Molecular Psychiatry (2005) 10, 213–219

www.nature.com/mp

ORIGINAL RESEARCH ARTICLE

Genetic polymorphisms of the *RGS4* and dorsolateral prefrontal cortex morphometry among first episode schizophrenia patients

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Polymorphisms of the gene encoding the regulator of G-protein signaling subtype 4 *(RGS4)* may confer risk for schizophrenia.[1] DNA microarray studies of postmortem brain samples have shown RGS4 underexpression in the dorsolateral prefrontal cortex (DLPFC, area 9), motor and visual cortices in schizophrenia patients relative to control subjects.[2] Underexpression of *RGS4* in DLPFC is pathophysiologically significant because DLPFC pathology in schizophrenia has been supported by neurocognitive,[3,4] structural[5] and functional[6,7] imaging, postmortem,[8] cellular[9,10] and molecular[11] pathological studies. For these reasons, we examined the association of DLPFC gray matter volume with *RGS4* polymorphisms in a series of antipsychotic-naïve first-episode schizophrenia patients and control subjects. We hypothesized that volumetric alterations of the DLPFC would be associated with *RGS4* polymorphisms and that these differences would be more pronounced in patients than in controls. We observed robust volumetric differences across the genotypes in the pooled sample of patients and control subjects; when separately analyzed, we observed differences within the patient group ($n = 30$) but not in control subject ($n = 27$) group. The findings suggest that *RGS4* polymorphisms may contribute to structural alterations in the DLPFC.
Molecular Psychiatry (2005) **10,** 213–219. doi:10.1038/sj.mp.4001562
Published online 21 September 2004

Keywords: *RGS4*; genetic polymorphisms; schizophrenia; neuroanatomy; MRI; psychosis; prefrontal cortex

In recent years, several candidate genes for schizophrenia have been reported.[12] One such gene is the regulator of G-protein signaling, subtype 4 (*RGS4*) that has been localized to 1q21–22, a locus linked to schizophrenia in a recent study.[13] Using DNA microarrays, reduced expression of the *RGS4* gene, but not other members of the RGS family of proteins, was initially noted in the dorsolateral prefrontal cortex (DLPFC, Brodmann's area 9), visual and motor cortices of schizophrenia patients compared to the matched control subjects and patients with major depressive disorder.[2] Subsequently, polymorphisms of *RGS4* have been investigated in five independent samples. Both case–control and family-based designs were employed in order to examine genetic associations. Modest associations were observed between *RGS4* gene polymorphisms and schizophrenia in two independently ascertained samples from Pittsburgh and the NIMH collaborative genetics initiative, and a trend was noted in a sample from India.[1] Recently, case–control associations have been observed in independent samples analyzed at Cardiff, UK[14] and Dublin, Ireland.[15] Together, these data provide congruent evidence for the suggestion that *RGS4* polymorphisms confer susceptibility to schizophrenia. However, the pathogenic mechanisms associated with the genetic variants are poorly understood. If genetic association studies are yoked with investigations of brain structure and function, it may be feasible to clarify such mechanisms.

The RGS family is a group of GTPase-activating proteins (GAPs) for heterotrimeric G-protein-subunits that negatively regulates G-proteins. Such regulation has been implicated in modulating the function of dopamine,[16] glutamate[17–19] and serotonin.[20] Reduced expression of RGS proteins could prolong signal transduction and may, thereby, result in the altered glutamatergic or dopaminergic states implicated in schizophrenia. Such neurotransmitter pathology has been demonstrated in the PFC in addition to other regions such as hippocampus and thalamus. Alterations in the PFC-regulated executive–cognitive

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Received 31 March 2004; revised 07 June 2004; accepted 28 June 2004

functions have been consistently reported in schizophrenia.[6] Additional evidence for prefrontal pathology in schizophrenia is provided by abnormalities in the *in vivo* membrane integrity and energy metabolism of the PFC suggested by the reductions in *N*-acetyl aspartate (NAA)[21] and phosphomonoesters (PMEs)[22,23] observed using proton (^{1}H) and phosphorus (^{31}P) magnetic resonance spectroscopy, respectively, decrements in slow wave sleep,[24] altered PFC metabolism[6,7] and reduced expression of genes and proteins associated with synaptic plasticity.[11] However, structural imaging findings have been inconsistent.[5] One way to tease apart such inconsistencies is to examine the genetic correlates of structural imaging findings.

In the present study, we examined the neurobiological correlates of *RGS4* polymorphisms among first-episode schizophrenia patients and control subjects. Our goal was to test the following hypotheses: (i) *RGS4* polymorphisms are associated with alterations in the DLPFC volume both in subjects with schizophrenia and healthy controls and (ii) the main effect of the polymorphisms is more pronounced in schizophrenia patients than in healthy subjects. The latter hypothesis is based on reports of replicated associations with several candidate genes, probable additive effect of these risk genes and interactions among them.[12] In order to test the hypotheses, we examined diagnosis $\times$ genotype interaction in the pooled sample of patients and controls covarying for gender and intracranial volume (ICV). Next, we examined the genotype effect covarying for diagnosis, gender and ICV. We, then, examined the diagnosis effect using gender and ICV as covariates. Subsequently, we examined within group differences across the genotypes. Finally, we examined the dose effect of alleles within each genotype in patients and controls.

Methods

Subjects

A series of consecutive first-episode, neuroleptic-naïve subjects with DSM IV[25] schizophrenia or schizoaffective disorders ($n=30$; male $=21$, female $=9$; mean age $=23.\ 73+7.71$ years) were recruited from in-patient and outpatient facilities of the Western Psychiatric Institute and Clinic, Pittsburgh. Healthy subjects ($n=27$; male $=14$, female $=13$; mean age $=24.74+7.23$ years) were recruited through local advertisements from the same neighborhoods as the patients. All patients and control subjects were reported to be of European-American ancestry. The groups did not differ with respect to their mean ages ($t=0.51$, $P=0.6$, NS) or gender ($\chi^2=1.98$, two-tailed $P=0.18$, NS). After fully explaining the experimental procedures, written informed consent was obtained from all participants. The Institutional Review Board of the University of Pittsburgh approved the study. The DSM IV[25] diagnoses were derived following consensus conference of senior diagnosticians (GLH, CSC, MSK and NRS) reviewing all data from the medical records and information from clinicians who had contact with the patients. All patients were followed up for at least six months in order to establish diagnostic stability. None of the subjects reported history of substance use disorder within the past month, serious medical or neurological illnesses, or mental retardation as defined in the DSM IV.[25]

MRI Scans

The MRI scans were obtained using a GE 1.5T whole body scanner (GE Medical Systems, Milwaukee, WI, USA). Details of image acquisition have been published earlier.[26] Dorsolateral prefrontal cortex (DLPFC) and intracranial volume (ICV) were measured using the NIH Image software (Version 1.56; US National Institutes of Health; http://rsb.info.nih.gov/nih-image/), which allows stripping of non-cerebral tissue and reliable segmentation of gray and white matter and cerebrospinal fluid.[27] The inter-rater reliability between two independent raters for DLPFC was 0.94 (for right DLPFC) and 0.88 (for the left DLPFC) and 0.88 for the ICV (intraclass correlation coefficient, ICC). DLPFC was defined as 10 slices anterior to the posterior border of the genu of the corpus callosum, the superior border was the superior frontal sulcus, the inferior border was the upper border of the Sylvian fissure posteriorly and the horizontal ramus of the Sylvian fissure anteriorly, the lateral boundary was the edge of the brain and the medial boundary was the line connecting the two most medial points of the superior frontal sulcus and the Sylvian fissure/horizontal ramus. We included frontal, temporal, parietal and occipital lobes, brain stem, cerebellum and the CSF around the brain for the ICV. Detailed methodology of DLPFC and ICV measurements has been published earlier.[26]

Genetic analysis

Genomic DNA was extracted from venous blood using the phenol–chloroform method. Polymerase chain reaction (PCR)-based assays were used to determine genotypes.[1] All assays were performed blind to clinical status. Of the 13 SNPs analyzed for transmission disequilibrium test (TDT), four SNPs (SNP1, 4, 7 and 18) were found to be associated with schizophrenia.[1]

Results

We examined the association of SNPs 1, 4, 7 and 18 with the DLPFC volumes. We observed a significant diagnosis $\times$ genotype interaction for SNP4 ($F(5,57)=3.95$, $P=0.004$) and SNP 18 ($F(5,57)=3.56$, $P=0.008$) for the left DLPFC and for SNP1 ($F(5,55)=2.70$, $P=0.032$), SNP4 ($F(5,57)=3.42$, $P=0.01$) and SNP18 ($F(5,57)=3.12$, $P=0.016$) for the right DLPFC volumes. In the pooled sample of patients and controls, there was a significant genotype effect; the left DLPFC volumes were significantly different across the groups on SNPs 4 and 18 after correcting for multiple comparisons (three genotypes

Table 1 Comparison of DLPFC volume across genotypes in the pooled sample of patients and control subjects (GLM Univariate ANCOVA)

SNP	*N*	*LDLPFC*					*RDLPFC*				
		Group difference			*Posthoc tests*[a]		*Group difference*			*Post hoc tests*[a]	
		Mean volume (in ml) (SD)	*F*	*P*[b]	*Comparison*	*P*	*Mean volume (in ml) (SD)*	*F*	*P*[b]	*Comparison*	*P*
SNP1											
A/A	16	10.60 (1.61)	(2,55) = 4.32	0.228	—	—	10.25 (1.85)	(2,55) = 3.04	0.684	—	—
A/G	20	11.47 (1.40)					10.99 (1.13)				
G/G	19	12.55 (2.07)					11.76 (1.89)				
SNP4											
T/T	24	10.75 (1.48)	(2,57) = 8.33	0.012	T/T < G/G	0.00004	10.46 (1.67)	(2,57) = 5.65	0.072	T/T < G/G	0.001
T/G	22	11.44 (1.54)			T/G < G/G	0.002	11.79 (1.44)			T/G < G/G	0.009
G/G	11	13.51 (1.87)					12.60 (1.59)				
SNP7											
A/A	17	10.66 (1.58)	(2,57) = 3.67	0.384	—	—	10.33 (1.83)	(2,57) = 2.02	1.0	—	—
A/G	20	11.47 (1.40)					10.99 (1.13)				
G/G	20	12.38 (2.16)					11.58 (2.02)				
SNP18											
A/A	25	10.83 (1.51)	(2,57) = 7.58	0.012	A/A < G/G	0.0001	10.51 (1.66)	(2,57) = 5.51	0.084	A/A < G/G	0.002
A/G	21	11.37 (1.54)			A/G < G/G	0.002	10.74 (1.46)			A/G < G/G	0.007
G/G	11	13.51 (1.87)					12.60 (1.59)				

[a]Bonferroni tests.
[b]Bonferroni corrected *P*-values.

in two sets of SNPs in linkage disequilibrium (LD) compared for left and right DLPFC volume differences). *Post hoc* Bonferroni tests revealed smaller volumes in subjects homozygous for allele T or A compared to the other groups (Table 1). Volumetric reductions in SNPs 4 and 18 was 20.43% for SNP4 and 19.84% for SNP18 between individuals *not* homozygous for allele G and those homozygous for allele G. Using ANCOVA, there were no main effects of diagnosis on DLPFC volumes (left: patients = 11.43 ± 1.99, controls = 11.68 ± 1.73, $F(1,55) = 0.00$, $P = 0.99$; right: patients = 10.98 ± 1.73, controls = 11.03 ± 1.78, $F(1,55) = 0.02$, $P = 0.9$).

A within-group comparison of DLPFC volumes in patients and controls across genotypes revealed significant differences in the DLPFC volumes in patients but not in control subjects (Table 2). After correcting for multiple comparisons (Bonferroni tests; three genotypes in two sets of SNPs in LD for volumetric alterations in left and right DLPFC), the genotype effect of SNP4 and 18 remained significant for both the left and right DLPFC volumes with a reduction in the volume by about 25% in those *not* homozygous for allele G compared to those homozygous for allele G. In addition, SNP1 also sustained its significance for the right but not the left DLPFC volume with a volume reduction of about 26% in those homozygous for allele A compared to those homozygous for allele G. Further, we examined the allele dose effect on the DLPFC volumes within each group. Using a general linear model we observed that with the addition of each non-G allele, the volume of DLPFC decreased linearly in patients but not in controls (after correcting for multiple comparisons) for SNP4 ($P = 0.0072$) and SNP18 ($P = 0.012$) on the left and SNP1 ($P = 0.0024$), SNP4 ($P = 0.012$) and SNP7 ($P = 0.022$) on the right. Healthy subjects showed significant results for SNP4 and SNP18 that did not survive correction for multiple comparisons (Figure 1).

Discussion

The main finding of our study was that *RGS4* polymorphisms are associated with alterations in DLPFC volumes among patients but not in controls. This observation in neuroleptic-naïve first-episode schizophrenia subjects suggests that the observed differences across genotypes may not be attributable to illness chronicity or treatment with antipsychotic medications. Illness chronicity[28] and antipsychotic administration[29] have been reported to alter the regional brain volumes. Specifically, smaller volumes were consistently observed in patients *not* homozygous for allele G on SNP4 and 18 but not in healthy controls; this supported our second hypothesis but not the first. Robust volumetric differences in patients but not in control subjects suggest interaction of *RGS4* polymorphisms with other illness-related variables, for example, other genetic variations or environmental

Table 2 Comparison of DLPFC gray matter volumes within patients across RGS4 genotypes

SNP	*N*	*LDLPFC*					*RDLPFC*				
		Group difference			*Posthoc tests*[a]		*Group difference*			*Posthoc tests*[a]	
		Mean volume (in cm³) (SD)	*F*	*P*[b]	*Comparison*	*P*	*Mean volume (in cm³) (SD)*	*F*	*P*[b]	*Comparison*	*P*
SNP1											
A/A	9	10.28 (1.36)	(2,28) = 5.67	0.12	—	—	9.66 (1.12)	(2,28) = 13.83	0.0012	T/T < C/C	0.00004
A/G	13	11.49 (1.50)					11.09 (1.25)			T/T < C/T	0.034
G/G	6	13.40 (2.53)					13.08 (1.21)			C/T < C/C	0.007
SNP4											
T/T	15	10.51 (1.22)	(2,30) = 10.96	0.0042	T/T < G/G	0.0002	10.13 (1.28)	(2,30) = 10.16	0.012	T/T < G/G	0.0004
T/G	11	11.55 (1.71)			T/G < G/G	0.006	11.19 (1.52)			T/G < G/G	0.018
G/G	4	14.57 (2.01)					13.55 (1.07)				
SNP7											
A/A	10	10.41 (1.35)	(2,30) = 3.31	0.624	—	—	9.86 (1.23)	(2,30) = 5.44	0.12	—	—
A/G	13	11.49 (1.50)					11.09 (1.25)				
G/G	7	12.79 (2.82)					12.37 (2.89)				
SNP18											
A/A	16	10.66 (1.31)	(2,30) = 9.76	0.012	A/A < G/G	0.0004	10.24 (1.31)	(2,30) = 9.07	0.012	A/A < G/G	0.001
A/G	10	11.42 (1.75)			A/G < G/G	0.006	11.13 (1.58)			A/G < G/G	0.019
G/G	4	14.57 (2.01)					13.55 (1.07)				

[a]Bonferroni tests.
[b]Bonferroni corrected *P*-values.

factors. A possible candidate gene for schizophrenia with which *RGS4* could be interacting is neuregulin (NRG1) because *RGS4* has been shown to exist as a single molecular complex with ErbB4 (an NRG1 receptor) in the cell membrane.[30]

While the association between RGS4 and schizophrenia has been replicated in independently ascertained samples, it is difficult to reconcile our results at individual polymorphisms with genetic association studies to date because different alleles of these SNPs have been associated with schizophrenia in different populations. In the initial report, the Pittsburgh sample revealed overtransmission of the G allele in all four SNPs,[1] which is in opposition to the alleles associated with smaller DLPFC volumes in our study. A recent replicate sample of Caucasians from Ireland has also shown association with the G alleles at SNPs 1 and 7, as well as a trend in SNP4, in cases with schizophrenia but not schizoaffective disorder.[15] However, reduced volumes in our study concurs with the associated SNPs in the NIMH sample, which is also a US-based sample and showed overtransmission of the A T A A alleles at SNPs 1, 4, 7 and 18, respectively. A subsequent replicate study in a large Caucasian sample from the UK also reported significant association for the A T A alleles at SNPs 1, 4 and 18, respectively.[14] Thus, the past research appears to indicate a positive association of RGS4 variants with schizophrenia, yet the associated alleles and haplotypes remain disparate between studies. Such variability in allelic associations with a disease phenotype is not unique for schizophrenia and RGS4. For example, although the Val/Met polymorphism of the COMT gene has been associated with schizophrenia in some studies, yet other studies have reported association with other SNPs and haplotypes.[31,32] Dysbindin has also been reported to show different allelic associations with schizophrenia.[33,34]

The precise RGS4 variants that primarily confer risk for schizophrenia are uncertain. However, it is possible that the risk is primarily conferred by hitherto unidentified variants. The functional significance of these alleles associated with schizophrenia is unknown to date. All four SNPs in the associated haplotype are non-coding SNPs, but SNPs 1, 4 and 7 are located in a 5′ region of the gene that may play a role in transcription regulation. Research is currently ongoing in our laboratories to identify the exact promoter region of this gene. Further, the linkage disequilibrium of all SNPs in this region is high, particularly between the four SNPs investigated here.[1] This is evident in our highly correlated results between SNPs 1 and 7 as well as SNPs 4 and 18, which are in very strong LD in all populations investigated to date. It is therefore possible that the volumetric changes seen in the DLPFC in this study are a result of a primary association with an unknown functional variant in strong LD with these SNPs.

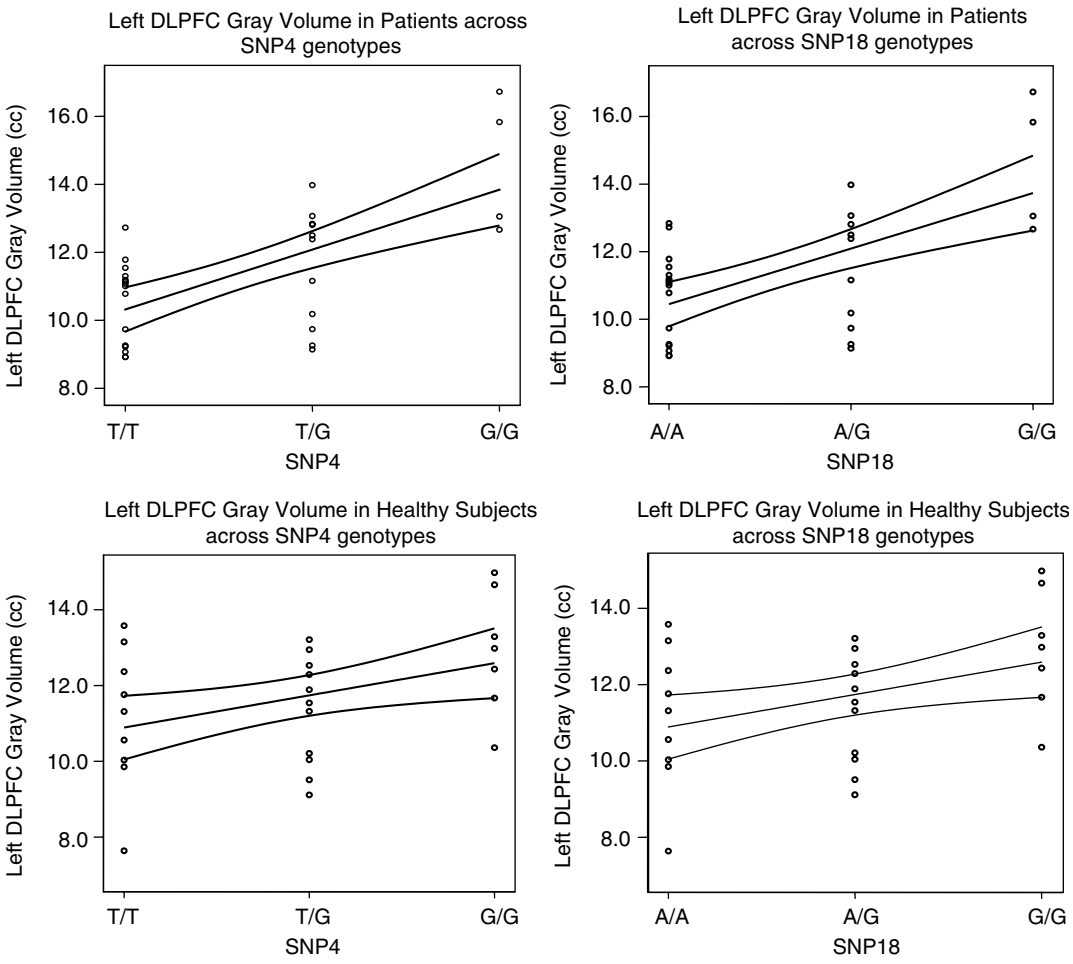

Figure 1 Allele dose effect of SNP4 and SNP18 on the DLPFC volume in schizophrenia patients and healthy subjects.

Although a substantial association between genetic variants and DLPFC gray matter volume was noted here, it is unclear if and how volumetric changes contribute to the pathogenesis of schizophrenia. Nevertheless, volumetric changes associated with a schizophrenia susceptibility gene within patients may explain inconsistencies in literature on the structural variations of DLPFC in schizophrenia. Furthermore, the subjects for this imaging study represent a subset of the entire schizophrenia patient pool used in the original association study. Examining the *in vivo* neurobiology in other samples where RGS4 associations have been reported (Wales and Dublin) may shed more light on the possible allelic heterogeneity.

This finding suggests that the functional effects of *RGS4* may be driven by SNPs 1 (for right DLPFC only), 4 and 18. However, a precise knowledge of the underlying mechanisms for DLPFC structural alterations is limited by a poor understanding of how these polymorphisms eventually result in volumetric alterations. Detailed structural genomics of *RGS4* gene has not been elucidated, yet. Current data suggest that these SNPs are located in the first intron and upstream and flanking regions. Relatively strong association of SNP4 and 18 may suggest that these polymorphisms may be in the putative promoter/regulator region or may determine splice variants, respectively. However, further work is needed to elucidate this.

Our data raise several important questions for future studies. Firstly, the association of *RGS4* polymorphisms and the allele dose effect on the DLPFC volumes suggest that the inconsistent findings on DLPFC volumetric differences in schizophrenia patients and healthy subjects may be due to the underlying genetic differences. Furthermore, different allelic backgrounds at *RGS4* and other risk genes may influence the case–control differences in MRI variables observed in other studies. Similar studies are required to examine this issue with other risk genes. Secondly, the relationship between the structural alterations and the functional and neurocognitive differences may not be straightforward. Further studies are being conducted to examine functional correlations of *RGS4* polymorphisms. Thirdly, although this study revealed an association between *RGS4* polymorphisms and DLPFC volumes, the

molecular pathways that lead to an alteration in the volumes is not known. *RGS4* has been reported to affect the neurodevelopmental processes[20] and abnormalities in neurodevelopment have been proposed to be one of the pathogenetic mechanisms of schizophrenia.[35] Thus, an abnormal neurodevelopmental process may underlie volumetric changes associated with *RGS4* polymorphisms. Future studies need to address this question and examine specific stages of neurodevelopment that are associated with the morphometric alterations. Fourthly, these associations raise the issue of specificity of these observations to DLPFC volume as opposed to other brain regions where *RGS4* is underexpressed. We are at present examining morphometric changes in other brain regions. Finally, diagnostic specificity of the association of *RGS4* polymorphisms and DLPFC volume also needs further examination. Although underexpression of *RGS4* transcripts was observed in schizophrenia but not in major depressive disorder,[2] it is not known if the MRI changes are specific to schizophrenia, too.

This study is limited by a relatively small sample size; type II errors cannot, therefore, be ruled out. Further studies are required to characterize *RGS4* expression patterns in other brain regions implicated in schizophrenia such as medial temporal structures, striatum and cerebellum. In addition, specific intracellular effector proteins that are affected and the range of environmental factors that influence gene expression also need further studies. Furthermore, heterogeneity in transmission distortions of alleles in different SNPs suggests a possibility of genetic heterogeneity of the disorder and needs to be examined further.

Acknowledgements

This work was supported through Grants NIMH/APIRE PMRTP MH19126 (KMP), R01 64023, K02 01180, NARSAD Established Investigator Award (MSK) and MH01489, MH56242 and MH53459 (VLN). We thank Dr Bernie Devlin, PhD and Dr Weiting Xie for their assistance in statistical analysis. We thank Drs Cameron S Carter, MD (CSC), Gretchen L Haas, PhD (GLH), Nina R Schooler, PhD (NRS), and the clinical core staff of the Center for the Neuroscience of Mental Disorders (MH45156) for their assistance in diagnostic and psychopathological assessments.

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