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Amygdala Volume in Offspring from Multiplex for Alcohol Dependence Families: The Moderating Influence of Childhood Environment and 5-HTTLPR Variation

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Abstract

Background: The increased susceptibility for developing alcohol dependence seen in offspring from families with alcohol dependence may be related to structural and functional differences in brain circuits that influence emotional processing. Early childhood environment, genetic variation in the serotonin transporter-linked polymorphic region (5-HTTLPR) of the SLCA4 gene and allelic variation in the Brain Derived Neurotrophic Factor (BDNF) gene have each been reported to be related to volumetric differences in the temporal lobe especially the amygdala

Methods: Magnetic resonance imaging was used to obtain amygdala volumes for 129 adolescent/young adult individuals who were either High-Risk (HR) offspring from families with multiple cases of alcohol dependence (N=71) or Low-Risk (LR) controls (N=58). Childhood family environment was measured prospectively using age-appropriate versions of the Family Environment Scale during a longitudinal follow-up study. The subjects were genotyped for Brain-Derived Neurotrophic Factor (BDNF) Val66Met and the serotonin transporter polymorphism (5-HTTLPR). Two family environment scale scores (Cohesion and Conflict), genotypic variation, and their interaction were tested for their association with amygdala volumes. Personal and prenatal exposure to alcohol and drugs were considered in statistical analyses in order to more accurately determine the effects of familial risk group differences.

Results: Amygdala volume was reduced in offspring from families with multiple alcohol dependent members in comparison to offspring from control families. High-Risk offspring who were carriers of the S variant of the 5-HTTLPR polymorphism had reduced amygdala volume in comparison to those with an LL genotype. Larger amygdala volume was associated with greater family cohesion but only in Low-Risk control offspring.

Conclusions: Familial risk for alcohol dependence is an important predictor of amygdala volume even when removing cases with significant personal exposure and covarying for prenatal exposure effects. The present study provides new evidence that amygdala volume is modified by 5-HTTLPR variation in High-Risk families.

Keywords: Amygdala; High-Risk; Alcohol Dependence; Childhood; Environment; 5-HTTLPR; BDNF

Introduction

Numerous studies have documented the neuropathological consequences of chronic alcohol consumption [1-3]. In addition, an emerging literature has identified familial loading for alcohol dependence as a factor influencing brain structure and function [4-8], including reduced amygdala volume in offspring from families with a high-density of alcohol dependent members [9,10]. Structural variation in the amygdala and other components of the limbic network involved in emotion regulation may provide a neurological substrate for excessive use of alcohol and development of Alcohol Dependence (AD).

Structural variation in the amygdala is likely to be influenced by genes that are responsible for neuronal growth and differentiation. One gene that has been studied extensively is the Brain-Derived Neurotrophic Factor (BDNF) gene [11-13]. Variation in BDNF has been associated with amygdala and hippocampal volume, with Met allele carriers showing smaller volume in some studies [14-16] though there are some negative reports [17-19] (Table 1).

Because epistatic effects between genes influence biological outcomes [20,21] including the rate of growth and development of specific brain regions, we considered genes that might interact with BDNF. Selection of an appropriate candidate gene that might interact with BDNF to alter amygdala volume was based on three criteria: (1) the gene is associated with alcohol dependence risk, (2) genetic variation in the gene is associated with differential response to environmental pressures, and (3) the gene has been associated with altered amygdala volume. Variation in the serotonin transporter (5-HTTLPR) gene has been associated with risk for alcohol dependence [22-24] and increased alcohol consumption in response to stress in both man [25,26] and in non-human primates [27]. Based on results from a 30 year longitudinal study, response to stressful life events and emergence of depressive symptoms appears to be influenced by 5-HTTLPR variation [28]. In that study individuals carrying the short (S) allele of the 5-HTTLPR were more likely to exhibit depressive symptoms in relation to stressful life events than homozygous long allele (L) carriers. Similarly, variation in the transporter-linked polymorphic region (5-HTTLPR) has been associated with greater amygdala neuronal activity in response to fearful stimuli among carriers of the S allele [29], as well as volumetric differences in the amygdala [30-34].

Only a few studies have investigated gene by environment

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Table 1A: Association of 5-HTTLPR with Temporal Lobe Volumes (Amygdala or Hippocampus) with and without Early Family Environmental Variation

Author	Sample	Childhood Environment	Gene/Allele	Brain Volume
Pezawas et al 2005 [30]	114 Controls	Not Included	5-HTTLPR S vs LL	↓ Right amygdala (gray) S carriers
Hickie et al 2007 [76]	45 MDD 16 Controls	Not Included	5-HTTLPR SS vs SL vs LL	↔ Total amygdala
Rao et al 2007 [77]	26 Controls	Not Included	5-HTTLPR SS vs LL	↔ Total amygdala
Scherk et al 2009 [32]	37 Bipolar 37 Controls	Not Included	5-HTTLPR S vs LL	↑ Right Amygdala
Beevers et al 2010 [78]	23 Females	Not Included	5-HTTLPR SS vs LL	↔ Total amygdala
Kobiella et al 2011 [33]	54 Controls	Not Included	5-HTTLPR S vs LL	↓ amygdala
Pezawas et al 2008 [31]	111 Controls	Not Included	5-HTTLPR S vs LL with BDNF Met vs Val/Val	↓ Right amygdala S allele with Val/Val
Frodl et al 2010 [35]	24 MDD 27 Controls	CTQ self report (Emotional Neglect)	5-HTTLPR S vs LL	↓ Hippocampus with SS or SL and Neglect
Hill et al present study	71 (62)* High-Risk 58 (41) Low-risk	Family Environment Scale - Cohesion	5-HTTLPR SS or LS vs LL	↓ Total amygdala SS or LS and High Risk for AD

*The number in parentheses is the number genotyped in the present study.

Table 1B: Association of BDNF with Amygdala Volume With and Without Early Family Environmental Variation

Author	Sample	Childhood Environment	Gene/Allele	Brain Volume
Frodl et al 2007 [17]	60 MDD Pts 60 Controls	Not Included	BDNF Met vs. Val/Val	↔ Total amygdala
Sublette et al 2008 [14]	55 Controls	Not Included	BDNF Met vs. Val/Val	↓ Total amygdala
Takahashi et al 2008 [18]	33 Schiz Pts 29 Controls	Not Included	BDNF Met vs. Val/Val	↔ Total amygdala
Montag et al 2009 [15]	87 Controls	Not Included	BDNF Met vs. Val/Val	↓ Right amygdala
Gatt et al 2009 [16]	89 Controls	Early Life Stress Questionnaire (High Stress)	BDNF Met vs. Val/Val	↓ Total amygdala ↑ Total amygdala
Gerritsen et al 2012 [19]	568 Controls	Life Threatening Events	BDNF Met vs. Val/Val	↔ Total amygdala
Hill et al present study	71 (71)* High-Risk 58 (58) Low-Risk	Family Environment Scale – Cohesion	BDNF Met vs. Val/Val	↔ Total amygdala

*The number in parentheses is the number genotyped in the present study.

interactions in predicting brain volumes. Gatt and colleagues [16] reported finding reduction of grey matter in the amygdala and hipocampus in association with childhood life stress and being a BDNF Met carrier. Frodl and colleagues [35] assessed adult patients in treatment for major depression (ages 18-65), finding that 5-HTTLPR S carriers who reported experiencing childhood stress had reduced hippocampus and prefrontal cortex volume. Those who were homozygous for the L allele who reported greater childhood stress had larger prefrontal volumes. In both studies childhood stress was evaluated retrospectively when subjects had reached adulthood. One study that evaluated life event experiences and variation in BDNF did not find differences in amygdala volume [19].

Families of alcoholics have been reported to undergo heightened stress because of the increased family conflict and lesser family cohesion seen within the family system [36]. Because chronic stress contributes to neuronal loss in regions of the limbic system [37], the study of childhood environment and its interaction with genetic variation may provide important clues concerning brain morphology and liability for alcohol dependence. We hypothesized that High-Risk offspring from multiplex alcohol dependence families might experience higher levels of family conflict and reduced family cohesion which might, in turn, result in smaller amygdala volume.

The present study obtained measures of family environment as part of a longitudinal study in which children/adolescents were evaluated annually and MRI imaging performed when the participants reached young adulthood. These data along with banked DNA made it possible to test the effects of the interaction of family environmental characteristics with candidate genes on amygdala volume. Because the candidate genes were chosen based on their effect on the neuronal growth and plasticity of amygdala networks [38] and their potential moderating effects on the impact of childhood adversity [16,35], the interaction between the genes and characteristics of the childhood family environment were tested for their effect on amygdala volume.

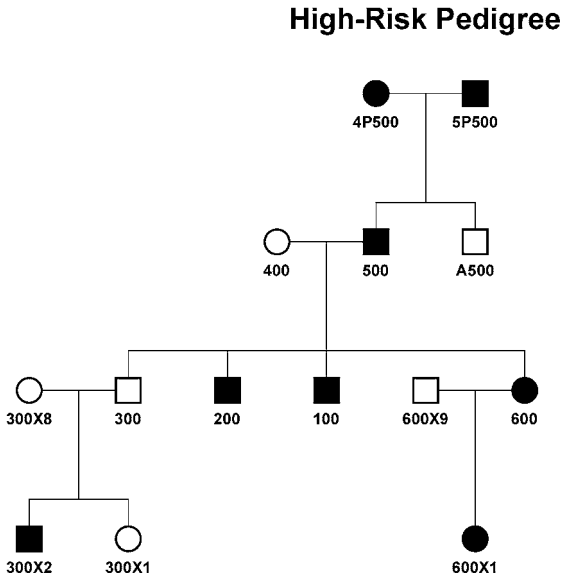

Figure 1: A High-Risk pedigree is shown. Note that while some offspring did not have parents with alcohol dependence as seen here, these offspring were also at increased risk for developing alcohol and other substance use disorders because of the high density of alcohol dependence in their extended family. Also, it should be noted that the study included all siblings of the male proband pair and their offspring as seen in this pedigree. The sister in this pedigree developed alcohol dependence following her pregnancy but did use alcohol while pregnant. Because some women did use alcohol during pregnancy, analyses were performed to adjust for this effect.

Materials and Methods

Participants

The sample included 71 High-Risk subjects from multiplex for alcohol dependence families and 58 Low-Risk control subjects (Table 2). The groups did not differ in mean age, socioeconomic status, gender, Body Mass Index (BMI) or hand preference (95.8% of the High-Risk and 93.1% of control subjects were right handed), though High-Risk offspring had larger intracranial Volume (ICV). All are participants in a larger longitudinal follow-up. This neuroimaging sub-sample had been followed an average of 7 years by the time they were scanned and have now been followed for clinical outcome an average of 11.1 years (± 5.93).

Risk Group Status

Multiplex AD families were identified through the presence of two adult brothers with AD. The multiplex sampling strategy used in this study resulted in a high-density of AD in the targeted pedigrees as previously described [39]. Offspring from the brother pairs and their siblings in the multiplex families provided the High-Risk offspring for the present report. Psychiatric status of the "marrying in" side of the offspring's family was also obtained. Due to the multiplex ascertainment strategy, the offspring had multiple first and second-degree relatives with alcohol dependence (mean = 4.49 ± 1.82). A typical pedigree is shown in (Figure 1). Even those offspring without an alcohol dependent parent were at high genetic risk for AD because of a large number of second-degree relatives with AD (mean = 3.92 ± 1.82).

Low-Risk control families were identified through newspaper advertisements and screening performed to insure that a pair of adult brothers without alcohol or drug dependence with offspring were available. Additionally, the parents and any additional siblings of the index pair were screened for absence of alcohol or drug dependence. This design provided minimal alcohol and drug dependence in these control families. Although the parental AD among Low-Risk offspring was infrequent in the target families (< 5%) it did occur where "marrying in" spouses had AD.

Prenatal Use of Substances by Mothers of the Offspring

Mothers of both High and Low-Risk offspring were administered a structured interview designed to assess quantity and frequency of use of alcohol, drugs and cigarettes during pregnancy. Although the assessment was retrospective, being obtained at the child's first longitudinal assessment, usually before the age of 12 years, the information obtained would appear to be valid. Comparison of prospective and retrospective data for drinking during pregnancy has shown retrospective data to be valid [40]. Moreover, follow-up of women for 4 and 5 years following their pregnancies has shown substantial reliability (r = 0.53 and 0.67, respectively) between reports obtained during pregnancy and those obtained following the pregnancy [41,42].

Personal History of Psychiatric Disorders

In order to control for variables other than familial/ genetic risk that might influence brain morphology, careful attention was paid to personal history of substance use and other psychiatric disorders along with prenatal exposure. Because the participants are enrolled in an ongoing longitudinal study that has followed youngsters from childhood through young-adulthood, extensive clinical information was available for determining if any psychiatric disorder including substance use disorder was present by the time the MRI assessment was performed. Children/adolescents were assessed yearly with the K-SADS [43] using separate interviews of parent and offspring to

Table 2: Characteristics of High and Low-risk Subjects by Age, Body Mass Index (BMI), Intracranial Volume (ICV) and Socioeconomic Status (SES) (Means and Standard Deviations)

	High-Risk N = 71		**Low-Risk N = 58**				
	Mean	S.D.	Mean	S.D.	F	df	p
Scan Age	18.21	4.20	17.71	5.79	0.33	1,127	NS
BMI	24.0	5.05	24.45	5.47	0.23	1,127	NS
ICV	1425.63	124.37	1348.53	123.32	12.36	1,127	0.001
SES*	41.83	11.69	44.53	10.82	1.82	1,127	NS
Age at First Visit	11.21	3.29	12.07	3.65	1.97	1,127	NS
Age at Last Visit	22.97	4.52	22.45	5.44	0.35	1,127	NS

Socioeconomic Status (SES) was determined using the Hollingshead Four Factor Index of Social Status [79].

determine the presence or absence of Axis I DSM-III diagnoses. (DSM-III was the current methodology at the initiation of the study in 1989). Young adults were assessed using the CIDI diagnostic instrument [44] to obtain DSM-IV Axis I diagnoses. There were no exclusion criteria applied based on presence of psychiatric disorder.

Family Environment Data

The Family Environment Scale (FES) [45] or a childhood version of the scale (CVFES) was administered to all offspring at their baseline assessment (mean age = 13.46 years, SD = 1.77). Alternate forms were used to insure that age appropriate versions were administered. The FES contains 90 true-false items designed to assess three dimensions within the family environment including relationships, personal growth, and system maintenance. The reliability and validity of the FES is well-supported, and an extensive body of research shows good internal consistency and stability for the FES subscales when applied to diverse samples [46]. In a sample of alcoholic and control families (N=356) internal consistency for the Cohesion and Conflict subscales was 0.76 and 0.72, respectively. The CVFES [47] is a 30 item pictorial, multiple choice measure designed for children ages 5-12 to assess conceptual dimensions identified in the FES. Three equivalent pictures representing a mother, father, a son and a daughter are depicted in cartoon characters. The pictures are identical except for one feature which indicates the FES concept being tested. The subscales of the CVFES and FES are identical. The test-retest coefficient for the subscale is 0.80.

MRI Structural Acquisition Methods

All subjects were scanned during adolescence/young adulthood on a GE 1.5 Tesla scanner located in the Department of Radiology MR Research Center. T1 weighted axial images with slice thickness of 1.5 mm were obtained using a 3 dimensional spoiled gradient recalled echo in the steady state (3D SPGR) (TE = 5, TR = 24, flip angle = 45 degrees, acquisition matrix = 192 X 256, NEX = 1, FOV = 24 cm). Slices were resliced in the coronal plane through the anterior commissures to provide a more reproducible guide for image orientation. Additionally, axial proton density and T2 weighted images were obtained covering the whole brain at a slice thickness of 5 mm, slice gap = 0 mm ([double echo spin echo, TE = 17 ms and 102 ms; TR = 3000 ms], acquisition matrix = 256 X 192, NEX = 1, FOV =24 cm). Obtaining the dual echo scan enabled us to adequately address segmentation. All scans were reviewed by a neuroradiologist where suspected structural abnormalities might be present.

Region of Interest Analysis

Images were transferred from the MR Research Center to a computer workstation in our neuroimaging laboratory and regions of interest drawn using BRAINS2 [48], a software that provides valid and reliable volume measurements of specific structures as well as a semi-automated tissue classification procedure. Two raters (SW and HC) blind to identity and risk group membership traced the amygdala and intracranial volumes (ICV) according to boundaries described previously [9]. Region of interest (ROI) manual tracings (approximately 7-10 slices) were performed in the coronal plane. Inter-rater reliability was above .90 for Total, Right and Left Amygdala volumes. Tracings of the right and left amygdala may be seen in (Figure 2).

Informed Consent and Safety Monitoring

The study has ongoing approval from the University of Pittsburgh Institutional Review Board. All participants signed informed consent documents after having the study explained to them. All subjects were screened to insure absence of ferromagnetic metal in or on their body. All female subjects were screened for the possibility of an early and unknown pregnancy using Icon® 25 hCG pregnancy kits.

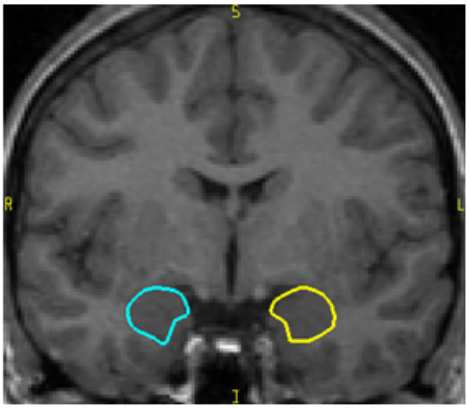

Figure 2: Outline of the right and left amygdala are shown in blue and yellow, respectively. Multiple slices were drawn to obtain the total volume of the amygdala. The typical amygdala structure required 7-10 slices to completely cover the entire structure.

Genotyping

The BDNF Val66Met was genotyped for the Single Nucleotide Polymorphism (SNP) rs6265 because of its known functional effects on BDNF activity [49] using methods previously described [6]. The 5-HTT-Linked Polymorphic Region (5-HTTLPR) was amplified using primer sequences to reveal the long (L) and short (S) variant [50]. To test for subtypes recently identified as necessary for more accurate triallelic characterization [50,51] a SNP, rs25531, was digested with the restriction endonuclease HpaII. All variants were visualized by agarose gel electrophoresis. Genotypes were determined using the L and S variation along with the rs25531 A or G nucleotide (La, Lg, Sa or Sg). The three genotypes were: (1) LL (LaLa), (2) LS (LaLg or LaS) (3) SS (LgLg, LgS, or SS).

Statistical Analysis

Linear Mixed Models (LMM) with random effects (SPSS version 20; SPSS, Chicago, Illinois) were used to investigate risk group differences in the volume of the amygdala. Because some families contributed multiple siblings, a family identifier was incorporated into the models as a random effect to handle familial correlations between subjects. Mixed effects models were used to investigate the association between the High and Low-Risk groups for Total, Left, and Right Amygdala volume using gender, risk status, as well as their interaction as fixed factors on amygdala volume, controlling for ICV.

The main and interactive effects of BDNF Val66Met, 5-HTTLPR, and childhood family environment on amygdala volume were also examined using LMM with a family identifier incorporated as a random effects variable. The family Cohesion and Conflict data from the FES/CVFES were entered as continuous variables in the mixed effects models. Genotypes were recoded for analysis so that Met/Met and Val/Met subjects formed one group and Val/Val the other. Similarly, the 5-HTTLPR genotypes S/S and L/S formed one subject group and were contrasted with those with the L/L genotype.

Intracranial volume, sex, and familial risk status were entered as covariates and retained in the model regardless of their significance. The main effects of 5-HTTLPR genotype ("L/L" vs. "any S"), BDNF genotype ("Val/Val" vs. "any Met") and family environment were tested along with all two way interactions. The family environment variables (Cohesion and Conflict) were evaluated in separate mixed effects models.

Results

Demographic information for the high and Low-Risk subjects (N=129) are presented in Table 2. A total of 71 subjects (38 males and 33 females) were offspring from families with multiple alcohol dependent members while 58 (26 males and 32 females) were offspring from Low-Risk control families. All participants are currently enrolled in a long-term longitudinal follow-up spanning childhood, adolescence, and young adulthood.

Because some participants had been administered the FES and others the CVFES, the concordance of values was checked using a set of 83 individuals who had both measures. Bivariate correlation analysis showed that scores on the FES and CVFES were significantly correlated (r=0.24, p =0.03). This provided justification for the use of either available measure resulting in 115 childhood measures of family environmental assessment of Conflict and Cohesion.

Prenatal Use of Substances

Mothers of both High and Low-Risk offspring were interviewed concerning their use of alcohol or drugs during pregnancy and found to be free of heavy use during pregnancy. Drinking among mothers in both groups was quite low. A total of 76.8% reported no drinking with an additional 23.2% drinking less than 1 drink per day (Median = 28.50; Mean = 98.1 + 40.3 SE drinks during pregnancy). A total of 2.7% reported using any drugs during pregnancy. Absence of cigarette use was reported by 76.4% of mothers for whom data was available (N=107). For those who smoked, the median number reported was 2700 cigarettes during the pregnancy. Smoking during pregnancy was significantly associated with familial risk group status (χ^2 =13.49, df =1, p,0.001) with 22 of the 25 mothers who smoked being members of High-Risk families.

Psychiatric Disorders

Chi square analyses were performed for disorders previously identified in our larger longitudinal study that differ by risk group to determine if significant differences in frequency were present that might impact brain morphology. A total of 119 subjects had been evaluated in childhood for the presence or absence of Attention Deficit Hyperactivity Disorder (ADHD). Significantly more of the High-Risk offspring had ADHD than did Low-Risk offspring (χ^2 = 5.58, df =1, p = 0.018, 12/67 versus 2/52). All 129 cases had data available for depression assessed in either childhood or young adulthood or both. An analysis was performed for the present sub-sample that included 19 cases of depression diagnosed by trained interviewers using either the KSADS for subjects evaluated in childhood (less than age 19) or the CIDI for those seen in young adulthood. Analysis showed that significantly more High-Risk offspring experienced an episode of Major Depressive Disorder (MDD) than did the Low-Risk control offspring (χ^2 = 5.15, df =1, p = 0.023). An analysis was also performed for substance use disorder, defined as any drug or alcohol abuse or dependence, to determine if risk group differences were present within this sub-sample. This analysis revealed significantly more SUD among High-Risk offspring than among the control offspring (χ^2= 5.29, df =1, p = 0.021).

Effect of Familial Risk on Amygdala Volume Controlling for Personal Diagnosis

Familial risk group differences were seen for total (p =0.006), right (p= 0.005) and left (p=0.012) amygdala volumes adjusting for gender and ICV. Because the presence of psychiatric problems in the offspring could influence amygdala volume, further analyses were performed in which individuals with the disorder were removed from the analysis to determine if familial risk would influence amygdala volume independent of personal diagnosis.

Effect of Sequential Removal of ADHD, Depression, and Substance Abuse Cases

There was almost complete collinearity between being High-Risk and having ADHD in this sample (12 out of 14 cases). Therefore, removing all 14 cases with re-analysis was needed to determine if risk status would continue to be associated with amygdala volume. The results of this analysis showed that risk remained significant (F=6.25, df =1, 62.22, p=0.015) after removing the ADHD cases and controlling for ICV and gender. Removal of the 19 cases meeting criteria for MDD with re-analysis also resulted in continued significance for the risk group effect on amygdala volume (F=4.20, df 1, 58.03, p =0.045) and controlling for ICV and gender.

Because exposure to alcohol and drugs could result in loss of tissue volume, an analysis was undertaken to remove those with greater likelihood of having significant neurotoxic exposures. Accordingly, 10 cases from the 129 that met criteria for either alcohol or drug dependence were removed and re-analysis performed. This analysis continued to reveal risk group difference (F=5.95, df 1, 62.02, p =0.018) controlling for ICV and gender. Because removal of alcohol and drug dependence cases along with those with any use or abuse would have resulted in a drastic reduction in available cases, removal of cases was restricted to those with dependence. Nevertheless, the results strongly suggest that the volumetric differences in amygdala seen were not the result of the potentially neurotoxic effects of alcohol.

Main Effects of Genes and Environment

A series of mixed effects analyses were conducted to evaluate associations between variation in BDNF and 5-HTT genes, family environment variables, and amygdala volume. Main effects of BDNF Val66Met status and 5-HTTLPR variants (LL vs. any S) did not predict amygdala volume nor did Cohesion and Conflict scale values.

Interaction Effects of Genes and Environment

All two-way interactions were tested between each gene, family environment (either Conflict or Cohesion) and risk status in mixed model analyses with gender and ICV as covariates. Statistical modeling included removal of non-significant two-way interactions while retaining all main effect variables (Tables 3A and 3B). A familial risk by 5-HTTLPR interaction was seen (p=0.002) with High-Risk S carriers showing the smallest amygdala volume (Figure 3). A familial risk by Cohesion effect was also seen (Figure 4). Greater family Cohesion was associated with an increased volume of the amygdala controlling for ICV and gender but only in Low-Risk control children (Figure 4). Family Conflict was not significantly associated with total amygdala volume in combination with either familial risk or genetic variation.

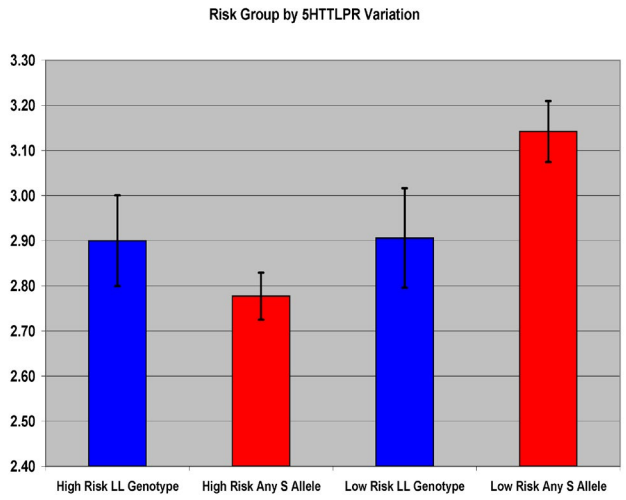

Figure 3: Amygdala volume is shown in association with the joint effects of having either a multiplex family background for alcohol dependence (High-Risk) or a family background without alcohol dependence (Low-Risk) and presence of either the homozygous LL 5-HTTLPR polymorphism variant or presence of at least one copy of the S allele of the 5-HTTLPR polymorphism.

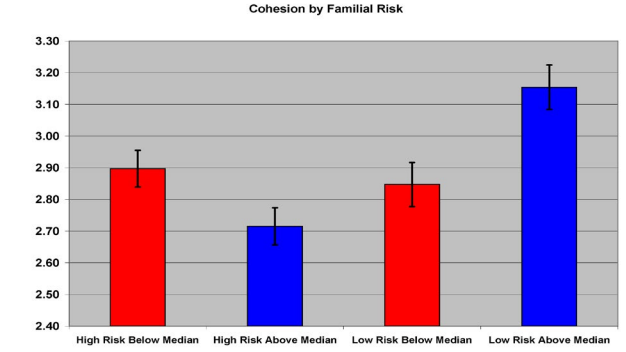

Figure 4: Amygdala volume is shown in association with High or Low-Risk family background and family cohesion during childhood. Note that higher levels of reported family cohesion are associated with greater amygdala volume but only among the low-risk control offspring.

Table 3A: The effect of 5HTTLPR variation, familial risk and childhood family environment-cohesion scale

	Numerator df	Denominator df	F	Significance
Risk	1	106.61	5.74	0.018
FES/CVFES Cohesion	1	107.55	3.15	0.079
Total ICV	1	79.68	17.35	<0.001
Gender	1	107.66	5.25	0.024
Risk by Cohesion	1	107.64	9.64	0.002

The main effect of BDNF and 5HTTLPR and their 2-way interactions were removed from the final model because there was a non-significant main effect of each gene and no significant interactions with Cohesion.

Table 3B: The effect of 5HTTLPR variation, familial risk and childhood family environment- conflict scale.

	Numerator df	Denominator df	F	Significance
Risk	1	83.12	4.03	0.048
5HTTLPR	1	75.53	0.46	NS
Total ICV	1	79.28	9.92	0.002
Gender	1	84.50	3.04	0.085
Conflict	1	84.92	0.50	NS
Risk by Conflict	1	84.99	1.69	NS
Risk by 5HTTLPR	1	76.43	4.49	0.037

The main effect of BDNF and its interaction with Conflict was removed from the model as neither was significant.

Discussion

Using an expanded sample of 129 High and Low-Risk adolescents/young adults, the current report replicates our previous findings [9] showing reduced amygdala volume among offspring from multiplex alcohol dependent families. The present report expands on this observation by investigating possible factors associated with this reduction by exploring childhood family environment (Cohesion and Conflict) and genetic variation in the 5-HTTLPR and BDNF genotypes. The present results find a significant interaction effect between familial risk for alcohol dependence and variation in the 5-HTTLPR polymorphism and amygdala volume. Also, the present results show that higher levels of reported family Cohesion is associated with greater amygdala volume but only in Low-Risk control children. No effect of scale score values of family Conflict was seen. The findings for family Conflict and Cohesion are somewhat unexpected. Neither increased scale score values of Conflict nor decreased Cohesion was related to amygdala volume in the High-Risk offspring.

Offspring of alcohol dependent individuals can be expected to have increased genetic risk for developing alcohol dependence but additionally are exposed to familial environmental characteristics that appear to be the result of parental alcohol dependence. Moos and Billings [36] early on noted that disrupted family relationships and dynamics in families of alcohol dependent individuals can have a negative impact on the adjustment of children that grow up in such environments. The Family Environment Scale was developed by Moos and colleagues [45] to measure family functioning by quantifying dimensions of negative family environment and support. Pillow et al. [52] have also suggested that family-specific stressors mediate the relationship between paternal alcoholism and adolescent substance use. Retrospective report of childhood stress associated with maladaptive family functioning appears to be highly associated with the onset of substance use disorders [53] and increased risk for lifetime alcohol dependence [54]. We are unable to explain the absence of a Family Environment Scale effect on amygdala volume in the High-Risk offspring. However, one explanation may that the critical window for these effects may occur between birth and school age. In the present study, family environment was first assessed at a median age of 11 years.

In the present study measures of family Cohesion in childhood was significantly associated with greater amygdala volume in control children. We find that Low-Risk offspring at an average age of 13.5 years who reported being reared in a family environment characterized by higher levels of family cohesion had the largest total amygdala volume when scanned at an average age of 19.5 years. This might be

expected in view of the stress buffering effects provided by greater family cohesion [55]. To our knowledge, the stress buffering effects of having a positive childhood family environment on brain morphology have not previously been studied. However, it is clear that the effects of a positive family environment in childhood have an effect on children's behavior problems [56]. Based on self-rated physical and mental health status, it appears that the influence of a positive childhood family environment can extend into middle age [57]. While the present results show that having a more cohesive childhood family is associated with greater volume of the amygdala in controls, this effect was not seen in the High-Risk offspring. It is unclear why greater family cohesion does not result in greater amygdala volume in High-Risk offspring as it does in control offspring. However, greater cohesion in the context of living in a High-Risk family may bring adversity. Early work by Wolin and colleagues [58] demonstrated that families that were able to ignore the presence of an alcohol dependent parent were healthier and less likely to transmit alcohol dependence to the next generation. Others describing family systems of alcohol dependent families have noted that those families that are enmeshed or too cohesive, often have members with unhealthy behaviors [59].

While allelic variation in BDNF, 5-HTTLPR or their interaction did not predict amygdala volume, a significant interaction between allelic variation in 5-HTTLPR and familial risk was associated with total amygdala volume. There is an extensive literature showing that early life stress is associated with morphological changes in the brain [60-62] with reduced volume associated with greater stress. High-risk offspring can be expected to experience increased stress due to often living in homes with alcohol dependent parents. Exacerbation of the outcome of having a negative environment has been shown to be associated with 5-HTTLPR variation with S allele carriers having smaller hippocampal volume [35]. Similarly those who are more prone to depression appear to develop episodes in the face of stress if they are S allele carriers [63].

The present findings appear to be inconsistent with previous studies showing that genetic variation in BDNF interacts with early family environmental characteristics to alter brain morphology. However, the two studies reporting an interaction between BDNF and family environment focused on negative environmental aspects such as higher levels of stress [16] or emotional or physical neglect [35] and did so using participants' retrospective report in adulthood. The present study had the advantage of prospectively collected family environment data which may explain the differing results.

The present findings are in accord with studies showing that 5-HTTLPR S allele carriers tend to have smaller amygdala volume than L carriers. The new finding from this study is that familial risk status interacts with S carrier status to provide significant reduction in amgydala volume. Because S allele carriers are more susceptible to environmental stress, it may be the case that High-Risk status which is often associated with living in a home with an alcohol dependent relative may make S allele carriers more vulnerable to reduction in amygdala volume. Although we did not observe a family environment interaction with the S allele, it is possible that presence of the S allele interacted with family stress at an earlier time in childhood prior to the entry of the child into our longitudinal study when family environment was measured.

The greater exposure to stressful family environments seen in homes of alcohol dependent individuals has been shown to be associated with biological variation between children of alcoholics and controls. Offspring of alcoholics tend to show elevated cardiovascular reactivity [64-68], increased cortisol response to aversive stimuli [69,70], and elevated baseline heart rate [71]. Additionally, heightened stress reactivity may represent a potential mechanism of vulnerability in individuals with a family history of AD that would amplify the effect of stressors on brain morphology [72,73]. Coupled with the fact that S carriers are more susceptible to stress, High-Risk offspring who are S carriers may be especially vulnerable.

Structural differences in the amygdala most likely emerge gradually across development as a consequence of disrupted family processes among vulnerable individuals, possibly laying the foundation for excessive alcohol use later in life. We are uncertain why the degree of reported family Cohesion may have been more instrumental in changing amygdala volume in conjunction with genetic variation than was Conflict. However, the importance of social networks in buffering stress and their effect on health has long been known [74]. Cohesive families are those that are more likely to support each other in the face of stressful life circumstances.

One limitation of our findings is that participants were not scanned at the time of the CVFES/FES administration so we do not know if the relationship between family Cohesion and Conflict and amygdala volume might have been found at that time. The lack of main effects of CVFES/FES based Cohesion and Conflict could be interpreted as being due to variation in test administration. Due to differing ages of entry into the study, it was necessary to administer alternate versions of the family environment scale, one that is most age-appropriate for children under 12 years of age, while the other is intended for adolescents and adults. Although this could have influenced results, we view this limitation as minimal because both versions were developed to tap the same concepts and contain the same scales [47]. In the larger longitudinal study from which this sample was drawn, a subset of youngsters received both instruments which allowed for comparison of values across tests which indicated good correspondence. Also, we observed that measures of Cohesion in control offspring had a predictable effect on amygdala volume. An alternate explanation is that levels of Conflict in High-Risk families appear to cluster at the high end of the scale and provide minimal individual variation. This may also explain the fact that higher levels of Cohesion had an effect on control children's amygdala volume but no effect in High-Risk children.

Another potential limitation was that significantly more alcohol and drug use occurred among the High-Risk offspring in comparison to controls. Substance use could have contributed to the smaller amygdala volume observed among the High-Risk offspring. This possibility is offset by previous observations of smaller amygdala volume in younger High-Risk offspring [9] who, on the whole, had not experienced significant substance use involvement. Moreover, an analysis was conducted removing those cases with alcohol and drug dependence with the same resulting outcome; High-Risk offspring had smaller amygdala volume than low risk controls independent of personal history of substance use disorder.

Additionally, prenatal use of substances by the mothers of these offspring is a potential concern. However, prenatal use of alcohol was minimal in this sample. Although the High-Risk offspring were selected through parental alcohol dependence, this study is based on families ascertained through fathers with alcohol dependence who additionally had a brother similarly diagnosed. Maternal alcohol dependence during pregnancy was not present. Also, maternal use of alcohol has proved to be of minimal significance in statistical analysis of cerebellar data acquired from this sample [75].

In summary, the present findings replicate an earlier report from

this lab showing reduced total, right and left amygdala volume in High-Risk offspring from multiplex families in an expanded sample. The new findings from the current report include the fact that carriers of the S variant of the 5-HTTLPR polymorphism from High-Risk families have reduced amygdala volume in comparison to those with an LL genotype. The positive effects of higher family Cohesion on amygdala volume seen in Low-Risk control offspring was not seen in High-Risk offspring. This may be due to differing effects of family cohesion in High-Risk families. Based on extant family process literature, there is a suggestion that alcohol dependent families with too great of cohesion may be more dysfunctional.

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