Magnetic Resonance Imaging Outcomes From a Comprehensive Magnetic Resonance Study of Children With Fetal Alcohol Spectrum Disorders

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Background: Magnetic resonance (MR) technology offers noninvasive methods for in vivo assessment of neuroabnormalities.

Methods: A comprehensive neuropsychological/psychiatric battery, coupled with MR imaging, (MRI), MR spectroscopy (MRS), and functional MRI (fMRI) assessments, were administered to children with fetal alcohol spectrum disorders (FASD) to determine if global and/or focal abnormalities could be identified, and distinguish diagnostic subclassifications across the spectrum. The 4 study groups included: (i) fetal alcohol syndrome (FAS)/partial FAS (PFAS); (ii) static encephalopathy/alcohol exposed (SE/AE); (iii) neurobehavioral disorder/alcohol exposed (ND/AE) as diagnosed with the FASD 4-Digit Code; and (iv) healthy peers with no prenatal alcohol exposure. Presented here are the MRI assessments that were used to compare the sizes of brain regions between the 4 groups. The neuropsychological/behavioral, MRS, and fMRI outcomes are reported separately.

Results: Progressing across the 4 study groups from Controls to ND/AE to SE/AE to FAS/PFAS, the mean absolute size of the total brain, frontal lobe, caudate, putamen, hippocampus, cerebellar vermis, and corpus callosum length decreased incrementally and significantly. The FAS/PFAS group (the only group with the 4-Digit FAS facial phenotype) had disproportionately smaller frontal lobes relative to all other groups. The FAS/PFAS and SE/AE groups [the 2 groups with the most severe central nervous system (CNS) dysfunction] had disproportionately smaller caudate regions relative to the ND/AE and Control groups. The prevalence of subjects in the FAS/PFAS, SE/AE, and ND/AE groups that had 1 or more brain regions, 2 or more SDs below the mean size observed in the Control group was 78, 58, and 43%, respectively. Significant correlations were observed between size of brain regions and level of prenatal alcohol exposure, magnitude of FAS facial phenotype, and level of CNS dysfunction.

Conclusions: Magnetic resonance imaging provided further validation that ND/AE, SE/AE, and FAS/PFAS as defined by the FASD 4-Digit Code are 3 clinically distinct and increasingly more affected diagnostic subclassifications under the umbrella of FASD. Neurostructural abnormalities are present across the spectrum. MRI could importantly augment diagnosis of conditions under the umbrella of FASD, once population-based norms for structural development of the human brain are established.

Key Words: Fetal Alcohol Spectrum Disorder, Magnetic Resonance Imaging, FASD 4-Digit Diagnostic Code.

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F ETAL ALCOHOL SYNDROME (FAS) is a permanent birth defect syndrome caused by maternal alcohol consumption during pregnancy. FAS is defined by growth deficiency, a unique cluster of minor facial anomalies, and central nervous system (CNS) dysfunction and/or structural brain abnormalities (Smith, 1979). The cognitive/behavioral problems in this condition stem from prenatal brain damage. Not all individuals with prenatal alcohol exposure present with measurable CNS dysfunction or structural brain abnormalities, and not all who present with measurable CNS dysfunction or structural brain abnormalities have FAS. Recently, the term fetal alcohol spectrum disorders (FASD) was coined to depict the spectrum of outcomes observed

among individuals with prenatal alcohol exposure. The degree of brain damage among individuals with prenatal alcohol exposure may vary from microcellular and neurochemical aberrations to gross structural anomalies. Similarly, cognitive/behavioral dysfunction varies along the full continuum from mild developmental delay or learning disabilities to global developmental disability (Astley et al., 2009b). The specificity of the FAS facial phenotype to prenatal alcohol exposure lends credence to the clinical judgment that the cognitive and behavioral dysfunction observed in individuals with FAS is due, at least in part, to brain damage caused by a teratogen (Aase et al., 1995; Astley and Clarren, 2001; Astley et al., 2002). Unfortunately, without the unique facial phenotype of FAS or at least a severe or clinically obvious expression of brain damage, the neurodevelopmental disabilities of an individual affected by prenatal alcohol exposure often go unrecognized and inappropriately served (Streissguth et al., 1993).

Many individuals with prenatal alcohol exposure exhibit cognitive difficulties and significant maladaptation that prevent them from leading productive, independent lives (Stratton et al., 1996; Streissguth et al., 2004). Across the population, the profile of cognitive dysfunction among individuals with prenatal alcohol exposure is highly variable, although there are some commonalities in functional compromise among subgroups, and conceptual models of overarching deficits have been proposed (Kodituwakku, 2007). However, no single behavioral phenotype specific to alcohol teratogenicity has been described. Without a behavioral phenotype specific to the teratogen alcohol, attributing an alcohol-exposed child's dysfunction to brain damage is often questionable at a clinical level (Aase et al., 1995). If indisputable evidence of brain damage (e.g., alterations in neurostructure, neurometabolites, and/or neuroactivation) could be found in these individuals, and linked to behavioral deficit, diagnostic efforts could be improved. The "disability" of these alcohol-exposed children would be clearly established, and help facilitate eligibility for needed services. Further, if specific alterations in neurostructure, neurometabolites, and/or neuroactivation could be linked to clinically meaningful, discrete neuropsychological deficits, development of appropriate intervention programs could be accelerated.

The overall goal of this research study was to determine if magnetic resonance (MR) imaging (MRI), MR spectroscopy (MRS), and/or functional MRI (fMRI) could serve as noninvasive methods for definitively identifying global and/or focal brain abnormality across the full continuum of FASD, and distinguish diagnostic subclassifications within the spectrum. The results of this comprehensive study are presented in 4 separate reports: MRI (presented here) and the neuropsychological/behavioral (Astley et al., 2009b), MRS (Astley et al., 2009c), and fMRI (Astley et al., 2009a) outcomes reported separately. The focus on FASD diagnostic methodology in this report directly responds to the following Research Recommendations for Diagnostic Criteria published in the Institute of Medicine report on FASD (Stratton et al., 1996)

research: (i) to evaluate the utility, reliability, and validity of schemes for classification and diagnosis, (ii) to identify potential structural or functional brain abnormalities and other neurobiological indices that may be associated with or distinguish FAS, alcohol-related neurodevelopmental disorder (ARND), or alcohol-related birth defect, and to relate these abnormalities and indices to cognitive and behavioral correlates.

Magnetic resonance imaging allows for very sensitive assessment of size, shape, spatial orientation, and even tissue composition of selected brain regions. Numerous FASD MRI studies have been conducted to date (Archibald et al., 2001; Bookstein et al., 2002b; Mattson et al., 2001; Miller et al., 1999; Riley et al., 1995; Sowell et al., 2001b, 2002a,b). Documented abnormalities include reduction in overall brain size, reduction in absolute size of selected brain regions (basal ganglia, caudate, cerebellum, and anterior/posterior regions of the corpus callosum), disproportionate reduction of the caudate, alterations in shape and spatial orientation of the corpus callosum, and white matter hypoplasia in the parietal and temporal lobes.

The majority of FASD MRI studies published to date have enrolled study groups diagnosed or classified as FAS, fetal alcohol effects (FAE), ARND, or prenatal exposure to alcohol (PEA) prior to the establishment of comprehensive, case-defined FASD diagnostic guidelines that are quickly becoming best practice (Astley, 2004; Bertrand et al., 2004; Chudley et al., 2005). The specific diagnostic criteria used to establish the FASD study groups (e.g., level of growth deficiency; type, number and severity of facial anomalies; breadth and magnitude of neuropsychological impairment; type of neurostructural anomaly present), providing confirmation that FASD diagnostic subgroups are clinically and statistically distinct from each other, are typically not reported. Absence of rigorous diagnostic methods can lead to diagnostic misclassification. Astley and Clarren (2000) and Hoyme and colleagues (2005) have both demonstrated that individuals diagnosed with FAS by a gestalt approach often lose that diagnostic classification when more rigorous diagnostic guidelines are applied. Misclassification impacts study validity and reduces the power of a study to detect clinically meaningful differences between FASD subgroups. If specific diagnostic features that define the FASD study groups are not clearly reported, this limits the ability to compare outcomes across studies.

In general, most FASD MRI studies have found significant differences between FAS and control groups, regardless of diagnostic system used; but have not always found differences between clinical subgroups on the fetal alcohol spectrum. In the current study, the sizes of brain regions were compared between 3 FASD diagnostic subgroups and a healthy control group with no prenatal alcohol exposure. An important goal of this MRI study was to determine if meaningful neurostructural differences do exist between FASD subgroups when the subgroups, including FAS, are rigorously case-defined (Astley, 2004) and confirmed to be clinically and statistically distinct from one another (Astley et al., 2009b).

MATERIALS AND METHODS

Subjects and Study Groups

The protocol was approved by the University of Washington Human Subjects Review Board. The goal of the study was to create 3 clinically distinct and increasingly less affected FASD study groups and 1 healthy unexposed control group that fell along the following ordinal scale: (i) significant brain damage/dysfunction with the FAS facial phenotype; (ii) significant brain damage/dysfunction without the FAS facial phenotype; (iii) mild to moderate brain dysfunction without the FAS facial phenotype, and (iv) healthy with no prenatal alcohol exposure. The 3 FASD groups were selected from among 1,200 patients previously diagnosed by an interdisciplinary team in the WA State FAS Diagnostic & Prevention Network (FAS DPN) of clinics using a practical, comprehensive diagnostic system called the FASD 4-Digit Code (Astley, 2004; Astley and Clarren, 2000). Briefly, the 4 digits of the FASD 4-Digit Code reflect the magnitude of expression of the 4 key diagnostic features of FASD, in the following order: (i) growth deficiency, (ii) characteristic FAS facial phenotype, (iii) CNS structural/functional abnormalities, and (iv) prenatal alcohol exposure (Fig. 1). The magnitude of expression of each feature was ranked independently on a 4-point Likert scale, with 1 reflecting complete absence of the FASD feature and 4 reflecting a strong "classic" presence of the FASD feature. Each Likert rank is specifically case-defined. There are 256 possible 4-digit diagnostic codes ranging from 1111 to 4444. Each 4-digit diagnostic code falls into 1 of 22 unique clinical diagnostic categories (labeled A through V). Seven of the 22 diagnostic categories (4-Digit Categories A–C and E–H) fall under the umbrella of FASD [A, FAS/alcohol exposed; B, FAS/alcohol exposure unknown; C, PFAS/alcohol exposed; E and F, static encephalopathy/alcohol exposed (SE/AE); and G and H, neurobehavioral disorder/alcohol exposed (ND/AE)]. The 3 FASD study groups in the current study represent these FASD diagnostic categories. This diagnostic system is currently being used
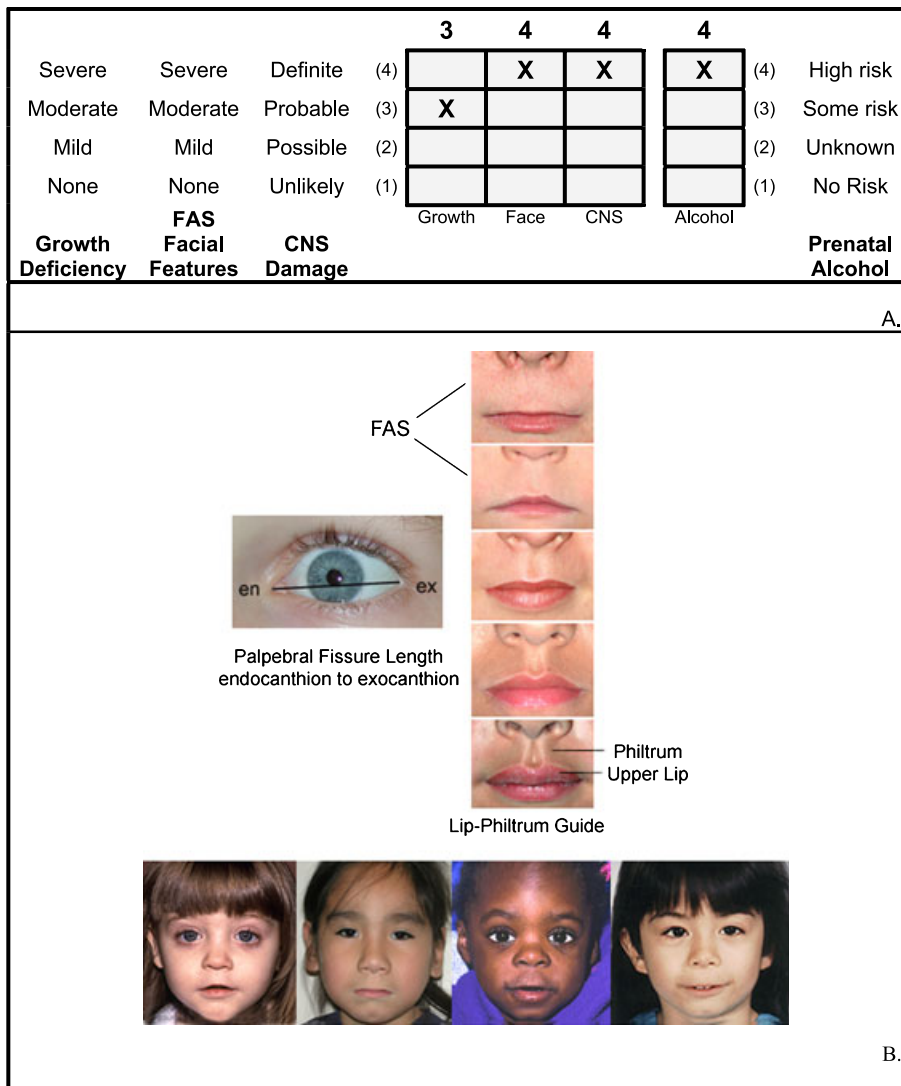
Fig. 1. (A) FASD 4-Digit Diagnostic Code grid. FASD is defined by growth deficiency, specific FAS facial features, evidence of CNS damage and prenatal alcohol exposure. The 4-Digit Code ranks each of these areas on 4-point, case-defined Likert scales. The 4-Digit Code (3444) inserted in the grid is 1 of 12 codes that meet the diagnostic criteria for FAS (Astley, 2004). (B) FASD 4-Digit Code FAS facial phenotype. The Rank 4 FAS facial phenotype determined with the 4-Digit Diagnostic Code requires the presence of all three of the following anomalies: (i) palpebral fissure length 2 or more SDs below the norm; (ii) smooth philtrum (Rank 4 or 5 on the Lip-Philtrum Guide), an (iii) thin upper lip (Rank 4 or 5 on the Lip-Philtrum Guide). Examples of the Rank 4 FAS facial phenotype for Caucasian, Native American, African American, and Asian American children are shown. © Susan Astley. FASD, fetal alcohol spectrum disorder; FAS, fetal alcohol syndrome; CNS, central nervous system.

by a wide variety of diagnostic teams in United States and other countries. The control population for this study was selected primarily from a large cohort of children enrolled at birth in a University of Washington study of typical development conducted through the Department of Speech and Hearing Sciences. With the enrollment of each child in the FAS/partial FAS (PFAS) group, a child matched on age (within 6 months), gender, and race was randomly identified and invited to enroll from the eligible SE/AE, ND/AE, and Control populations. The enrollment goal was 80 subjects (20 per group).

The study enrollment procedure produced a sample of 81 children of diverse ethnicity. The age range (8 to 15.9 years) included the broadest age range of children that could be administered a comparable psychometric assessment battery and be reasonably capable of participating in the MR scanning. Each of the 4 study groups had 16 to 24 subjects successfully balanced on age, gender, and race. The 61 children with FASD were highly representative of the entire clinic sample of 1,200 from which they were drawn. The clinic population of 1,200 was 43% female, 51% Caucasian, with 40% between 8 and 15.9 years of age. The 4-Digit Codes of all 81 children were presented in the neuropsychological/behavioral report for this study (Astley et al., 2009b). The diagnostic features specific to each group are as follows:

1. Children in Group 1 had a 4-Digit diagnosis of FAS/PFAS (e.g., 4-Digit Diagnostic Categories A, B, C: with Growth Ranks 1–4, Face Ranks 3–4, CNS Ranks 3 and/or 4, and Alcohol Ranks 2–4) (Fig. 1). Alcohol Rank 2 (unknown exposure) could only be present if the child had a diagnosis of full FAS because the Rank 4 FAS facial features are so specific to prenatal alcohol exposure (Astley and Clarren, 1996; Astley et al., 2002). As the only clinical difference between FAS and PFAS in this study was the presence of growth deficiency in the former, FAS and PFAS were combined. In summary, children in Group 1 had severe cognitive/behavioral dysfunction and the FAS facial phenotype.
2. Children in Group 2 had a 4-Digit diagnosis of SE/AE (e.g., 4-Digit Diagnostic Categories E and F: with Growth Ranks 1–4, Face Ranks 1–2, CNS Ranks 3 and/or 4, and Alcohol Ranks 3–4). In summary, children in Group 2 had severe cognitive/behavioral dysfunction, comparable with Group 1, but did not have the FAS facial phenotype.
3. Children in Group 3 had a 4-Digit diagnosis of ND/AE (e.g., 4-Digit Diagnostic Categories G and H: with Growth Ranks 1–4, Face Ranks 1–2, CNS Rank 2, and Alcohol Ranks 3–4). In summary, children in Group 3 had prenatal alcohol exposure like Groups 1 and 2, but had only mild to moderate cognitive/behavioral dysfunction, and did not have the FAS facial phenotype.
4. Children in Group 4 (Healthy Controls/No Alcohol Exposure) were selected based on parental report that the child was healthy, had no academic concerns, and no prenatal alcohol exposure (e.g., 4-Digit Diagnostic Category V: with Growth Ranks 1–2, FAS Face Ranks [no restrictions], CNS Rank 1, Alcohol Rank 1). In summary, these were nonexposed, healthy, average to high-functioning controls.

Using the FASD terminology introduced by Stratton and colleagues (1996), the SE/AE and ND/AE groups most closely reflected the severe and mild expressions of ARND, respectively. A comprehensive analysis of the between-group differences of these diagnostic features is presented in the neuropsychological/psychiatric report for this study (Astley et al., 2009b).

Within our FASD participants, 1 subject with PFAS had agenesis of the corpus callosum (ACC) and 1 subject with FAS had hypogenesis of the corpus callosum (HCC). That these subjects had callosal abnormalities was known prior to study enrollment. Indeed these 2 subjects with ACC/HCC were the only documented cases of ACC/HCC in the 2,040 patients with prenatal alcohol exposure diagnosed to date at the WA State FAS DPN

clinics. MRIs were typically only available when clinically indicated (e.g., evidence of neurological abnormalities). As such, only 204 (10%) of the 2,040 patients evaluated at the FAS DPN had a previous MRI evaluation summarized in their medical record and 76% of the 204 MRI evaluations were interpreted as normal by the patient's neuroradiologist. Although ACC/HCC has been observed in individuals with FASD (Riley et al., 1995), ACC/HCC is not specific to prenatal alcohol exposure. The prevalence of ACC among developmentally disabled populations is estimated to be 2 to 3 per 100 (Jeret et al., 1986). Thus, a causal link between ACC/HCC and prenatal alcohol exposure in these 2 individuals should not be assumed nor can it be ruled out. As all current FASD diagnostic guidelines (Astley, 2004; Bertrand et al., 2004; Chudley et al., 2005; Hoyme et al., 2005) list ACC/HCC as one of the many types of structural abnormalities that meet the CNS criteria for a FAS/PFAS diagnosis, it would be clinically invalid to exclude ACC/HCC from the FAS/PFAS study group. While they might represent a very small fraction of all alcohol-exposed patients evaluated in the FAS DPN clinic, they represent 2 of all 41 patients with FAS/PFAS from the FAS DPN clinic who met the 8- to 15-year-old eligibility criteria for enrollment into this study.

Study Participation

Participation in the study involved 5 visits over a 4- to 6-week study period. The psychological and sociodemographic data were collected during visits 1 and 2. The MR data were collected during visits 3 and 4. Outcomes of the psychological assessments were shared with the caregivers on visit 5, and submitted to the child's medical record with caregiver consent.

Sociodemographic and Clinical Assessments

A comprehensive sociodemographic and health/medication history of each child was obtained by parent interview and record review. Information included birth data, growth, and all prenatal and lifetime exposures and adverse events. For subjects with FASD, most information was obtained at the time of their FASD diagnostic evaluation. All controls had a reported absence of prenatal alcohol exposure. All children had a standardized digital facial photograph taken at the time of enrollment. The facial photographs were analyzed using the FAS Facial Analysis Software (Astley, 2003) to document the magnitude of expression of the FAS facial phenotype (Astley and Clarren, 1996). A more detailed methodology and analysis of the sociodemographic and FASD diagnostic outcomes, including prenatal alcohol exposure histories, were presented in the neuropsychological/behavioral report from this study (Astley et al., 2009b).

Neuropsychological and Psychiatric Assessments

A detailed description of the assessment battery and a comprehensive analysis of the between-group differences in neuropsychological outcome were presented in the neuropsychological/behavioral report for this study (Astley et al., 2009b). Briefly, a comprehensive, standardized assessment battery was administered to each child and their primary caregiver by a psychologist masked to group assignment. The assessment battery was designed to capture the domains of potential neuropsychological impairment seen as the result of the typically diffuse brain damage arising from alcohol teratogenesis (Bertrand et al., 2004; Chudley et al., 2005; Kodituwakku, 2007; Mattson and Riley, 1998; Olson et al., 1998; Roebuck et al., 1999). The neuropsychological/behavioral outcomes served to profile the study groups and confirm the groups were clinically and statistically distinct from one another; fundamental to the interpretation of the MR outcomes. The neuropsychological data will also be used to

assess correlations between brain structure and function. These correlations will be reported separately.

It is important to note that the mean full scale IQ of the healthy Control group (123 ± 7 SD) was higher than the population-based mean of 100 ± 15 SD. This was anticipated as children with potential threats to brain development were screened out and recruitment was through a University community. Other population-based MRI and FASD-MRI studies enrolling healthy controls have reported mean FSIQs ranging from 110 to 127 (Bookstein et al., 2002b; Haier et al., 2004; Sowell et al., 2008; Waber et al., 2007). Most FASD-MRI studies do not report the IQ or neuropsychological profile of their healthy control population. In spite of the Control group's relatively high IQ, however, many of the memory, executive function, language, and adaptive behavior scores assessed for this Control group were, on average, solidly within normal limits compared with age peers (Table 1) (Astley et al., 2009b). The ND/AE group had a mean FSIQ (99.2 ± 11.3 SD) equivalent to the population-based mean, despite their multiple prenatal/postnatal risk factors and significant adaptive/behavioral deficits.

MR Scanner

All scans (MRI, MRS, and fMRI) were acquired using a General Electric 1.5 Tesla scanner in the Diagnostic Imaging Sciences Center at the University of Washington.

MRS and fMRI

The MRS (Astley et al., 2009c) and fMRI (Astley et al., 2009a) components of this study were reported separately. Briefly, MRS was used to measure the concentrations of neurometabolites, including: (i) choline, a marker of cell membrane stability and myelination, (ii) N-acetyl aspartate, a neuronal or axonal marker, and (iii) creatine, a marker of metabolic activity, in selected brain regions. fMRI was used to assess neuroactivation in selected brain regions during performance of N-back working memory tasks.

Structural MRI Acquisition

An initial sagittal series was obtained first for orientation of subsequent series (Echo Time (TE) = 8, Time to Repetition (TR) = 400, flip angle = 25°, Field of View (FOV) = 24 × 24, matrix = 256 × 160, thickness = 4.0 mm, gap = 1.0 mm]. A high resolution 3-D T1-weighted fast gradient echo image of the whole brain was then performed in the axial plane [TE = 3, TR = 18, FOV = 24 × 18, matrix = 256 × 256, number of excitations (NEX) = 2, thickness = 1.5 mm, no gap]. This acquisition allowed images to be reformatted into any plane, which, in turn, allowed measurement of each structure in the plane that was optimal for its visualization. This also allowed brain scans to be realigned to ensure that all scans were measured in a standardized format. An exact midsagittal slice was reconstructed, allowing for area measures of corpus callosum and cerebellar vermis. Total scanning time for the structural series was approximately 15 minutes. Each scan was clinically reviewed by the neuroradiologist (masked to group assignment) to determine if there were any gross structural abnormalities.

Structural MRI Image Processing

Magnetic resonance imaging measures were performed on a PC workstation using the MEASURE program (The Johns Hopkins University, Baltimore, MD) (Barta et al., 1997). Each scan was rotated in 3-D space so that the axial images were parallel to the line connecting the anterior and posterior commissures and perpendicular to the interhemispheric fissure (ac–pc plane). Coronal slices were reconstructed with a thickness of 0.9375 mm and positioned perpendicular to the ac–pc plane. Based on prior FASD MRI literature (Archibald et al., 2001; Mattson et al., 2001; Miller et al., 1999; Sowell et al., 2001b), volume measurements focused on the hippocampus, caudate, putamen, frontal lobe, gray matter and white matter of the frontal lobe, and total brain volume from the 1.5 mm spoiled gradient recalled echo in steady state (SPGR). Area measures included cerebellar vermis and its subregions, corpus callosum and its subregions, and total brain area in the midsagittal slice. For the hippocampus, caudate, putamen, and frontal lobe volume measures, area of each structure was outlined manually, using a mouse-controlled cursor, in each slice. Areas within each slice were calculated, summed across slices, and multiplied by slice thickness, resulting in approximate structure volumes. Semi-automated methods were used to measure total brain volume and a stereology point-counting method was used to measure gray and white matter within the frontal lobe. Specific methods of measurement for each structure are mentioned below.

Hippocampus. The rules for defining boundaries of the hippocampus were developed by Aylward and colleagues (1999). Briefly, measurement was made on the reconstructed coronal slices, began in the most posterior coronal slice in which the hippocampus was viewed. Boundaries of the hippocampus were traced manually, with the choroid fissure as the superior boundary, the inferior temporal horn of the lateral ventricle as the lateral boundary, and the white matter of the parahippocampal gyrus as the inferior boundary. The hippocampus formed a natural boundary around the edge of the medial temporal lobe. Both the alveus and the subiculum were included in hippocampal measurements. Anteriorly, when a clear demarcation between the hippocampus and amygdala was not seen coronally, the sagittal view was consulted to determine the border between the hippocampus and amygdala. Interrater reliability for the hippocampus yielded an intraclass correlation of 0.92.

Basal Ganglia. Volumes of putamen and caudate were obtained on the axial images using rules previously described (Aylward et al., 1997a). Briefly, measurement of putamen and caudate began in the most inferior slice in which these structures were clearly separated by the internal capsule. Measurement continued in a superior direction until the body of the caudate was no longer observed. The borders of the caudate were defined laterally by the anterior limb of the internal capsule and medially by the frontal horn or body of the lateral ventricle. The borders of the putamen were defined laterally by the external capsule. At more inferior levels, the medial borders of the putamen were defined by the globus pallidus; at more superior levels, the medial borders were defined by the internal capsule. Intrarater reliability yielded intraclass correlations of 0.99 for both caudate and putamen. Interrater reliability yielded intraclass correlations of 0.97 for caudate and 0.94 for putamen.

Frontal Lobe Volume. The procedure used to measure the volume of the frontal lobes (Aylward et al., 1997b) and of the gray and white matter within the frontal lobes was based on the identification of sulcal-gyral landmarks on the surface of a 3-D reconstruction of the 1.5 mm coronal slices. For frontal lobe measures, this 3-D reconstruction which could be viewed in any orientation, was used to identify the precentral gyrus and sylvian fissure. Using the MEASURE software, the surfaces of these regions were "painted" on the 3-D reconstruction. The brain imaging data were then resliced in the axial plane, starting at the most superior level, and the paint (which remains on the surface of the recreated slices) was used to guide as the rater cut away portions of the brain posterior to the paint. Interrater reliability yielded an intraclass correlation of 0.99 for frontal lobe volume. After the frontal lobe was thus identified, a stereological point-counting method was used to measure gray and white volumes within the frontal lobe, as described by Aylward and colleagues (1995). Interrater reliability yielded an intraclass correlation of 0.92 for the frontal cortex.

Table 1. Sociodemographic and FASD 4-Digit Diagnostic Code Profiles of the 4 Study Groups

	Groups				Statistics			
					ANOVA			Chi-squared test
	1. FAS/PFAS[a]	2. SE/AE	3. ND/AE	4. Control	Overall	Post hoc	A priori LT	
Characteristic	n = 20	n = 24	n = 21	n = 16	F (p)[b]	Duncan	F (p)[c]	Chi (p)
Gender: n (%)								
Female	10 / 50.0	8 / 33.3	10 / 47.6	8 / 50.0				1.7 (0.63)
Age at enrollment in years: mean (SD)	12.7 / 2.4	12.2 / 2.0	12.4 / 2.3	12.4 / 2.7	0.1 (0.96)		0.1 (0.79)	
Race: n (%)								
Caucasian	12 / 60.0	11 / 45.8	12 / 57.1	13 / 81.3				[d]5.0 (0.17)
African American	6 / 30.0	4 / 16.7	6 / 28.6	2 / 12.6				
Native American	2 / 10.0	7 / 29.2	2 / 9.5	0 / 0.0				
Other	0 / 0.0	2 / 8.3	1 / 4.8	1 / 6.3				
Growth rank from 4-Digit Code: n (%)								
1. None	10 / 50.0	15 / 62.5	13 / 61.8	15 / 93.7				[e]10.3 (0.02)
2. Mild	2 / 10.0	2 / 8.3	6 / 28.6	1 / 6.3				
3. Moderate	5 / 5.0	3 / 12.5	1 / 4.8	0 / 0.0				
4. Severe	3 / 15.0	4 / 16.7	1 / 4.8	0 / 0.0				
Current height percentile: mean (SD)	33.5 (31.6)	30.4 (40.7)	34.7 (30.9)	60.6 (34.2)	2.7 (0.05)	132,24	5.2 (0.03)	
Current weight percentile: mean (SD)	51.7 (33.4)	34.2 (50.4)	31.2 (46.6)	67.6 (24.5)	1.5 (0.22)		1.7 (0.19)	
Face Rank from 4-Digit Code: n (%)[c]								
1. None	0 / 0.0	4 / 16.7	7 / 33.3	10 / 62.5				
2. Mild	0 / 0.0	20 / 83.3	14 / 66.7	6 / 37.5				
3. Moderate	4 / 20.0	0 / 0.0	0 / 0.0	0 / 0.0				
4. Severe	16 / 80.0	0 / 0.0	0 / 0.0	0 / 0.0				
Facial D-Score	1.2 (0.9)	−0.4 (0.9)	−0.8 (0.7)	−1.5 (0.9)	31.6 (0.000)	1,23,4	84.4 (0.000)	
CNS Ranks 1–3 from 4-Digit Code								
Level of functional impairment: n (%)								
1. None	0 / 0.0	0 / 0.0	0 / 0.0	16 / 100				[g]31 (0.000)
2. Moderate	0 / 0.0	[f]3 / 12.5	21 / 100	0 / 0.0				
3. Severe	20 / 100	21 / 87.5	0 / 0.0	0 / 0.0				
CNS Rank 4 from 4-Digit Code								
Structural/Neurologic abnormality: n (%)								
Present	13 / 65.0	6 / 25.0	0 / 0.0	0 / 0.0				[h]26 (0.000)
Microcephaly (OFC ≤ −2 SD): n, %	10 / 50.0	2 / 8.3	0 / 0.0	0 / 0.0				
Alcohol Rank from 4-Digit Code: n (%)								
1. No exposure	0 / 0.0	0 / 0.0	0 / 0.0	16 / 100				
2. Unknown exposure	1 / 5.0	0 / 0.0	0 / 0.0	0 / 0.0				
3. Confirmed exposure, level: moderate or Unk	7 / 35.0	12 / 50.0	11 / 52.4	0 / 0.0				
4. Confirmed exposure, level: high	12 / 60.0	12 / 50.0	10 / 47.6	0 / 0.0				
Alcohol use prior to pregnancy								
Days per week: mean (SD), range	5.4 (1.7), 2–7	4.0 (2.2), 1–7	5.3 (2.1), 1–7	0.9 (1.0), 0–3	19.8 (0.000)	123,4	31.0 (0.000)	
Most drinks/occasion: mean (SD), range	23.1 (24.8), 8–78	19.8 (26.3), 2–96	12.7 (7.7), 4–24	1.7 (1.5), 0–5	3.8 (0.018)	123,4	8.7 (0.005)	

Table 1. (Continued)

Characteristic	Groups 1. FAS/PFAS[a] n = 20 Mean	SD	2. SE/AE n = 24 Mean	SD	3. ND/AE n = 21 Mean	SD	4. Control n = 16 Mean	SD	Statistics ANOVA Overall F (p)[b]	Post hoc Duncan	A priori LT F (p)[c]	Chi-squared test Chi (p)
Alcohol use during pregnancy												
Days per week: mean (SD), range	5.5 (1.7)	3–7	3.9 (2.1)	1–7	5.3 (2.1)	1–7	0 (0)	0–0	35.9 (0.000)	123,4	35.8 (0.000)	
Most drinks/occasion: mean (SD), range	11.6 (7.1)	5–24	14.1 (8.9)	3–26	11.7 (7.3)	4–24	0 (0)	0–0	14.2 (0.000)	123,4	17.8 (0.000)	
Drank all 3 trimesters: n (valid %)	13	77	13	59	7	50.0	0	0.0				21 (0.000)
Neuropsychological selected tests												
WISC-III: FSIQ (ss)	77.5	14.4	79.3	10.5	99.2	11.3	123.9	6.5	67.6 (0.000)	12,3,4	183.4 (0.000)	
VABS: adaptive behavior composite (ss)	59	17.5	55	14.2	65.4	21.1	95.3	12.3	21.1 (0.000)	123,4	46.3 (0.000)	
D-KEFS: Tower, Total Achievement (ss)	7.6	2.3	8.3	2.5	9.6	2.1	10.8	2.1	7.0 (0.000)	12,23,34	20.5 (0.000)	
VMI: Total (ss)	76.2	12.7	81.4	9.2	90.9	11.8	102.7	12.9	17.8 (0.000)	12,3,4	51.6 (0.000)	

Chi2: chi-square test across four study groups, unless otherwise specified. D-KEFS: Delis-Kaplan Executive Function System, Tower, Total Achievement (Delis et al., 2000). Duncan: Multiple comparison range test. Reported if the overall ANOVA is statistically significant. Commas separate groups with homogeneous means at $p < 0.05$. F: F statistic. LT: ANOVA unweighted linear trend. OFC: occipital frontal circumference. Overall: Overall assessment of between-group means using ANOVA. p: p value. SD: standard deviation. Unk: unknown. VABS: Vineland Adaptive Behavior Scales, Adaptive Behavior Composite (Sparrow et al., 1984). VMI: Beery Buktenica Developmental Test of Visual-Motor Integration (Beery, 1997). WISC-III Full Scale IQ (Wechsler, 1996).

[a]Six of the 20 subjects in Group 1 had full FAS using the 4-Digit Code. Ten of the 14 PFAS had Rank 4 Faces but received a diagnosis of PFAS because they had no growth deficiency (Growth Rank 1).
[b]Between-groups, df = 3; within-groups, df = total sample size − 4.
[c]Between-groups linear term df = 1; within-groups df = total sample size − 4.
[d]Caucasian versus not Caucasian.
[e]No growth deficiency versus mild to severe growth deficiency.
[F]All 3 children with moderate functional impairment had structural evidence of brain abnormality (microcephaly).
[G]FAS/PFAS versus SE/AE ($\chi^2 = 7.1$, $p = 0.008$).
[H]FAS/PFAS versus SE/AE ($\chi^2 = 9.6$, $p = 0.002$).

Total Brain Volumes. Regional volumes were divided by total brain volume to allow correction for overall brain size. Total brain volume was measured using semi-automated thresholding procedures for segmenting brain tissue from cerebral spinal fluid (CSF) on the 1.5 mm axial images. Briefly, this procedure allowed the user to set the contrast such that all pixels above a certain value were highlighted, thus eliminating CSF (which in these images was black). A mask of this region was saved, and a command of "erode" was executed that "shrunk" the highlighted areas by a specified number of pixels. This performed automatic cutting of "bridges" between highlighted brain and nonbrain (e.g., dura and muscle) tissue regions. The cursor was then placed inside the brain region, and an "isolate blob" command then identified only those pixels that were connected (in any plane) with the pixel at the cursor location. This allowed the brain to be isolated from nonbrain tissue. Finally, a "dilate" command was executed, which restored pixels that were eroded previously, but no further than the original mask. Raters modified the procedure to ensure that only nonbrain tissue was removed. Intrarater reliability for obtaining brain volumes with this procedure yielded an intraclass correlation of 0.99. At each step, modifications were made manually to ensure inclusion of all brain tissue and exclusion of nonbrain regions.

Midsagittal Area Measures. The 3-D brain was rotated so that the interhemispheric fissure was perfectly positioned in the vertical plane. As described above, the slices were reformatted from the 1.5 mm axial series, yielding sagittal slices that were 0.9375 mm thick. The sagittal slice yielding the clearest visualization of the cerebral aqueduct was selected for the midsagittal measures. Midsagittal area measures were performed for cerebellar vermis and its 3 subsections (Lobules I–V, Lobules VI–VII, and Lobules VIII–X), corpus callosum and 5 subregions, and total brain (Aylward and Reiss, 1991; Aylward et al., 1994; Reiss et al., 1991). Interrater reliability for obtaining midsagittal areas yielded intraclass correlations ranging from 0.87 to 0.94. Midsagittal area of the total brain was also measured to allow correction of the corpus callosum and cerebellar vermis measures for overall brain size. The corpus callosum (CC) length (cm) was the distance from the most anterior and posterior borders of the CC that intersected with a line perpendicular to the ac–pc plane. The CC was transected into 5 equiangular regions: (i) genu, (ii) anterior body, (iii) posterior body, (iv) isthmus, and (v) splenium, using the mammillary body as a reference point (Riley et al., 1995).

MRI Hypotheses

In this report the analyses focus on the MRI comparisons between the 4 study groups. The following primary hypotheses were derived from the current MRI FASD literature (Archibald et al., 2001; Astley and Clarren, 2001; Bookstein et al., 2002b; Mattson et al., 2001; Miller et al., 1999; Sowell et al., 2001b). Note again that the neuropsychological/psychiatric report for this study (Astley et al., 2009b) confirmed that the 4 study groups were clinically distinct and increasingly more affected progressing across the 4 groups from the Controls to the FAS/PFAS.

1. The mean absolute and/or relative size of the brain as a whole and its regions would become increasingly smaller progressing across the 4 study groups from Controls to ND/AE to SE/AE to FAS/PFAS.
2. The mean absolute and/or relative size of the brain as a whole and its regions would be significantly smaller in the FAS/PFAS group (with the FAS facial phenotype) compared with the SE/AE group (without the FAS facial phenotype). This hypothesis specifically addressed a common clinical question: did individuals with FASD and the FAS facial phenotype have more severe brain abnormality than individuals with FASD and no FAS facial phenotype?

3. The prevalence of subjects with 1 or more brain regions 2 or more SDs below the mean size of the Control group would increase significantly progressing from the ND/AE group to the SE/AE group and to the FAS/PFAS group.

Statistical Analysis

The statistical analyses used to confirm the 4 study groups, effectively balanced on age, gender, and race, were described and presented in the neuropsychological/psychiatric report (Astley et al., 2009b).

Primary Analyses. ANOVA was used to determine if differences in mean size of brain regions existed among the 4 study groups. If significant differences existed, the Duncan post hoc range test was used to identify which group mean differed. The Duncan test makes pairwise comparisons using a stepwise procedure. Mean was ordered from highest to lowest, and extreme differences were tested first. The Duncan test sets a protection level for the error rate for the collection of tests and identifies homogeneous subsets of mean that are not different from one another at the $p = 0.05$ level. To specifically test primary hypothesis 1, an a priori test for linear trend was included in the ANOVA to determine if the mean size of selected brain regions became increasingly smaller progressing across the 4 study groups from Control to ND/AE to SE/AE to FAS/PFAS. To test primary hypothesis 2, an a priori contrast (*t*-test) between the FAS/PFAS and SE/AE groups was specified in the ANOVA. To test primary hypothesis 3, a chi-squared test for trend was used to compare the prevalence of structural anomalies across the 3 FASD groups relative to the Control group. Two-tailed *p* values of 0.05 were used throughout the analyses.

Secondary Analyses. Secondary analyses using Pearson Correlations coefficients and ANOVA tests for linear trends were conducted to determine if the size of brain regions decreased with increasing quantity, frequency, and/or duration of reported prenatal alcohol exposure between the 3 FASD groups. Two adjustments were used throughout the analysis of the MRI data: (1) adjustment for total brain volume (or midsagittal area) and (2) inclusion or exclusion of the 2 subjects in the FAS/PFAS group with ACC/HCC (Fig. 2A and 2B). Adjustment 1: to determine if some brain regions were disproportionately reduced in size. Given that overall brain size was often reduced among individuals with prenatal alcohol exposure, relative measures of brain regions were computed by dividing the volume of the region by total brain volume, or the midsagittal area of the brain region by the midsagittal area of the total brain. The terms "relative" and "absolute" were used to distinguish the 2 types of measures. Adjustment 2: the 2 subjects with ACC/HCC in FAS/PFAS group had CCs that were substantially smaller than the CCs of the other members of that group. The midsagittal area of the CC was 0.3 cm^2 in the subject with agenesis and 1.95 cm^2 in the subject with hypogenesis (Figs. 2A and 2B). These are 6 and 42%, respectively, of the mean midsagittal area of the CC (4.64 cm^2) for the remaining 18 members of the FAS/PFAS group. Inclusion of these 2 subjects in the FAS/PFAS group could influence some between-group differences (particularly differences in the mean midsagittal area of the CC). As such, it was important to confirm statistically significant group differences were not driven primarily by these 2 lowest measures (which represent outliers in the FASD sample). Although the size of the CC for the subject with ACC was a statistical outlier (more than 2 SDs below the mean of the FAS/PFAS group), it would not be clinically valid to exclude this case from the study. All current published FASD diagnostic guidelines (Astley, 2004; Bertrand et al., 2004; Chudley et al., 2005; Hoyme et al., 2005) list ACC as an example of a CNS structural abnormality that meets the CNS diagnostic criteria for FASD. Thus, all analyses were conducted
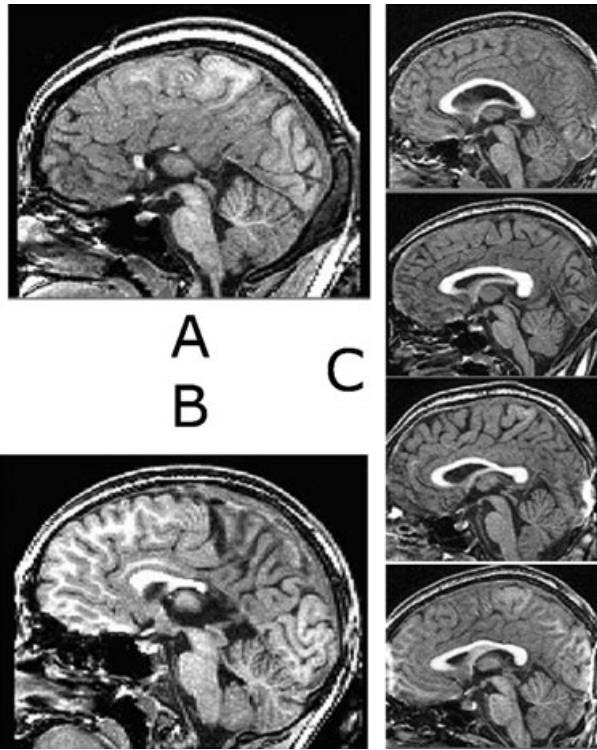
Fig. 2. Two subjects with (**A**) agenesis and (**B**) hypogenesis of the corpus callosum in the fetal alcohol syndrome (FAS)/partial FAS group. (**C**) Variability in corpus callosum shape among Controls with no prenatal alcohol exposure.

with inclusion of these 2 subjects. If their exclusion resulted in a statistical outcome that was discordant with the statistical outcome when the 2 subjects were included, both analyses are presented.

Power/Sample Size. This study had 80% power or greater to detect the following effect sizes at a two-tailed alpha level of 0.05: (i) a difference in 2 means equal to or greater than the SD of the mean difference; (ii) a correlation coefficient of 0.30 or greater; and (iii) a 35-point or greater difference in proportions between 2 groups.

RESULTS

Size of Brain Regions Across the 4 Groups (Primary Hypotheses 1–2)

Total Brain. The mean total brain volume decreased significantly progressing across the 4 study groups from Control to ND/AE to SE/AE to FAS/PFAS (Table 2). The linear trend remained significant with exclusion of the Control group, confirming that total brain size decreased incrementally across the 3 FASD groups. Indeed, all significant linear trends by brain region, reported in Table 2, remained significant with exclusion of the Control group. The mean total brain volume of the FAS/PFAS group was significantly smaller (11% smaller) than the mean of the Control group. The prevalence of microcephaly (occipital frontal circumference ≤ -2 SD) was significantly higher in the FAS/PFAS group with the FAS facial phenotype than in the SE/AE group without the FAS facial phenotype (83.1% vs. 16.7%;

$\chi^2 = 7.6$, two-tailed $p = 0.006$) (Astley et al., 2009b). This group difference was not influenced by the 4-Digit Code diagnostic criteria for CNS abnormality. The CNS criteria for FAS, PFAS, and SE/AE were identical (e.g., presence of structural and/or significant functional abnormality). Was brain size reduced simply because individuals with FASD were typically growth deficient? Although FAS is the only FASD subclassification in the 4-Digit Code that requires the presence of some level of growth deficiency (height and/or weight below the 10th percentile), all 3 FASD groups were significantly shorter in stature (but not lower in weight) than the Controls (Table 1). Despite the growth deficiency among the FASD, there was no significant correlation between height or weight percentile and total brain size across the 81 subjects.

Frontal Lobe. Absolute Volume: The mean absolute volume of the frontal lobe decreased incrementally and significantly, progressing across the 4 study groups from Controls to FAS/PFAS (Table 2). The mean absolute volume of the frontal lobe for the FAS/PFAS group was significantly smaller (11–18% smaller) than each of the other groups.

Relative Volume: The mean relative volume of the frontal lobe decreased significantly progressing across the 4 study groups from Control to FAS/PFAS (with and without inclusion of the 2 subjects with ACC/HCC). The mean volume of the frontal lobe for the FAS/PFAS group with the 2 subjects with ACC/HCC removed was 352.3 (62.1 SD). When the subjects with ACC/HCC were included in the FAS/PFAS group, no significant pair-wise group differences were observed. When the 2 subjects with ACC/HCC were excluded from the FAS/PFAS group, the mean relative volume of the frontal lobe for the FAS/PFAS group was significantly smaller (6–9% smaller) than each of the 3 other groups.

Gray/White Matter: The mean absolute volume of white and gray matter in the frontal lobe decreased significantly progressing across the 4 study groups from Control to ND/AE to SE/AE to FAS/PFAS. The mean absolute volume of the frontal gray matter for the FAS/PFAS group was also significantly smaller (19% smaller) than the mean of the SE/AE group. The mean percent white matter and percent gray matter in the frontal lobes were comparable across the 4 study groups.

Caudate. Absolute Volume: The mean absolute volume of the caudate decreased significantly progressing across the 4 study groups from Control to FAS/PFAS. The mean absolute volume of the caudate in both the FAS/PFAS and SE/AE groups were comparable with one another but significantly smaller (on average 23 and 19% smaller, respectively) than the mean of the Control group (Table 2).

Relative Volume: The mean relative volume of the caudate in both the FAS/PFAS and SE/AE groups were comparable with one another and significantly smaller (12 and 14%, respectively) than the mean of the Control group. No differences in absolute or relative size were observed between the right and left caudate.

Table 2. Mean Size of Brain Regions Across the 4 Study Groups

Brain Region	1. FAS/PFAS n	Mean	SD	2. SE/AE n	Mean	SD	3. ND/AE n	Mean	SD	4. Control n	Mean	SD	ANOVA Overall F (p)[b]	Post Hoc Duncan[a]	A Priori Contrasts LT F (p)[c]	Groups 1vs2 T (p)[a]
Total brain volume (cm³)	20	1217.8	183.4	24	1284.7	180.0	21	1305.0	95.1	16	1370.5	99.3	3.2 (0.03)	123,234	9.3 (0.003)	1.5 (0.14)
Total brain midsagittal area (cm²)	20	6.7	0.8	24	7.1	0.8	21	7.2	0.4	14	7.6	0.7	5.0 (0.003)	12,234	14.1 (0.000)	2.1 (0.04)
Frontal lobe volume (cm³)[d]	19	346.1	61.7	23	385.7	59.7	20	391.3	41.1	16	419.8	45.6	5.7 (0.001)	1,234	16.0 (0.000)	2.4 (0.02)
Frontal lobe/total brain[d]	17	0.283	0.03	23	0.301	0.03	20	0.300	0.02	16	0.306	0.02	3.0 (0.03)	1,234	5.4 (0.02)	2.0 (0.05)
Frontal lobe/total brain	19	0.286	0.03	23	0.301	0.03	20	0.300	0.02	16	0.306	0.02	1.9 (0.14)		4.5 (0.04)	
Frontal lobe gray matter volume (cm³)	19	190.6	38.1	22	216.2	33.7	20	220.8	28.6	15	235.4	28.3	5.7 (0.001)	1,234	15.5 (0.000)	2.5 (0.01)
Frontal lobe % gray matter	19	0.550	0.04	22	0.564	0.03	20	0.563	0.04	15	0.559	0.03	0.7 (0.53)		0.5 (0.50)	
Frontal lobe white matter volume (cm³)	19	155.4	28.6	22	167.5	31.8	20	171.0	22.9	15	186.0	24.7	3.5 (0.002)	123,234	10.3 (0.002)	1.4 (0.17)
Frontal lobe % white matter	19	0.450	0.04	22	0.436	0.03	20	0.437	0.04	1	0.441	0.5	0.7 (0.53)		0.5 (0.50)	
R. Caudate volume (cm³)	19	3.8	0.5	24	3.9	0.9	21	4.5	0.4	16	4.8	0.5	12.2 (0.000)	12,34	33.7 (0.000)	0.7 (0.46)
L. Caudate volume (cm3)	19	3.7	0.5	24	3.9	0.9	21	4.4	0.6	16	4.8	0.6	10.2 (0.000)	12,3,4	29.4 (0.000)	0.9 (0.36)
Total caudate volume (cm³)	19	7.4	1.0	24	7.8	1.7	21	8.8	0.9	16	9.6	1.1	11.6 (0.000)	12,34	32.8 (0.000)	0.9 (0.39)
Total caudate/total brain (10⁻²)	19	0.618	0.09	24	0.608	0.12	21	0.679	0.78	16	0.705	0.86	4.6 (0.005)	12,23,34	10.5 (0.002)	0.4 (0.73)
Caudate + putamen volume (cm³)	19	14.0	2.3	24	15.2	2.6	21	16.4	1.8	16	17.2	2.0	7.2 (0.000)	12,23,34	8.8 (0.04)	1.8 (0.08)
R. Putamen volume (cm³)	19	3.3	0.8	24	3.7	0.9	21	3.8	0.6	16	3.8	0.7	2.3 (0.08)		5.2 (0.03)	
L. Putamen volume (cm³)	19	3.3	0.8	24	3.8	0.7	21	3.8	0.7	16	3.7	0.7	1.8 (0.15)		2.7 (0.11)	
Total putamen volume (cm³)	19	6.6	1.5	24	7.4	1.6	21	7.6	1.2	16	7.6	1.3	2.9 (0.04)	12,234	4.4 (0.04)	2.0 (0.04)
Total putamen volume (cm³)[d]	17	6.7	1.5	24	7.4	1.6	21	7.6	1.2	16	7.6	1.3	1.7 (0.18)		3.4 (0.07)	
Total putamen/total brain (10⁻²)	19	0.546	0.12	24	0.582	0.12	21	0.584	0.09	16	0.550	0.08	0.8 (0.53)		0.1 (0.91)	
R. Hippocampus volume (cm³)	20	2.9	0.5	24	3.0	0.4	21	3.1	0.5	16	3.4	0.5	5.1 (0.003)	123,4	14.7 (0.000)	1.0 (0.29)
L. Hippocampus volume (cm³)	20	2.9	0.5	24	2.9	0.4	21	3.1	0.4	16	3.4	0.6	4.5 (0.006)	123,4	12.6 (0.001)	0.5 (0.63)
Total hippocampus volume (cm³)	20	5.7	1.0	24	5.9	0.8	21	6.2	0.8	16	6.8	1.0	5.1 (0.003)	123,4	14.6 (0.000)	0.8 (0.43)
Total hippocampus/total brain (10⁻²)	20	0.473	0.07	24	0.470	0.09	21	0.478	0.08	16	0.501	0.07	0.6 (0.64)		1.3 (0.26)	
CC: midsagittal area (cm²)[d]	20	4.12	1.3	24	4.45	0.8	21	4.35	0.7	16	4.64	0.7	1.0 (0.38)		2.3 (0.13)	
CC: Region 1 (genu) (cm²)	20	1.15	0.4	24	1.21	0.3	21	1.19	0.2	16	1.34	0.2	1.5 (0.22)	123,234	3.6 (0.06)	
CC: Region 2 (cm²)	20	0.74	0.2	24	0.76	0.2	21	0.72	0.1	16	0.76	0.2	0.2 (0.90)		0.1 (0.92)	
CC: Region 3 (cm²)	20	0.63	0.2	24	0.64	0.1	21	0.63	0.1	16	0.70	0.1	0.6 (0.58)		1.4 (0.25)	
CC: Region 4 (cm²)	20	0.55	0.2	24	0.62	0.1	21	0.61	0.2	16	0.63	0.2	0.8 (0.48)		1.6 (0.21)	
CC: Region 5 (splenium) (cm²)	20	1.04	0.4	24	1.21	0.3	21	1.18	0.3	16	1.22	0.2	1.5 (0.23)		2.4 (0.13)	
CC: Length (cm)[d]	18	6.75	0.7	24	6.75	0.8	21	6.99	0.4	16	7.41	0.5	3.9 (0.01)	123,4	9.9 (0.002)	0.1 (0.99)
CC: Length (cm)	20	6.26	1.8	24	6.75	0.8	21	6.99	0.4	16	7.41	0.5	3.9 (0.01)	123,234	11.5 (0.001)	1.6 (0.13)
CC/brain midsagittal area	20	0.614	0.18	24	0.631	0.12	21	0.602	0.09	14	0.611	0.10	0.3 (0.83)		0.1 (0.80)	
CC/brain midsagittal area, Region 1 genu	20	0.171	0.05	24	0.171	0.04	21	0.165	0.03	16	0.175	0.02	0.3 (0.85)		0.1 (0.84)	
CC/brain midsagittal area, Region 2[d]	18	0.117	0.02	24	0.108	0.04	21	0.100	0.02	16	0.099	0.02	1.5 (0.22)		5.4 (0.02)	
CC/brain midsagittal area, Region 2	20	0.111	0.04	24	0.108	0.04	21	0.100	0.02	16	0.099	0.02	0.7 (0.57)		2.0 (0.16)	
CC/brain midsagittal area, Region 3	20	0.092	0.03	24	0.092	0.02	21	0.088	0.02	16	0.091	0.02	0.2 (0.93)		0.1 (0.71)	
CC/brain midsagittal area, Region 4	20	0.082	0.03	24	0.089	0.02	21	0.085	0.02	16	0.084	0.02	0.4 (0.44)		0.0 (0.96)	
CC/brain midsagittal area, Region 5	20	0.154	0.06	24	0.172	0.04	21	0.163	0.03	14	0.161	0.04	0.8 (0.49)		0.1 (0.79)	
Total CV: midsagittal area (cm²)	20	7.4	0.8	24	8.4	1.6	21	7.9	1.3	16	8.4	1.6	3.4 (0.02)	13,234	5.2 (0.03)	2.7 (0.009)
CV: lobules I–V midsagittal area (cm²)	20	2.9	0.4	24	3.3	0.5	21	3.1	0.4	16	3.4	0.5	5.4 (0.002)	13,23,24	8.2 (0.005)	3.3 (0.002)
CV: lobules VI–VII midsagittal area (cm²)	20	2.1	0.4	24	2.4	0.6	21	2.4	1.0	16	2.4	0.5	1.1 (0.35)		1.9 (0.18)	
CV: lobules VIII–X midsagittal area (cm²)	20	2.5	0.3	24	2.7	0.7	21	2.4	0.3	16	2.7	0.4	2.5 (0.07)		1.6 (0.21)	
Total CV/Brain midsagittal area	20	1.1	0.2	24	1.2	0.2	14	1.1	0.2	14	1.1	0.1	0.9 (0.41)		0.1 (0.79)	

CC, corpus callosum; CV, cerebellar vermis; L, left; R, right; T: t statistic from ANOVA a priori contrast; FAS, fetal alcohol syndrome; PFAS, partial FAS; SE, static encephalopathy; AE, alcohol exposed; ND, neurobehavioral disorder.

Duncan: multiple comparison range test; commas separate groups with homogeneous means at $p < 0.05$. LT: ANOVA unweighted linear trend across the four study groups. All significant linear trends remain significant with exclusion of the Control group.

[a]Only reported when overall ANOVA is statistically significant.
[b]Numerator degrees of freedom = 3; denominator df = total sample size minus 4.
[c]Numerator degrees of freedom = 1; denominator df = total sample size minus 4.
[d]Two subjects with agenesis and hypogenesis of the corpus callosum removed from FAS/PFAS group.

Putamen. Absolute Volume: The mean absolute volume of the putamen decreased significantly progressing across the 4 study groups from Control to FAS/PFAS (Table 2). This trend was no longer statistically significant ($F = 3.4$; df: 3,74; $p = 0.07$) after removal of the 2 subjects with ACC and HCC. The mean volume of the putamen for the FAS/PFAS group was significantly smaller (13% smaller) than the mean of the Control group. When the 2 subjects with ACC/HCC were excluded from the FAS/PFAS group, the mean volume of the putamen was 12% smaller than the mean of the Control group. This difference was no longer statistically significant ($t = 1.8, p = 0.08$).

Relative Volume: The mean relative volume of the putamen was comparable across all 4 study groups. No differences in absolute or relative size were observed between the right and left putamen.

Hippocampus. Absolute Volume: The mean absolute volume of the hippocampus decreased significantly progressing across the 4 study groups from Control to FAS/PFAS (Table 2). The mean absolute volumes of the hippocampus for each of the 3 FASD groups were significantly smaller (9–16% smaller) than the mean of the Control group.

Relative Volume: The mean relative volume of the hippocampus was comparable across the 4 study groups. No differences in absolute or relative size were observed between the right and left hippocampus.

Corpus Callosum. Absolute Area: The midsagittal area of the CC and each of the 5 subregions were 6–15% smaller in size (although not significantly) in each of the FASD groups relative to the Control group (Table 2). Exclusion of the 2 subjects with ACC/HCC did not alter these outcomes. When the 3 alcohol-exposed groups were combined, the mean midsagittal area of the genu (region 1) was significantly smaller (9%) (mean: 1.22 cm^2, 0.2 SD) relative to the Control group (mean: 1.34 cm^2, 0.2 SD) ($t = 2.0$; $p = 0.03$). This difference remained significant with exclusion of the 2 subjects with ACC/HCC. The mean absolute length of the corpus callosum decreased significantly progressing across the 4 study groups from Control to FAS/PFAS (with and without the ACC/HCC). The mean length of the corpus callosum for the FAS/PFAS group was significantly shorter (16%) than the mean of the Control group. The mean length of the corpus callosum for the FAS/PFAS group remained significantly shorter (9%) than the mean of the Control group after removal of the 2 subjects with ACC/HCC. Visual inspection of the CCs revealed substantial variability in the shape of the CC, even within the Control group (Fig. 2C).

Relative Area: The mean relative midsaggital area of the CC and its 5 subregions (after adjustment for total brain midsaggital area) were comparable across all 4 groups. When the 2 subjects with ACC/HCC were removed from the FAS/PFAS group, a significant increase in the relative size of Region 2 was observed, progressing across the 4 study groups from the Control group to the FAS/PFAS group.

Cerebellar Vermis. Absolute Area: The mean absolute midsagittal areas of the cerebellar vermis (CV) and lobules I–V were significantly smaller in the FAS/PFAS group (12 and 15%, respectively) than the mean of each area in the Control group (Table 2). Removal of the 2 subjects with ACC/HCC had no impact on these outcomes.

Relative Area: The mean relative midsagittal area of the CV and its 3 regions were comparable across all 4 groups.

Radiologist Review of MRI. Visual inspection of the study MRIs by the neuroradiologist on the investigative team identified the following prevalence of structural abnormalities: Control ($n = 0$, 0.0%), ND/AE ($n = 0$, 0.0%), SE/AE ($n = 3$, 12.5%), FAS/PFAS ($n = 3$, 15.0%). Three subjects in the FAS/PFAS presented with the following anomalies: 1 ACC, 1 HCC, and 1 with a 13 mm lesion in the left cerebellar hemisphere and slight deformity of the fourth ventricle. Three subjects in the SE/AE group presented with Chiari 1 malformations. Only the ACC and HCC were known to be present prior to the subjects' enrollment in the study. The neuroradiologist was masked to the subject's FASD diagnosis and study group assignment.

Size of Brain Regions Between FASD Subjects With and Without the FAS Facial Phenotype (Primary Hypothesis 2)

The mean absolute size of the frontal lobe, putamen, hippocampus, and CV were significantly smaller in the FAS/PFAS group than the SE/AE group (Table 2). The children with FAS/PFAS also had disproportionately smaller frontal lobes than the children with SE/AE (Table 2, Fig. 3). It is important to note that the only diagnostic feature that distinguishes the FAS/PFAS group from the SE/AE group in this study is the presence of the FAS facial phenotype in the former. Both groups had comparably severe CNS dysfunction (Astley et al., 2009b). The FAS Facial *D*-Score is a continuous measure of the magnitude of expression of the FAS facial features: the higher the *D*-Score, the more severe the expression of the FAS facial features (Astley and Clarren, 2001). The size of the following brain regions decreased significantly (Pearson Correlation Coefficients with two-tailed p values < 0.05) as the FAS Facial *D*-Score increased: frontal lobe volume (absolute size: −0.261, relative size: −0.215); midsagittal area of cerebellar vermis (absolute size: −0.299); and caudate volume (absolute size: −0.362, relative size: −0.262).

Structural Abnormality Correlations With FASD Group and CNS Function (Primary Hypothesis 3)

The number of subjects in each FASD study group, with 1 or more brain regions that were 2 or more SDs below the mean of the Control group, increased significantly as one advanced from the ND/AE to the SE/AE to the FAS/PFAS group (χ^2 for trend: 4.8, $p = 0.03$) (Fig. 4A).
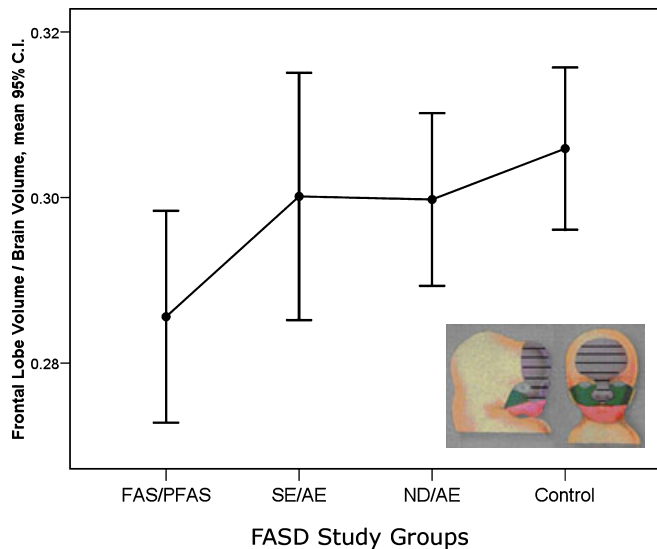
Fig. 3. The relative volume (cc) of the frontal lobe was significantly smaller in the fetal alcohol syndrome (FAS)/partial FAS (PFAS) group (after exclusion of the 2 subjects with agenesis of the corpus callosum/ hypogenesis of the corpus callosum) compared with each of the other study groups. The FAS/PFAS group is the only group with the full FAS facial phenotype. Morphogenesis of the middle and upper face is heavily influenced by signals emanating from the forebrain to the frontonasal prominence (Marcucio et al., 2005). The frontonasal prominence is the striped region in the insert depicting a 5-week (left) and 10-week (right) fetus (Moore et al., 1994). C.I.: confidence interval; FASD, fetal alcohol spectrum disorders; SE, static encephalopathy; AE, alcohol exposed; ND, neurobehavioral disorder; FAS, fetal alcohol syndrome; PFAS, partial FAS.

Importantly, this demonstrated that even when the FAS facial phenotype was missing and the level of brain dysfunction was mild to moderate (4-Digit CNS Rank 2 in ND/AE group), there was a significant increased risk of underlying structural brain abnormality relative to the Control group (Fisher exact test, $p = 0.005$). Further evidence of structural brain abnormality along the full continuum of mild to severe brain dysfunction was observed by dividing the 81 subjects into 3 groups based on their 4-Digit CNS Rank: Rank 1 (no dysfunction), Rank 2 (mild to moderate dysfunction), and Rank 3 (severe dysfunction). The absolute size of all brain regions (with the exception of the CC, putamen, and CV) decreased significantly and incrementally progressing from CNS Rank 1 to 2 to 3. The only measures of the CC that decreased significantly with increasing CNS Rank were CC length and CC region 1 midsagittal area. Figure 4B serves to illustrate one of these findings, the significant decrease in the mean absolute volume of the caudate progressing from CNS Rank 1 to 2 to 3 (ANOVA overall $F = 13.5$; df: 2,77; $p < 0.001$: unweighted linear trend $F = 25.8$; df: 1,74; $p < 0.001$). The Duncan range test confirmed that all 3 mean caudate volumes were significantly distinct from one another.

Size of Brain Regions and Alcohol Exposure

Reported prenatal alcohol exposure patterns (frequency, quantity, and duration) across the 3 FASD study groups are
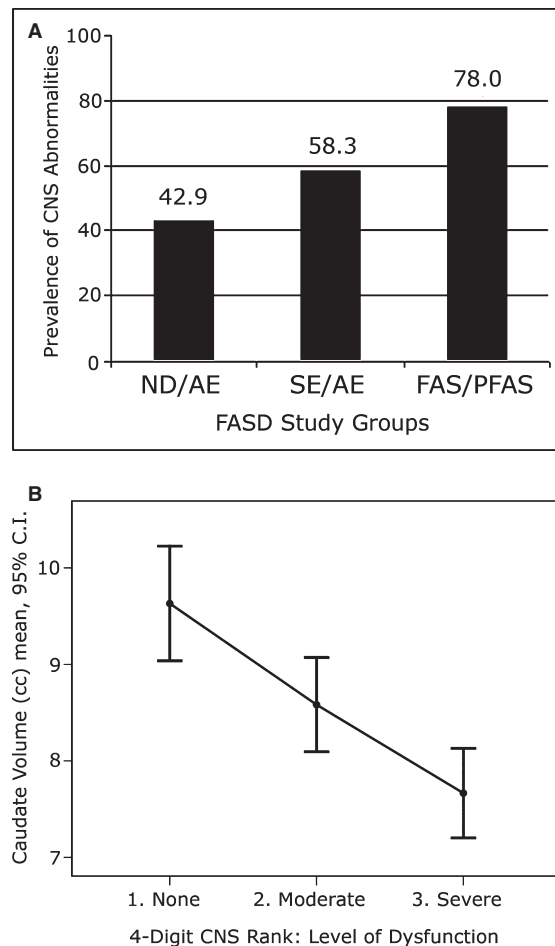
Fig. 4. (**A**) Prevalence of subjects in each FASD study group that had 1 or more brain regions, 2 or more SDs below the mean size observed in the Control group. (**B**) Mean absolute caudate volume decreased as a global measure of brain function (4-Digit Code CNS Rank) increased in impairment. C.I., confidence interval; FASD, fetal alcohol spectrum disorder; SE, static encephalopathy; AE, alcohol exposed; ND, neurobehavioral disorder; FAS, fetal alcohol syndrome; PFAS, partial FAS; CNS, central nervous system.

presented in detail in the neuropsychological/psychiatric report for this study (Astley et al., 2009b) and briefly in Table 1. Of the 65 subjects with FASD, 64 had confirmed prenatal alcohol exposure and one with full FAS had an unknown exposure. More detailed information on quantity, frequency, and/or trimester of alcohol use was available on 53 of the 65 alcohol-exposed subjects. The size of various brain regions decreased significantly and incrementally among the subjects with FASD with increasing frequency, quantity, and/or duration of reported alcohol exposure.

Days Per Week. Significant inverse correlations (Pearson Correlation Coefficients with p values < 0.05) were observed between the average number of days per week of drinking during pregnancy and the size of the following brain regions: midsagittal area of the brain (−0.306), absolute (−0.435) and relative (−0.306) volumes of the hippocampus, and CC length (−0.327).
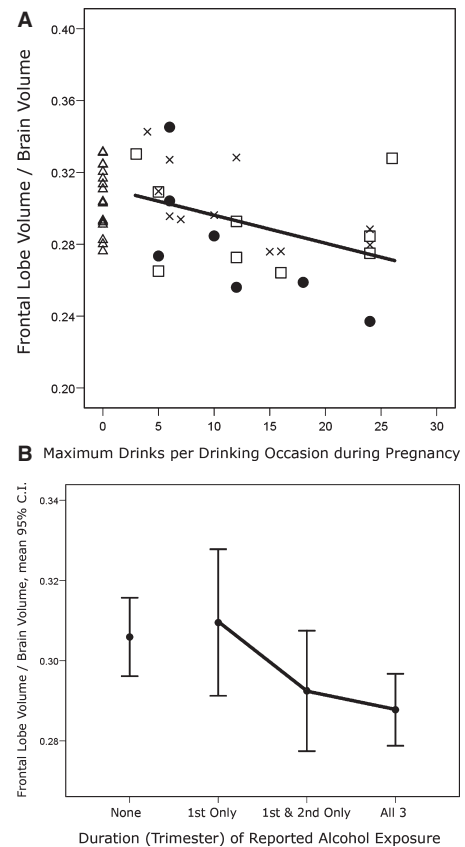
Maximum Drinks Per Drinking Occasion. Significant inverse correlations were also observed between the maximum number of alcoholic drinks consumed per drinking occasion during pregnancy and the size of the following brain regions: relative volume of the frontal lobe (−0.430) (Fig. 5*A*), relative volume of the caudate (−0.373), and the absolute (−0.463) and relative (−0.538) volumes of the hippocampus.

Trimester of Exposure. A significant linear decrease in the mean relative volume of the frontal lobe was also observed when the FASD study sample was divided into groups based on their duration of exposure [exposure through first trimester only ($n = 10$, mean: 0.31, SD: 0.03); through the second trimester only ($n = 9$, mean: 0.29, SD: 0.02); and through all 3 trimesters ($n = 32$, mean: 0.28, SD: 0.02); ANOVA overall $F = 3.1$; df: 2,50; $p = 0.05$; unweighted linear trend, $F = 6.1$; df: 1,50; $p = 0.017$] (Fig. 5*B*). Post hoc comparison tests confirmed that the mean relative volume of the frontal lobe was significantly smaller among those with 3 trimesters of exposure relative to those with exposure through just the first trimester. Interestingly, the mean relative volume of the frontal lobe of the unexposed Control group (mean: 0.31, SD: 0.02, $n = 16$) was comparable with the 2 FASD groups with exposure through the first and second trimesters, but was significantly larger than the group with exposure through all 3 trimesters.

DISCUSSION

Overall, all 3 primary hypotheses were supported in this study. *Hypothesis 1:* progressing across the 4 study groups from Controls to ND/AE to SE/AE to FAS/PFAS, the mean absolute size of most brain regions decreased significantly in size. The frontal lobe and caudate were disproportionately reduced in size. *Hypothesis 2:* even though the FAS/PFAS and SE/AE groups had comparably severe levels of brain dysfunction (CNS Rank 3), those with the FAS facial phenotype had significantly smaller frontal lobes, frontal lobe gray matter volume, and CV. *Hypothesis 3:* the risk of underlying structural abnormalities increased linearly as one advanced from ND/AE to SE/AE to FAS/PFAS, relative to Controls. Most notably, 43% of subjects in the ND/AE group had 1 or more brain regions that were 2 or more SDs below the mean of the Control group, despite only mild to moderate brain dysfunction, no FAS facial phenotype, and the absence of microcephaly. MRI provided further validation that ND/AE, SE/AE, and FAS/PFAS, as defined by the FASD 4-Digit Code, are 3 clinically distinct and increasingly more affected diagnostic subclassifications under the umbrella of FASD. The significant inverse correlations between quantity, frequency, and timing of alcohol exposure and size of selected brain regions provided further evidence of a potential causal association between alcohol exposure and structural brain abnormality observed in this study population. It is important to note that the findings reported in this study are reflective of the 4-Digit Code definitions used to generate the

Fig. 5. (**A**) The relative volume of the frontal lobe decreased significantly with increasing maximum number of alcohol drinks per drinking occasion during pregnancy among subjects with FASD (Pearson Correlation Coefficient −0.430, $p < 0.02$). (**B**) A significant linear decrease in the mean relative volume of the frontal lobe was also observed when the FASD study sample was divided into groups based on their duration of exposure (exposure through first trimester only, $n = 10$, mean: 0.31, SD: 0.03; through the second trimester only, $n = 9$, mean: 0.29, SD: 0.02; and through all 3 trimesters, $n = 32$, mean, 0.28, SD: 0.02) (ANOVA overall $F = 3.1$; df: 2,50; $p = 0.05$; unweighted linear trend, $F = 6.1$; df: 1,50; $p = 0.017$). The unexposed Control group is plotted for visual comparison. C.I., confidence interval; FASD, fetal alcohol spectrum disorder. Circle = FAS/PFAS, square = SE/AE, X = ND/AE, triangle = control.

FASD study groups. While all FASD diagnostic systems (Astley, 2004; Bertrand et al., 2004; Chudley et al., 2005; Hoyme et al., 2005) share some common clinical terminology (e.g., FAS and PFAS), the criteria used to define these diagnostic classifications are not the same.

FASD Diagnostic Classification Systems

When comparing outcomes across published FASD MRI studies, it is important to note that not all investigations use the same FASD diagnostic criteria to establish their FASD study groups. In many fields, readers of research data can draw their own conclusions about the comparability of diagnostic subgroups because the diagnostic criteria are widely established, or in newer fields of study, the diagnostic methods would be presented in sufficient detail. At present the FASD MRI literature lacks the diagnostic detail necessary

when a field has not yet settled on common diagnostic standards. Most FASD MRI studies to date have classified their subjects into 1 or more of the following groups: (i) an alcohol-exposed group with FAS facial features (FAS), (ii) an alcohol-exposed group without FAS facial features (e.g., ARND or FAE); or (iii) a group in which all FASD subgroups are combined into 1 group with PEA. Criteria used to define FAS groups are typically described qualitatively (e.g., known prenatal alcohol exposure, craniofacial anomalies consistent with FAS, prenatal or postnatal growth deficiency or both, and CNS dysfunction). Criteria used to define ARND groups are also described qualitatively (e.g., exposed to high levels of alcohol but display few or none of the facial features characteristic of FAS, and do not show growth retardation). But these qualitative descriptions do not adequately convey what goes into the classifications of FAS, ARND, or FAE. Missing are parameters such as what level of growth deficiency must be present, what level of CNS dysfunction must be present, which FAS facial features must be evident, and how severe the facial anomalies had to be. Without this information, it is not clear if clinically distinct study groups have been established or how they are clinically distinct. Nor can it be determined if the FAS/FASD group in 1 study is comparable with the FAS/FASD group in another study. As the field has matured, there is now clear consensus that more rigorous diagnostic methods should be used and several more rigorous methods have been published (Astley, 2004; Bertrand et al., 2004; Chudley et al., 2005). Two groups of investigators (Astley and Clarren, 2000; Hoyme et al., 2005) have demonstrated in 2 large clinical populations that the majority (50–87%) of FAS diagnoses rendered using gestalt diagnostic methods did not meet FAS criteria when more rigorous diagnostic criteria were applied.

To help bridge the gap between the different diagnostic terms used in the FASD literature, our ND/AE and SE/AE groups would be most comparable with mild and severe ARND, respectively. Our FAS/PFAS group will differ from other groups labeled FAS or PFAS if the diagnostic criteria used to establish the groups differ. Our FAS/PFAS, SE/AE, and NE/AE groups span the entire continuum of FASD. The effort here is to describe groups across the full fetal alcohol spectrum and to retain sufficient statistical power to detect clinically meaningful differences between diagnostic subgroups. Combining possible subtypes into 1 larger alcohol-exposed group may obscure actual differences between these subtypes. In the current study, most neuroimaging differences would not have been revealed if the 3 FASD study groups had been combined in any way and compared with the Controls. We suggest the following questions as standards for review and interpretation of current and future literature on neuroimaging and behavioral outcomes in FASD: (i) What diagnostic criteria are used to establish the study groups? (ii) Has the magnitude of growth deficiency, FAS facial features, CNS structural/functional abnormality, and alcohol exposure been reported to confirm that study groups are clinically

distinct? (iii) When significant differences between groups are not observed, does the study have clinically distinct groups and sufficient sample size/power to support null findings? and (iv) When outcomes between FAS groups differ between studies, is this because outcomes differ or the definition of FAS differs?

MRI FASD Literature

In general, the FASD MRI literature documents significant reductions in the size of many but not all brain regions studied, when comparing full FAS to a healthy control group. The current study supports and extends these findings. The FASD MRI literature also documents marginal effects in PEA, FAE, or ARND groups relative to FAS or healthy controls, but these differences are rarely statistically significant. The current study provides definitive evidence that when these nondysmorphic FASD clinical subgroups are more rigorously defined and subdivided (e.g., SE/AE and ND/AE) (Astley, 2004), significant differences are observed not only when each of these FASD groups is compared with a healthy control group, but also when each is compared with the other. These findings provide compelling evidence that clinically meaningful and distinct subgroups do exist under the umbrella of FASD, and at least 2 distinct subgroups exist under the broad classification ARND (e.g., SE/AE and ND/AE). Key outcomes in the current study are discussed below and compared with the FASD MRI literature, with emphasis on findings specific to FASD clinical subgroups and how subgroups are clinically defined.

Total Brain. In the current study, total brain size became incrementally smaller progressing across the 4 study groups from Control to FAS/PFAS. This was influenced, in part, by the criteria used to construct the 4 groups. Microcephaly is a sufficient but not necessary CNS criterion for diagnosis of FAS, PFAS, and static encephalopathy/alcohol-exposed subjects. Of note, however, is the finding that the prevalence of microcephaly was fourfold greater in the FAS/PFAS group relative to the SE/AE group. Both groups were required to meet the same criteria for CNS damage/dysfunction (CNS Rank 3 and/or 4). The only difference between the criteria for the 2 groups was that the FAS/PFAS group had to have FAS facial features (Face Ranks 3 or 4) and SE/AE group had to have normal facial features (Face Ranks 1 and 2). Thus, the fact that microcephaly was far more prevalent in the group with the FAS facial features is a further evidence that 4-Digit Code definition of the FAS face is a marker for underlying structural abnormality. The impact of alcohol on reduction of overall brain size is well documented in the animal and human FASD literature (Archibald et al., 2001; Hoyme et al., 2005; Mattson and Riley, 1995; Sowell et al., 2001b; Stratton et al., 1996). The current study provided definitive evidence of the continuum of effects across the full spectrum, not just among those with FAS.

Frontal Lobe. The most notable and novel finding in the current study was a significant reduction in absolute and relative volume of the frontal lobe in the FAS/PFAS relative to all other groups including the SE/AE group. The significant difference observed between FAS/PFAS and SE/AE indicates individuals with FASD and the FAS facial features have smaller frontal lobes than those with FASD, but no facial features, despite the fact that both groups had comparably severe cognitive/behavioral impairment (4-Digit CNS Rank 3: 3 or more domains of function, 2 or more SDs below the mean) (Astley et al., 2009b). Archibald and colleagues (2001) using a study sample of 14 FAS and 12 PEA by gestalt diagnosis and 41 controls and Sowell and colleagues (2001b) using a subset of the Archibald study sample, 14 FAS and 7 PEA by gestalt diagnosis and 21 controls, were unable to detect significant differences in absolute or relative measures of frontal lobe volumes between FAS, PEA, and controls. Further analysis of the Sowell and colleagues (2001b) study population (Sowell et al., 2002a) using surface-based image analysis revealed a significant reduction in the absolute volume and a significant increase in the relative volume of the frontal lobe, when the 14 FAS and 7 PEA were combined and compared with 21 controls. Wass and colleagues (2001) reported significant reductions in the absolute size of the frontal cortex associated with prenatal alcohol exposure in an ultrasonographic study of 167 pregnant women. The percent of fetuses with a frontal cortex below the 10th percentile increased from 4% for nonexposed fetuses to 23% for heavily exposed fetuses.

Frontal Lobe Gray–White Matter. Although we observed significantly smaller absolute volumes of white matter and gray matter in the frontal lobes among subjects with FAS/PFAS relative to Controls, the proportion of the frontal lobe that contained white matter did not vary significantly across the 4 study groups. These findings are consistent with the literature. Archibald and colleagues (2001) identified a significant proportional reduction of white matter across the entire cerebrum among subjects with a gestalt diagnosis of FAS relative to Controls. When their analysis was repeated for each lobe (frontal, parietal, temporal, and occipital), a significant proportional reduction of white matter was only observed in the parietal lobe. When subsets of these children were further assessed by Sowell and colleagues (2001b, 2002a) proportional reductions in white matter were observed in the left hemisphere perisylvian cortices of the temporal and parietal lobes (not in the frontal lobes) among alcohol-exposed subjects relative to Controls.

Caudate. The caudate, lenticular nuclei, some subthalamic nuclei, and the substantia nigra are subcortical gray matter structures that form the basal ganglia (Cote and Crutcher, 1990). The caudate nucleus has extensive neural connections to the frontal lobes and is thought to mediate higher cognitive and executive function. Like the frontal lobe, the absolute and relative volume of the caudate was significantly reduced in the current study but unlike the frontal lobe this reduction in size was observed in both the FAS/PFAS and SE/AE groups relative to the Control group. Archibald and colleagues (2001) reported that the absolute and relative size of the caudate was significantly reduced in 14 children with a gestalt diagnosis of FAS relative to 41 controls. But no significant caudate reductions were observed among their group of 12 children with PEA. Cortese and colleagues (2006) assessed caudate size in an fMRI study with a very small sample of children (7 with FAS and 4 with FAE by gestalt diagnosis, and 4 controls). They reported significantly smaller absolute, but not relative, caudate volumes in the FAS group relative to controls, but the unconventional use of one-tailed p values increased the probability of achieving statistical significance. No caudate differences were detected between their FAE and control groups. It is noteworthy that no significant differences in intracranial volume were detected between their FAS, FAE, and control groups.

Putamen. The putamen (part of the lenticular nuclei) together with the caudate form the striatum. The putamen is innervated by neurons from the primary motor, premotor, supplementary motor, and somotosensory cortices, whereas the caudate receives input from frontal eye fields and association areas of the frontal and parietal lobes (Cote and Crutcher, 1990; Giedd et al., 1994). In the present study, the absolute but not relative volume of the putamen was significantly smaller in the FAS/PFAS group relative to the SE/AE, ND/AE, and Control groups. Mattson and colleagues (1996) in a study of 6 children with FAS (by gestalt diagnosis) reported that the absolute but not relative volume of the lenticular nuclei was significantly smaller relative to 7 controls. Archibald and colleagues (2001) reported no significant absolute or relative reduction in the size of the lenticular nucleus among 14 FAS or 12 PEA (by gestalt diagnosis) when compared with 41 controls.

Hippocampus. The hippocampus is a structure of the limbic system involved in learning, memory storage, and retrieval (Eichenbaum et al., 1992). In the present study, the mean absolute volume of the hippocampus decreased significantly, progressing across the 4 study groups from Control to FAS/PFAS. In contrast, Archibald and colleagues (2001) reported a disproportionate sparing of the hippocampus in an otherwise hypoplastic brain among 14 FAS participants (by gestalt diagnosis) relative to 41 controls.

Corpus callosum. The CC is a large bundle of nerve fibers connecting the 2 hemispheres of the brain. CC deficits have been linked to deficits including intellectual functioning, learning, memory, executive function, and attention. In this study, the absolute midsagittal area of the genu (Region 1) and the length of the CC were comparably small across the FASD groups and significantly smaller relative to the Control group. No significant differences were observed after adjustment for midsagittal brain area. Riley and colleagues

(1995) reported significant decreases in the absolute midsagittal area of the CC and regions 1, 3, 4, and 5 among 11 alcohol-exposed subjects (9 with FAS by gestalt diagnosis) relative to 12 controls. After adjustment for midsaggital brain area, regions 1 (genu), 4, and 5 (splenium) remained significantly smaller but overall midsagittal area did not. The more severe outcomes reported by Riley and his colleagues were not due to differences in diagnostic methodology, as our outcomes reflected our entire alcohol-exposed group. It does appear the alcohol-exposed group in the study by Riley and colleagues was more severely impaired than our alcohol-exposed group. They had a substantially lower FSIQ and were drawn from a sample with a higher estimated prevalence of ACC (3/44 or 6.8%). In a more recent study, Sowell and colleagues (2001a) observed the size, shape, and location of the CC among 20 alcohol-exposed subjects (13 with FAS by gestalt diagnosis) and 21 controls. Again, in contrast to our findings, they reported significant reductions in the absolute midsagittal area of the CC and all 5 subregions in their alcohol-exposed group relative to their control group. Their more severe findings could be explained by the much higher prevalence of microcephaly in their alcohol-exposed population (they report "most" were microcephalic). Only 18% of our alcohol-exposed group was microcephalic. After adjustment for brain volume, only the splenium in their alcohol-exposed group remained significantly smaller. Their key finding was a significant anterior-inferior displacement of the splenium in the alcohol-exposed relative to controls. Based on their published Figs. 2 and 3 illustrating this effect, this finding would appear to be commensurate to our finding of a shorter CC. Autti-Ramo (2000) reported that CC was significantly shorter (7.4%), the midsagittal area significantly smaller (13.8%), and the width of the splenium significantly smaller (12%) among 17 alcohol-exposed subjects (5 with FAS by gestalt diagnosis) relative to 17 controls. These differences were no longer significant after adjustment for midsagittal area of the brain. Forty-one percent of their alcohol-exposed population was microcephalic. Bookstein and colleagues (2002a,b) reported a variety of shape but not size differences in the CC of subjects with gestalt diagnoses of FASD versus controls. It is important to remember that brain regions are inherently highly variable in shape and size, even in typically developing individuals (Steen et al., 2007). This is clearly evident in our Control population. They present with extraordinary variability in CC shape (Fig. 2C) despite their high level of cognitive function and absence of alcohol exposure. One final comparison with the literature is warranted. Miller and colleagues (1999) reported that the absolute size of the CC was significantly larger in ethanol-exposed nonhuman primates relative to unexposed controls. The number of axons (not the axon size or myelin thickness) was significantly greater, especially in the rostral half of the CC (anterior to where the fornix joins the CC). It is important to note that total brain volume was comparable between the exposed and unexposed groups, no animal had microcephaly, and the alcohol-exposed animals had mild to moderate cognitive dysfunction with possibly only 1 animal having full FAS. Several human studies, including ours, have reported sparing of some anterior callosal regions in their alcohol-exposed group. We observed a significant relative sparing of CC Region 2. Riley and colleagues (1995) observed significant absolute and relative sparing of CC Region 2 and Sowell and colleagues (2001a) reported relative but not absolute sparing of anterior callosal regions.

What appears to be 1 recurring theme across several FASD studies is that the CC is reduced in length and the regions most impacted are the genu and splenium. These findings are consistent with the developmental spectrum of ACC. CC abnormalities are present in a multitude of syndromes and disease entities (Paul et al., 2007). ACC is not specific to prenatal alcohol exposure. ACC is present in 2 to 3 per 100 individuals with general developmental disability (Jeret et al., 1986). The CC is the major commissure forming a junction between the cerebral hemispheres. The formation of the CC starts with the development of the genu in week 11; the body, isthmus and splenium develop at a later stage through week 20 of gestation (Paul et al., 2007). If the normal developmental process is disturbed, the CC may be absent completely or partially ("hypogenetic"). As the developmental process starts from the anterior part and progresses from front to rear, when the CC is hypogenetic, it is usually the posterior portion that is affected the most (the posterior body and the splenium) (Fig. 2B).

Cerebellar vermis. The cerebellum is located at the base of the brain and is involved in motor, cognitive, and affective functions (Vidal et al., 2006). The absolute but not the relative midsagittal area of the CV and lobules I–V were significantly smaller in the FAS/PFAS group relative to the Controls and SE/AE in the current study. Sowell and colleagues (1996) reported a significant reduction in the absolute midsagittal area of lobules I–V in a group of alcohol-exposed subjects (6 FAS and 3 PEA by gestalt diagnosis) relative to 24 controls. They did not report if these findings remained significant when adjusted for overall brain size. More recently, O'Hare and colleagues (2005) reported significant reductions in the midsagittal area of lobules I–V and the VIII–X in a group of alcohol-exposed subjects (14 FAS and 7 PEA by gestalt diagnosis) relative to 21 controls. After correction for brain size, lobule I–V remained significantly reduced in size. Goodlett and colleagues (1990), using an animal model, demonstrated that the number of Purkinje cells was significantly reduced in earlier maturing regions of the CV (lobules I–V and VIII–X) after a single critical day of neonatal alcohol exposure. Notably, the Purkinje cells in the later maturing neocerebellar vermis (lobules VI and VII) were apparently spared.

Prevalence of CNS Structural Abnormalities Among FASD Clinical Subgroups

From a FASD diagnostic perspective, it is interesting to note how many subjects within all 3 FASD clinical subgroups

had 1 or more brain regions, 2 or more SDs below the mean of the Control group. Although we reported the prevalence of anomalies in our FASD study groups using our research control group as the reference, this would not be an appropriate practice in a clinical setting. Norms for the size of brain regions adjusted for gender and age must be established using large, representative, population-based samples, rather than small, convenient research control samples. The National Institutes of Health (NIH) MRI Study of Normal Brain Development (Waber et al., 2007) is a landmark study that is documenting structural brain development and behavior longitudinally from birth to young adulthood in a large population-based sample of healthy children targeted to the United States 2000 census distribution.

Validation of the FASD 4-Digit Diagnostic Code

This study further validated (Astley and Clarren, 1996, 2000, 2001; Astley et al., 2002) the measurement scales and procedures for diagnostic classification used in the FASD 4-Digit Diagnostic Code. Statistically significant differences in neuropsychological outcomes (Astley et al., 2009b) and measures of brain structure were observed between the FAS/PFAS, SE/AE, and ND/AE groups, confirming that these groups reflect 3 clinically distinct and increasingly more affected diagnostic subclassifications under the umbrella of FASD. The prevalence/severity of structural brain abnormality increased significantly as dysfunction increased from CNS Rank 1 (no dysfunction) to Rank 2 (mild to moderate dysfunction) to Rank 3 (severe dysfunction). Indeed, when these CNS Ranks were first defined 10 years ago (Astley and Clarren, 2000), the underlying principle was that as the magnitude and breadth of functional impairment increased, the probability of underlying structural abnormality would increase. It is for this reason that the 4-Digit CNS Ranks 1, 2, and 3 were labeled "unlikely," "possible," and "probable" underlying CNS abnormality, respectively (Fig. 1). The finding that the subjects in the FAS/PFAS group with the 4-Digit FAS facial phenotype had significantly smaller frontal lobes than the SE/AE group with no FAS facial phenotype provides the most compelling data yet that this facial phenotype truly is a marker for underlying brain abnormality (Fig. 3). Several animal and clinical studies have documented correlations between midline facial anomalies and underlying brain abnormality caused by prenatal alcohol exposure (Astley and Clarren, 1996, 2001; Johnston, 1975; Sulik, 2005; Sulik and Johnston, 1982; Sulik et al., 1981, 1984). Morphogenesis of the middle and upper face is heavily influenced by signals emanating from the forebrain. Marcucio and colleagues (2005) report that a role of Sonic hedgehog (Shh) in the forebrain is to regulate Shh expression in the face, and that together, these Shh domains mediate patterning within the frontonasal prominence (Fig. 3) and proximodistal outgrowth of the middle and upper face.

The primary limitation of this study is the presence of other prenatal and/or postnatal risk factors, in addition to alcohol, that are capable of adversely impacting brain development. Clinical records for this FASD study sample documented that up to 83% of the mothers reportedly smoked during pregnancy, up to 67% reportedly used illicit drugs during pregnancy, and over 75% of the children were in foster/adoptive care (Astley et al., 2009b). While the significant dose–response associations observed between alcohol exposure and neurostructural alterations in this study provided compelling evidence of alcohol's potential causal role, one cannot rule out the impact of the other risk factors.

In conclusion, the results of this study confirm that significant differences in the sizes of many brain regions exist between children with FASD and their healthy peers. More importantly, when the FASD 4-Digit Code is used to create clinically distinct diagnostic subgroups under the umbrella of FASD (FAS/PFAS, SE/AE, and ND/AE), statistically significant differences in neurostructure are identified between the FASD diagnostic subgroups. Structural abnormalities were prevalent across the full spectrum of FASD. MRI could importantly augment the diagnosis of FASD, once population-based norms for structural development of the human brain are established.

ACKNOWLEDGMENTS

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