not significantly differ in anterior or posterior volumes (p's > 0.834). All effect sizes for FraX anterior and posterior insular volume reduction were moderately large to large.

3.2.2. Main effects of hemisphere (i.e., asymmetry) and gender

There was a significant main effect of hemisphere (i.e., asymmetry between left and right insular volumes) ($p = 0.013$) that was unrelated to group ($p = 0.575$). Because insular asymmetry was found to be unrelated to group no further hemispheric analyses were conducted. There was no main effect of gender for total, anterior or posterior (p's > 0.563) insular volume.

4. Discussion

The current study was designed to examine insular volumes in individuals with FraX as compared to non-FraX individuals who were matched for IQ and age, and age-matched healthy controls. The inclusion of both control groups in this study was important for determining whether between-group differences in insular volumes could be related to FraX-specific genetic factors rather than general cognitive disability.

Due to the well-described behavioral phenotype associated with FraX, and symptoms of hyperarousal and gaze aversion in particular (Reiss and Dant, 2003), it was expected that FraX subjects would have significantly reduced insular volumes compared to both healthy and developmentally delayed controls. As expected, total insular volumes were significantly reduced in FraX compared to both HC and DD after controlling for total brain volume. When insular sub-regions were inspected, posterior insular volume was significantly reduced in FraX relative to both the DD and HC groups. Anterior insular volumes were significantly reduced in FraX com-

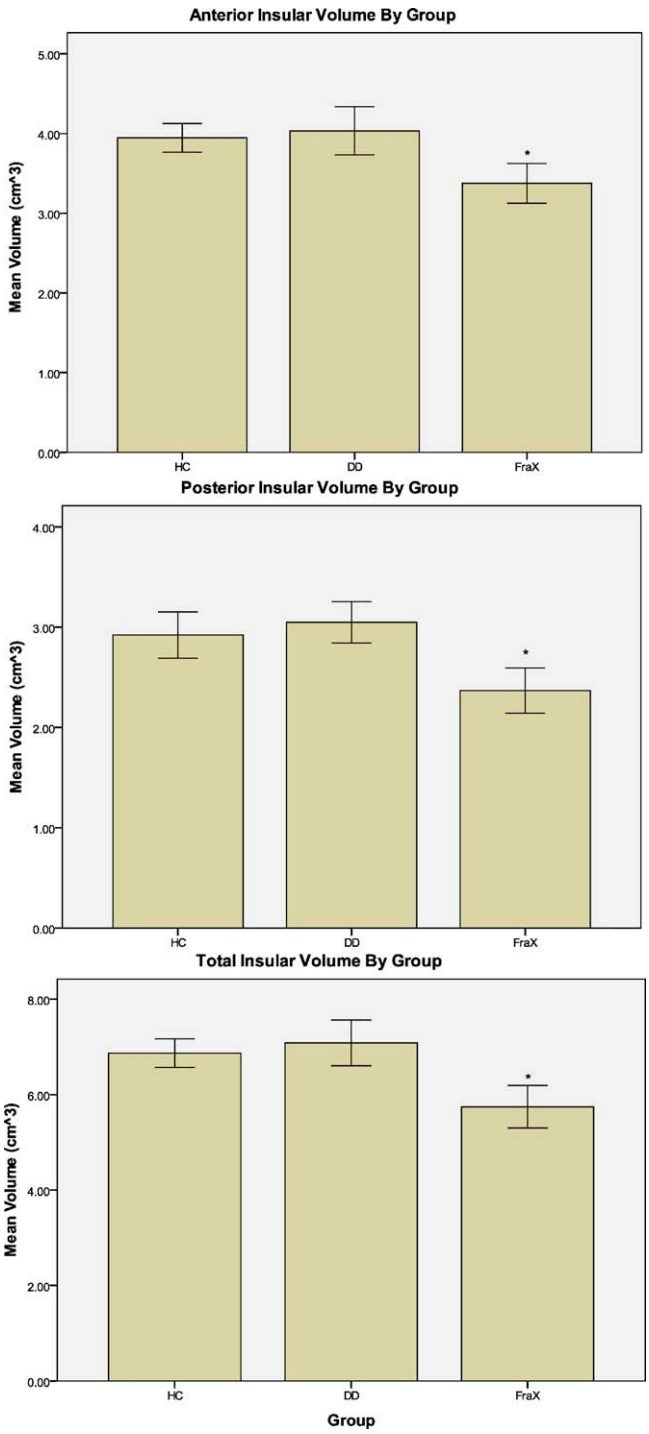

Fig. 1. Insular volume means by region. Graphs show mean insular volumes (in cm^3; adjusted for total brain volume) for anterior, posterior and total ROIs across groups. Since there were no significant group related hemispheric effects, the left hemisphere and right hemisphere volumes were averaged for each ROI. Error bars represent ±1 SEM. *Denotes significant volume reduction in FraX compared to DD and HC (p's < 0.042) (note: FraX vs. HC was a trend difference in anterior insula, $p = 0.087$).

pared to DD, while the difference between FraX and HC volumes approached significance. The trend difference in anterior insular volume between HC and FraX, rather than statistically significant differences, is most likely due to the small sample size though other reasons cannot be ruled out at this time. Since DD volumes were similar to HC volumes, our results indicate that insular volume reductions in FraX are associated with specific, aberrant neurodevelopmental processes occurring in this condition, or the interaction of these FraX-specific neurobiological factors with common environmental influences.

As a sensory integrative region and a part of the central autonomic network, the insula has a critical role in anxiety manifestation and aversive behaviors. Indeed both males and females with the full *FMR1* mutation express varying degrees of social anxiety, hyperarousal, and aversion to eye gaze (males especially) (Reiss and Dant, 2003). Heightened insular activation in response to exposure to eyes and faces may be a contributing factor to these symptoms as previous studies in non-FraX populations have shown increased insular responses to anxiety stimuli (Wright et al., 2003; Stein et al., 2007; Rauch et al., 1997; Etkin and Wager, 2007). Simmons et al. (2006) correlated this insular activation with a simultaneous decrease in superior temporal sulcus (STS) activation, and associated this functional pattern with the anticipation of aversive visual stimuli. Most importantly, previous work from our lab has shown increased insular activation in both males and females with FraX in response to eye-gaze stimuli (Garrett et al., 2004; Watson et al., 2008) while the current data shows significant insular volume reductions in the same population. The increased insular activation in FraX to eye-gaze stimuli was found along with reduced STS activation, the precise pattern of functional activation associated with the anticipation of aversive visual stimuli. Aversion to eye-gaze and anxiety are well documented phenotypic characteristics of FraX (Schneider et al., 2009; Watson et al., 2008; Garrett et al., 2004; Reiss and Dant, 2003; Tsiouris and Brown, 2004; Hagerman, 2002; Riddle et al., 1998; Bennetto and Pennington, 1996). Therefore, it is likely that insular volume reduction reported here is related to both the pattern of functional activation associated with the anticipation of aversive visual stimuli as well as eye gaze aversive behavior characteristic of individuals with FraX.

Along with total insular volume reduction, both anterior and posterior insular volumes were reduced in FraX compared to DD and HC. While it was anticipated that the anterior insula would be significantly reduced, the findings here suggest an equal functional role for the posterior insula that was not predicted by the Paulus–Stein model of anxiety. Current insular models of anxiety place little or no emphasis on the posterior region. The anatomical segmentation method described here is the first to characterize the posterior insula based on its pattern of connectivity, and such a method can help establish the expanded functional role of the posterior insula in anxiety. Based on its connectivity, the posterior insula is involved in physical somato-visceral sensory integration (Craig, 2003; Paulus and Stein, 2006). The morphometric change in this region may indicate that individuals with FraX have disrupted primary interoceptive processing, or exaggerated interoceptive processing, which may significantly contribute to the altered interoceptive prediction signal in FraX, particularly towards faces and eye-gaze. FraX individuals may then try to suppress emotional and interoceptive responses which serves to reinforce the process of worry (via avoidance behavior) but also prevents extinction (Borkovec and Roemer, 1995). The results here suggest that the involvement of posterior insula in anxiety may be underestimated by Paulus and Stein model (2006)Paulus and Stein (2006). Our data also indicate that posterior insular cortex function, along with its connectivity to other brain regions, may be as important as the anterior insula in contributing to anxious states.

Previous voxel-based morphometry studies have shown insular volume reduction in children and adults with FraX (Hoeft et al., 2008; Gothelf et al., 2008). However, whole brain automated approaches are more exploratory, which then requires further studies with a more directed approach towards targeted regions of interest. The current results from the targeted morphometric approach give further support to the significance of previous findings. The fact that reduced insular size is observed in children with

FraX as young as 1–3 years of age (Hoeft et al., 2008) also suggests that this neuroanatomical finding is an early neurodevelopmental aberration that is maintained throughout development. Given the high frequency of autistic behaviors in individuals with FraX (Reiss and Dant, 2003), it is also intriguing that recent studies of insular function and connectivity implicate this region in the development of autism spectrum disorders (Uddin and Menon, 2010; Ebisch et al., 2010).

The data presented here also strongly support the concept of analysing the insula in a sub-regional manner based on its connectivity to other brain regions, as opposed to viewing the region as a whole (Cohen et al., 2010). The current data provides structural evidence for both anterior and posterior insular involvement in the increase of anxiety and aversion to eye-gaze in FraX. This is an important finding considering the aberrant insular functional activation pattern seen in FraX in response to eye-gaze stimuli (Watson et al., 2008; Garrett et al., 2004). However, it should be noted that behavioral measures were not available in the current study sample. This is mainly due to the fact that the measurement of anxiety is difficult in individuals with intellectual disability, particularly in FraX (Sullivan et al., 2007). Furthermore, nearly all studies assessing behavioral symptoms and physiological metrics find strong indications of increased anxiety in FraX individuals (Schneider et al., 2009). While behavioral data (e.g., anxiety measures) were not directly available for correlation with insular volumes in this study, previous data from our lab concerning increased insular activity in FraX individuals in response to eye-gaze stimuli (Garrett et al., 2004), the well-documented phenotype of eye-gaze aversive behavior in FraX individuals, and the extensive literature on insular involvement in anxiety helps support the conclusions drawn here. As for the small sample sizes reported here, it is important to note that significant results were found despite reduced statistical power. The finding of total insular volume reduction in FraX is also supported by previous studies using voxel-based morphometry (Hoeft et al., 2008; Gothelf et al., 2008). The current study should be replicated with a larger sample size. Future research into FraX, and other populations with significant anxiety symptoms, should focus on how discrete measures of anxiety or hyperarousal relate to the anatomy and function of specific insular subregions.

Acknowledgements

The author would like to thank Dr. Murray Ettinger, who assisted with advice and proof-reading of the manuscript. This study was supported by NIMH grants R01MH050047 and R01MH064708 (Dr. Reiss), and T32MH019908 (Drs. Reiss and Cohen). Funding was also provided by the generosity of the Canel Family Fund.

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