Convergent Genomic Studies Identify Association of *GRIK2* and *NPAS2* with Chronic Fatigue Syndrome

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Abstract

Background: There is no consistent evidence of specific gene(s) or molecular pathways that contribute to the pathogenesis, therapeutic intervention or diagnosis of chronic fatigue syndrome (CFS). While multiple studies support a role for genetic variation in CFS, genome-wide efforts to identify associated loci remain unexplored. We employed a novel convergent functional genomics approach that incorporates the findings from single-nucleotide polymorphism (SNP) and mRNA expression studies to identify associations between CFS and novel candidate genes for further investigation. *Methods:* We evaluated 116,204 SNPs in 40 CFS and 40 nonfatigued control subjects along with mRNA expression of 20,160 genes in a subset of these subjects (35 CFS subjects and 27 controls) derived from a population-based study. *Results:* Sixty-five SNPs were nominally associated with CFS (p < 0.001), and 165 genes were differentially expressed (≥4-fold; p ≤ 0.05) in peripheral blood mononuclear cells of CFS subjects. Two genes, glutamate receptor, ionotropic, kinase 2 *(GRIK2)* and neuronal PAS domain protein 2 *(NPAS2),* were identified by both SNP and gene expression analyses. Subjects with the G allele of rs2247215 *(GRIK2)* were more likely to have CFS (p = 0.0005), and CFS subjects showed decreased *GRIK2* expression (10-fold; p = 0.015). Subjects with the T allele of rs356653 *(NPAS2)* were more likely to have CFS (p = 0.0007), and *NPAS2* expression was increased (10-fold; p = 0.027) in those with CFS. *Conclusion:* Using an integrated genomic strategy, this study suggests a possible role for genes involved in glutamatergic neurotransmission and circadian rhythm in CFS and supports further study of novel candidate genes in independent populations of CFS subjects.

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Introduction

Recent years have witnessed a surge in interdisciplinary efforts to delineate the pathophysiology of chronic fatigue syndrome (CFS), a debilitating disorder of unknown etiology with no known lesions, biomarkers or laboratory tests for diagnosis [1]. Neuroendocrine and immune system deregulation as well as perturbations in cognitive, sleep and metabolic functions have been documented in CFS [1–3]. Further, twin, family and candidate gene studies support a moderate genetic contribution to the development of CFS [4–6]. Gene expression and candidate gene association studies support the association of several genes with CFS [4, 7–10], but candidate gene studies remain limited by the poor understanding of CFS pathophysiology. Despite progress, there is no consistent evidence of specific gene(s) or molecular pathways that contribute to CFS pathogenesis, provide insights for therapeutic intervention or contribute to diagnosis.

Polymorphisms are more likely to impact on gene expression than to change protein coding sequences, suggesting that gene expression may be an early manifestation of a complex phenotype [11]. Genetic associations supported by independent molecular evidence are more compelling than those identified by a single approach. Along these lines, analyses of genetic linkage or association results combined with mRNA expression have revealed promising candidates for schizophrenia [12, 13], obesity [14], cancer treatment outcome [15] and drug sensitivity [16]. Recently, the convergent functional genomics approach, which integrates multiple independent lines of evidence, was used successfully to prioritize viable candidate genes and blood biomarkers for multiple neuropsychiatric diseases, including bipolar disorder, psychosis and mood disorders [17–21].

To begin the process of identifying novel candidates for further study in CFS, we utilized a novel convergent functional genomics approach in a proof of principle exploration, by combining unbiased studies of polymorphisms and mRNA expression using subjects recruited from a population-based study. Since there are no known lesions in CFS, gene expression analysis was performed using total RNA from peripheral blood mononuclear cells (PBMCs), in keeping with the hypothesis that changes in PBMC expression reflect systemic changes due to illness [22]. We identified glutamate receptor, ionotropic, kinase 2 *(GRIK2)* and neuronal PAS domain protein 2 *(NPAS2)*, genes involved in glutametergic neurotransmission and circadian rhythm regulation, respectively, to be consistently associated with CFS in independent genomic analyses. These previously unrecognized associations should provide exciting new hypotheses in the study of CFS.

Materials and Methods

Subjects and Illness Classification

This study adhered to the human experimental guidelines of the US Department of Health and Human Services and the Helsinki Declaration. The Centers for Disease Control and Prevention (CDC) Human Subjects Committee approved the study protocol, and all subjects gave informed consent.

The subjects in this study were from the Wichita CFS Surveillance Study, a population-based study conducted between 1997 and 2000 [23]. From December 2002 to July 2003, 227 subjects were recruited from this surveillance cohort for a 2-day in-hospital study to reevaluate symptoms and exclusionary medical and psychiatric conditions and to collect samples for molecular epidemiologic studies. In brief, 43 subjects meeting the criteria for CFS and 60 control subjects were identified. CFS was diagnosed using the criteria of the 1994 International Research Case Definition [24] as recommended by the International CFS study group [25] and currently used by the CDC [23]. To identify exclusionary medical conditions, a standardized medical history and physical examination, review of current medications and routine blood and urine clinical laboratory tests were performed as recommended by the International CFS Study Group [23, 25]. Exclusionary psychiatric conditions were identified by specifically trained, licensed psychiatric interviewers using the Diagnostic Interview Schedule. The single-nucleotide polymorphism (SNP) association study was restricted to 40 CFS and 40 control Caucasian subjects because of limited power to detect associations in other racial groups. We also restricted the gene expression analysis to the subjects for whom RNA microarray data were available (35 CFS subjects and 27 controls).

Isolation of DNA and RNA

Peripheral blood was collected in 8-ml vacutainer tubes containing citric acid (BD, N.J., USA), and PBMCs were immediately isolated on Lymphocyte Separation Medium (Organon Teknika, N.C., USA). PBMCs were stored in liquid nitrogen under conditions designed to maintain viability. Both DNA and RNA were extracted from the same PBMC sample by a Trizol DNA/RNA extraction protocol (Invitrogen, Carlsbad, Calif., USA). Genomic DNA was amplified using the Genomiphi DNA amplification kit (Amersham Biosciences, Piscataway, N.J., USA). Amplified DNA was quantified by TaqMan PCR with primers and probe for β-globin (2 copies of β-globin/cell and 5 pg of DNA per cell) prior to genotyping. Contaminating DNA from RNA preparations was removed by digesting it with 2 U of DNase I (GeneHunter Corporation, Tenn., USA) at 37°C for 15 min. The quality and quantity of the RNA preparation was determined using a Bioanalyzer 2100 (Agilent, Calif., USA).

SNP Association Study

The Affymetrix GeneChip Mapping 100K Set (split into 2 array sets referred to as 50K *Xba*I and 50K *Hin*dIII) was used to examine 116,204 SNPs according to the manufacturer's protocol

(Affymetrix, Santa Clara, Calif., USA). GeneChip images were analyzed using GeneChip® DNA Analysis Software (version 3.0, Affymetrix). The quality control measures used to assess chip reliability are shown in online suppl. table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000326692). Briefly, samples with call rates below 92% were repeated. DNA provided by Affymetrix was included with each batch of samples as a positive control. Concordance of this positive control with known genotypes always exceeded 99%, and the sex of each study sample was correctly identified by heterozygosity of the X chromosome. Additionally, 31 SNPs were replicated on both *Xba*I and *Hin*dIII arrays to ensure that samples were not switched; the median concordance between these 31 SNPs on the 2 arrays was 100% for each sample. SNPs that were monomorphic in this data set (n = 9,323) were removed, along with SNPs with call rates of less than 80% (n = 1,864). An additional 2,363 SNPs located on the X chromosome were not included in this analysis. The remaining 102,654 polymorphisms were assessed to determine if the observed genotype frequencies were consistent with Hardy-Weinberg equilibrium using χ^2 tests. Markers that deviated from Hardy-Weinberg equilibrium (p < 0.05) in the control subjects were excluded. In total, 93,508 autosomal SNPs passed all quality control measures and proceeded to further analysis.

χ^2 tests were used to assess allelic and genotypic associations between a marker and CFS in empirically defined CFS cases and controls. Potentially confounding factors, including age, sex, body mass index and depression history, were examined for association with CFS, but no adjustments were needed. All p values were estimated using 10,000 Monte Carlo simulations under the null hypothesis of no association. To examine if population stratification was present in the sample, the genomic control method [26] was used.

Gene Expression

Gene expression was evaluated using the MWG 20K human array A (MWG Biotech, Ebersberg, Germany), which contains oligonucleotide features representing 20,160 genes. The laboratory procedure used in the expression study has been described previously [27]. Briefly, DNase I-treated total RNA (1 μg) was reverse transcribed into biotinylated cDNA for hybridization to microarrays using the Ventana Discovery system and ChipMap™ kit (Ventana Medical Systems, Ariz., USA). Hybridization signals were detected using antibiotin antibodies conjugated to resonance light-scattering RLS™ gold particles and a GSD-501 scanner (Genicon Sciences Corporation, Calif., USA). Signal intensity was quantified using ArrayVision™ RLS image analysis software (Genicon Sciences).

Microarray data analysis was conducted using ArrayTrack version 3.4.0 (http://www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack/). The intensity data were normalized using a global median scale to 1,000 and then log$_2$ transformed. Genes differentially expressed between CFS and controls were assessed using a significance criteria of p < 0.05 (Welch test) and a fold change >4 with an average gene intensity filtering of 1,000 across all 62 hybridizations. Molecular function and associated pathways of the differentially expressed genes were extracted using Gene Ontology For Function Analysis (GOFFA), Kyoto Encyclopedia of Genes and Genomes (KEGG) and PathArt (proprietary pathway database from Jubilant Biosystems Ltd.) as implemented in ArrayTrack. Genes with scores yielding p values <0.01

Table 1. Demographic characteristics of the subjects in the study

	Controls (n = 40)	CFS subjects (n = 40)
Female, %	90.0	82.5
Age, years (mean ± SD)	50.6 ± 7.4	50.7 ± 8.7
BMI (mean ± SD)	29.3 ± 5.3	29.4 ± 4.5
Current MDD, %	0.0	7.5
Lifetime MDD, %	5.0	15.0
Lifetime MDDM, %	15.0	22.5

BMI = Body mass index; MDD = major depressive disorder; MDDM = major depressive disorder with melancholia.

were considered biologically relevant by GOFFA. We also used Ingenuity Pathways Analysis (IPA; Ingenuity Systems Inc., Calif., USA) to identify molecular networks among the differentially expressed genes.

Post hoc Analysis and Multiple Test Correction

Fisher's combined probability test was used to incorporate the SNP association and gene expression results for each gene that reached significance independently in each experiment. A Bonferroni correction was then applied to account for the total number of possible tests.

Results

SNP Association Study

Subjects with CFS did not differ significantly from nonfatigued controls with respect to age, sex, body mass index or history of major depressive disorder or melancholic depression (table 1). A genomic control correction was used to evaluate potential population stratification. The mean (0.74) and median (0.29) χ^2 values for over 50,000 unassociated SNPs were calculated and suggested no evidence that population stratification confounded the results. Allelic association tests revealed 3,234 SNPs associated with CFS with a p value <0.05 and 65 SNPs with a p value <0.001 (table 2). Regions of interest included chromosomes 1, 2, 6, 7, 10, 11, 12 and 13 and an extended area of association on chromosome 19. Thirty-five of these SNPs are located in or near 33 unique genes, and the remainder are located near expressed sequence tags (n = 10) or in regions with no known genes (n = 20; table 2).

Several associated polymorphisms reside in or near known genes of particular relevance to CFS (table 2). For example, in *GRIK2*, the C allele of rs2247218 and the G

Table 2. Allele frequencies and location of SNPs associated with CFS (p < 0.001)

Chromo-some	SNP ID	Gene/expressed sequence tag Genome Build 36.3EST	Genotype	Frequency, n		p value	
				controls	CFS subjects	genotype	allele
1	rs2105239	unknown	AA	17 (44.7)	4 (10.3)	0.0006	0.0003
			AG	18 (50.0)	24 (61.5)		
			GG	2 (5.3)	11 (28.2)		
	rs1157819	NPC-A5	AA	9 (24.3)	1 (2.8)	0.0053	0.0006
			AT	15 (40.5)	11 (30.6)		
			TT	13 (35.1)	24 (66.7)		
	rs10489599	*FBXO42*	CC	6 (17.7)	1 (3.3)	0.0043	0.0006
			CT	14 (41.2)	5 (16.7)		
			TT	14 (41.2)	24 (80.0)		
	rs6694861	*MAGI3*	GG	25 (62.5)	11 (29.0)	0.0041	0.0008
			GA	13 (32.5)	18 (47.4)		
			AA	2 (5.0)	9 (23.7)		
2	rs6710681	BF513477	CC	14 (35.0)	3 (7.7)	0.0014	0.0002
			CT	19 (47.5)	17 (43.6)		
			TT	7 (17.5)	19 (48.7)		
	rs10496982	unknown	AA	37 (97.4)	29 (72.5)	0.0047	0.0003
			AC	1 (2.6)	8 (20.0)		
			CC	0 (0.0)	3 (7.5)		
	rs2715898	DA324672	GG	2 (5.3)	14 (38.9)	0.0006	0.0002
			GA	15 (39.5)	12 (33.3)		
			AA	21 (55.3)	10 (27.8)		
	rs356653	*NPAS2*	CC	29 (74.4)	16 (41.0)	0.0017	0.0007
			CT	10 (25.6)	17 (44.4)		
			TT	0 (0.0)	6 (15.4)		
	rs6721414	DA335567	CC	6 (15.4)	2 (5.9)	0.0024	0.0008
			CG	18 (46.2)	5 (14.7)		
			GG	15 (38.5)	27 (79.4)		
3	rs10510985	unknown	AA	6 (15.4)	17 (43.6)	0.0007	0.0002
			AG	16 (41.0)	19 (48.7)		
			GG	17 (43.6)	3 (7.7)		
	rs1377828	DA709814	CC	6 (15.4)	0 (0.0)	0.0009	0.0006
			CA	21 (53.9)	13 (32.5)		
			AA	12 (30.8)	27 (67.5)		
	rs4894505	BG218013	TT	15 (37.5)	29 (72.5)	0.0029	0.0008
			TG	18 (45.0)	10 (25.0)		
			GG	7 (17.5)	1 (2.5)		
4	rs2389957	unknown	GG	18 (46.2)	9 (22.5)	0.0029	0.0005
			AG	20 (51.3)	19 (47.5)		
			AA	1 (2.6)	12 (30.0)		
	rs10516187	*AFAP1*	TT	0 (0.0)	2 (5.0)	0.0011	0.0006
			TG	3 (7.7)	14 (35.0)		
			GG	36 (92.3)	24 (60.0)		
5	rs33013	*MSH3*	GG	26 (65.0)	12 (30.0)	0.0019	0.0006
			GA	13 (32.5)	21 (52.5)		
			AA	1 (2.5)	7 (17.5)		
	rs10514211	*RASGRF2*	AA	28 (71.8)	40 (100.0)	0.0005	0.0006
			AG	11 (28.2)	0 (0.0)		
			GG	0 (0.0)	0 (0.0)		
	rs3797302	*TCERG1*	CC	0 (0.0)	3 (8.6)	0.004	0.0009
			CG	2 (5.6)	10 (28.6)		
			GG	32 (94.1)	22 (62.9)		

Table 2 (continued)

Chromo-some	SNP ID	Gene/expressed sequence tag Genome Build 36.3EST	Genotype	Frequency, n		p value	
				controls	CFS subjects	genotype	allele
6	rs2047179	unknown	TT	25 (67.6)	15 (40.5)	0.0032	0.0003
			TC	11 (29.7)	11 (29.7)		
			CC	1 (2.7)	11 (29.7)		
	rs10498968	unknown	GG	5 (12.8)	1 (2.6)	0.0006	0.0005
			GC	20 (51.3)	8 (20.5)		
			CC	14 (35.9)	30 (76.9)		
	rs2247218	*GRIK2*	CC	2 (5.6)	11 (29.7)	0.0012	0.00007
			CT	11 (30.6)	17 (45.9)		
			TT	23 (63.9)	9 (24.3)		
	rs2247215	*GRIK2*	AA	23 (57.5)	9 (22.5)	0.0025	0.0005
			AG	15 (37.5)	22 (55.0)		
			GG	2 (5.00)	9 (22.5)		
	rs6915865	unknown	TT	6 (16.2)	20 (50.0)	0.0055	0.0007
			TC	16 (43.2)	12 (30.0)		
			CC	15 (40.5)	8 (20.0)		
	rs4714468	DQ141194	AA	0 (0.0)	2 (5.1)	0.0011	0.0007
			AG	4 (10.0)	15 (38.5)		
			GG	36 (90.0)	22 (56.4)		
	rs9320409	*KLHL32*	TT	15 (38.5)	4 (10.0)	0.0022	0.0008
			TC	19 (48.7)	21 (52.5)		
			CC	5 (12.8)	15 (37.5)		
7	rs3801293	*SHFM1*	TT	0 (0.0)	2 (5.0)	0.0011	0.0008
			TC	2 (5.6)	13 (32.5)		
			CC	34 (94.4)	25 (62.5)		
	rs4245562	BX111274	TT	15 (37.5)	25 (67.6)	0.0016	0.0009
			TC	17 (42.5)	12 (32.4)		
			CC	8 (20.0)	0 (0.0)		
	rs10499740	unknown	TT	4 (5.3)	12 (30.8)	0.0063	0.0009
			TC	10 (27.8)	17 (43.6)		
			CC	22 (61.1)	10 (25.6)		
	rs723886	unknown	GG	25 (62.5)	12 (30.0)	0.0074	0.001
			GT	11 (27.5)	16 (40.0)		
			TT	4 (10.0)	12 (30.0)		
8	rs4236780	unknown	CC	21 (52.5)	34 (85.0)	0.0027	0.0006
			CG	16 (40.0)	6 (15.0)		
			GG	3 (7.5)	0 (0.0)		
	rs543736	LOC100131813	GG	15 (40.5)	31 (79.5)	0.0014	0.0007
			GA	16 (43.2)	5 (12.8)		
			AA	6 (16.2)	3 (7.7)		
	rs1499646	unknown	CC	5 (12.5)	1 (2.6)	0.006	0.0009
			CT	14 (35.0)	5 (12.8)		
			TT	21 (52.5)	33 (84.6)		
9	rs871024	*MTAP*	AA	16 (42.1)	6 (16.2)	0.015	0.0006
			AC	13 (34.2)	11 (29.7)		
			CC	9 (23.7)	20 (54.1)		
	rs4978076	unknown	CC	17 (43.6)	6 (16.2)	0.0046	0.0006
			CT	16 (41.0)	14 (37.8)		
			TT	6 (15.4)	17 (46.0)		
	rs10511961	*PIP5K1B*	CC	12 (31.6)	2 (5.3)	0.0012	0.0007
			CG	20 (52.6)	18 (47.4)		
			GG	6 (16.8)	18 (47.4)		

Table 2 (continued)

Chromo-some	SNP ID	Gene/expressed sequence tag Genome Build 36.3EST	Genotype	Frequency, n		p value	
				controls	CFS subjects	genotype	allele
10	rs10509412	*CFLP1*	GG	12 (31.6)	2 (5.26)	0.0035	0.0003
			GT	15 (39.5)	14 (36.8)		
			TT	11 (29.0)	22 (57.9)		
	rs10509958	*TECTB*	AA	22 (61.1)	11 (35.5)	0.0031	0.0004
			AG	13 (36.1)	10 (32.3)		
			GG	1 (2.78)	10 (32.3)		
	rs726817	*FRA10AC1*	CC	1 (2.5)	6 (15.8)	0.0054	0.0006
			CT	10 (25.0)	17 (44.7)		
			TT	29 (72.5)	15 (39.5)		
	rs1325904	*C10orf59*	CC	19 (47.5)	30 (79.0)	0.0093	0.0008
			CT	15 (37.5)	7 (18.4)		
			TT	6 (15.0)	1 (2.6)		
11	rs10500964	unknown	TT	3 (8.1)	0 (0.0)	0.0009	0.0001
			TC	8 (21.6)	1 (2.6)		
			CC	26 (70.3)	38 (97.4)		
	rs734640	*OTOG*	CC	8 (21.6)	0 (0.0)	0.0021	0.0004
			CT	13 (35.1)	8 (22.9)		
			TT	16 (43.2)	27 (77.1)		
	rs10500965	unknown	GG	26 (66.7)	39 (97.5)	0.0002	0.00006
			GT	10 (25.6)	1 (2.5)		
			TT	3 (7.7)	0 (0.0)		
	rs10501068	CN274762	GG	10 (25.0)	1 (2.7)	0.0019	0.0003
			GT	19 (47.5)	13 (35.1)		
			TT	11 (27.5)	23 (62.2)		
	rs10501376	*DTX4*	CC	38 (100.0)	29 (74.4)	0.0015	0.0005
			CG	0 (0.0)	9 (23.1)		
			GG	0 (0.0)	1 (2.56)		
	rs1881470	unknown	GG	7 (18.9)	0 (0.0)	0.0018	0.0005
			GC	14 (37.8)	7 (20.6)		
			CC	16 (43.2)	16 (43.2)		
	rs10488767	*ARHGAP20*	GG	10 (27.8)	20 (57.1)	0.0037	0.0009
			AG	16 (44.4)	14 (40.0)		
			AA	10 (27.8)	1 (2.9)		
12	rs10505778	*GRIN2B*	AA	22 (55.0)	7 (8.86)	0.0012	0.0002
			AG	14 (35.0)	18 (22.8)		
			GG	4 (10.0)	14 (77.8)		
	rs10506025	*PPFIBP1*	AA	7 (18.4)	23 (59.0)	0.0009	0.0001
			AG	21 (55.3)	13 (33.3)		
			GG	10 (26.3)	3 (7.69)		
	rs1144418	*FLJ41278*	CC	30 (76.9)	20 (52.6)	0.0068	0.0004
			CA	8 (20.5)	8 (21.1)		
			AA	1 (2.56)	10 (26.3)		
	rs4931109	unknown	TT	3 (7.5)	0 (0.0)	0.0033	0.0009
			TC	13 (32.5)	4 (10.0)		
			CC	24 (60.0)	36 (90.0)		
	rs167337	*SCN8A*	CC	0 (0.0)	3 (7.9)	0.0042	0.0009
			CT	4 (10.0)	12 (31.6)		
			TT	36 (90.0)	23 (60.5)		

Table 2 (continued)

Chromo-some	SNP ID	Gene/expressed sequence tag Genome Build 36.3EST	Genotype	Frequency, n		p value	
				controls	CFS subjects	genotype	allele
13	rs7994531	BG220650	TT	0 (0.0)	6 (17.1)	0.0004	0.0001
			TC	6 (17.1)	16 (45.7)		
			CC	29 (82.9)	13 (37.1)		
	rs1931035	unknown	AA	0 (0.0)	6 (15.8)	0.0025	0.0001
			AG	4 (10.0)	9 (23.7)		
			GG	36 (90.0)	23 (60.5)		
	rs1359536	unknown	TT	38 (95.0)	27 (69.2)	0.0045	0.0005
			TC	2 (5.0)	7 (18.0)		
			CC	0 (0.0)	5 (12.8)		
	rs547571	*HS6ST3*	AA	0 (0.0)	1 (2.50)	0.0003	0.0002
			AG	3 (7.50)	16 (40.0)		
			GG	37 (61.7)	23 (57.5)		
	rs1555589	*CLYBL*	AA	24 (77.4)	18 (45.0)	0.0123	0.0004
			AG	6 (19.4)	13 (32.5)		
			GG	1 (3.23)	9 (22.5)		
	rs7325773	CA425896	CC	31 (79.5)	18 (45.0)	0.0012	0.0006
			CG	8 (20.5)	17 (42.5)		
			GG	0 (0.0)	5 (12.5)		
	rs10507556	unknown	AA	2 (5.3)	0 (0.0)	0.0051	0.001
			AG	8 (21.1)	1 (2.6)		
			GG	28 (73.7)	38 (97.4)		
14	rs2372200	unknown	GG	0 (0.0)	5 (13.5)	0.0026	0.0004
			GA	7 (20.0)	16 (43.2)		
			AA	28 (80.0)	16 (43.2)		
	rs3759688	*SIX6*	GG	28 (77.8)	39 (100.0)	0.0015	0.0008
			GT	7 (19.4)	0 (0.0)		
			TT	1 (2.8)	0 (0.0)		
17	rs6503623	*KRTHA3A*	AA	27 (69.2)	38 (97.4)	0.0017	0.0004
			AG	9 (23.1)	1 (2.56)		
			GG	3 (7.69)	0 (0.0)		
19	rs400322	*LILRB4*	AA	0 (0.0)	6 (16.2)	0.0032	0.0001
			AG	5 (13.9)	11 (29.7)		
			GG	31 (86.1)	20 (54.1)		
	rs10500321	*NLRP11*	CC	0 (0.0)	2 (5.1)	0.0021	0.0008
			CT	4 (10.0)	14 (35.9)		
			TT	36 (90.0)	23 (59.0)		
	rs2059152	*NLRP11*	AA	0 (0.0)	2 (5.1)	0.0028	0.001
			AG	4 (10.0)	14 (35.9)		
			GG	36 (90.0)	23 (59.0)		
	rs382958	*NLRP13*	CC	3 (7.89)	13 (37.1)	0.0051	0.0002
			CT	11 (29.0)	10 (28.6)		
			TT	24 (63.2)	12 (34.3)		
21	rs1399592	*KCNJ6*	GG	6 (16.2)	15 (40.5)	0.0038	0.0004
			GT	15 (40.5)	18 (48.7)		
			TT	16 (43.2)	4 (10.8)		

Mapping of SNPs to respective chromosomes, genes or expressed sequence tags was accomplished with NCBI Genome Build 36.3. Values in parentheses represent percentages.

allele of rs2247215 were more common in CFS cases (50.0%) than in controls (24.0%; 0.00007 < p < 0.0005); both SNPs are in linkage disequilibrium. In glutamate receptor, ionotropic, N-methyl-D-aspartate 2B *(GRIN2B)*, the G allele of rs10505778 was more common in CFS subjects (59.0%) than in controls (27.5%; p = 0.0002). We also observed associated polymorphisms in 3 immune response genes, namely NLR family, pyrin domain-containing *(NLRP)* 11 and 13 and leukocyte immunoglobulin-like receptor, subfamily B, member 4 *(LILRB4)*, which are located in a 1.28-megabase region of chromosome 19, which also contains several SNPs associated with CFS. The A allele of rs400322 *(LILRB4)* was more common in CFS subjects (31.1%) than in controls (6.9%; p = 0.0001), and the C allele of rs382958 *(NLRP13)* was also more common in CFS subjects (51.4%) than in controls (22.4%; p = 0.0002). In *NLRP11,* the C allele of rs10500321 and the A allele of rs2059152 were also associated with CFS (0.001 < p < 0.0008). Finally, the T allele of rs356653, an SNP located in *NPAS2,* a gene implicated in circadian regulation, was more common in subjects with CFS (p = 0.0007).

Gene Expression Analysis

The demographics of those subjects for whom we obtained gene expression data did not differ significantly from those included in the association study described above (data not shown). One hundred and sixty-five genes were differentially expressed between CFS subjects and controls (online suppl. table 2). Once again, genes of particular relevance to CFS were identified in this analysis. For example, hypocretin receptor 1 *(HCRTR1)* was one of the highly upregulated genes (nearly 20-fold; p = 0.004) in CFS subjects in this study. Glutamate receptor, metabotropic 1 *(GRM1)* was upregulated 4.9-fold (p = 0.046), and *GRM4* was downregulated more than 10-fold (p = 0.0093). *GRIK2* was also downregulated (10-fold; p = 0.015). The mRNA expression of killer cell immunoglobulin-like receptor, 2 domains, long cytoplasmic tail 4 and 5 *(KIR2DL4* and *KIR2DL5)* was 5 times lower for both in CFS subjects compared to controls (p = 0.024 and p = 0.030, respectively). Hypoxia-inducible factor 1, alpha subunit *(HIF1A)* was downregulated 6-fold (p = 0.026) in CFS subjects. Finally, *NPAS2* was upregulated (nearly 10-fold; p = 0.027) in CFS subjects. However, only *GRIK2* and *NPAS2* were associated with CFS in both the SNP association and mRNA expression experiments.

Differentially expressed genes were then evaluated using GOFFA, KEGG, PathArt and IPA to explore gene function and molecular networks. Among these genes, GOFFA yielded 42 genes (p < 0.01) with a known molecular function, including *GRIK2* and *NPAS2* (online suppl. table 2). KEGG analysis of these 42 genes identified 5 pathways (p < 0.05), including neuroactive ligand-receptor interaction (HSA04080; *APLNR, EDNRB, GRIK2, GRM1, GRM4, HCTRR1, OPRM1, TAAR9*), taste transduction (HAS04742; *TAS2R10, TAS2R9*), leukocyte transendothelial migration (HSA04670; *ACTN1, CTNNA1, CXCR4, PXN*), adherens junctions (HSA04520; *ACTN1, CTNNA1, SORBS1*) and regulation of actin cytoskeleton (HSA04810; *ACTN1, CFL1, SPTAN1*). Similarly, PathArt analysis identified several pathways, notably chemotaxis *(CXCR4, PXN)*, amyloid beta-peptide *(NPAS2, PXN)* and cell-cell *(GRIK2, GRIM1, GRIM4, HCRTR1)* signaling pathways. IPA organized the differentially expressed genes into 5 molecular networks (scores ≥20 including a minimum of 10 focus molecules; online suppl. table 3).

Two genes, *GRIK2* and *NPAS2*, reached significance independently in the SNP association and gene expression studies. We used Fisher's combined probability test to incorporate the SNP association and gene expression results for these two genes. Because the two polymorphisms in *GRIK2* that were associated with CFS were in linkage disequilibrium and were therefore not independent, only the result from rs2247218 was used. The results of this meta-analysis increased the magnitude of the associations for both *GRIK2* (p = 0.000016) and *NPAS2* (p = 0.00022). However, 3,593 genes were common to both the SNP and expression analyses and are expressed in both the blood and brain according to the Body Atlas data implemented in MetaCore (GeneGo). Using a stringent Bonferroni correction for the number of possible genes tested, *NPAS2* (p = 0.55) did not retain significance while *GRIK2* (p = 0.054) remained suggestive of an association.

Discussion

Convergent functional genomics allowed the identification of novel candidate genes, *GRIK2* and *NPAS2,* involved in glutamatergic neurotransmission and the circadian rhythm, respectively, that are potentially associated with CFS. Polymorphisms in *GRIK2* were associated with CFS, and expression of *GRIK2* mRNA was decreased in CFS subjects. Also, a polymorphism in *NPAS2* was more common in subjects with CFS, while *NPAS2* expression was increased in CFS subjects. A recent computational study suggested that genes that are highly differentially

expressed are more likely to harbor disease-associated DNA variants. By determining differential expression ratios (DERs) using all of the data sets (>476) in the human Gene Expression Omnibus database, Chen et al. [28] demonstrated that genes with a DER >0.55 are 2.5 times more likely to harbor disease-associated genetic variants than constitutively expressed genes. We used the FitSNP database developed by Chen et al. [28] to determine DER values for *GRIK2* and *NPAS2*. High DER values (>0.55) for both *GRIK2* (0.56) and *NPAS2* (0.64) lend another level of analytical support to our conclusion that these genes warrant further examination as potential contributors to CFS pathophysiology. These associations must be confirmed in independent populations of subjects with CFS.

It is interesting to note that both *GRIK2* and *NPAS2* have been reported to be associated with impaired cognition as well as memory and sleep, 2 of the hallmark symptoms of CFS [29, 30]. The association of these two genes with some of the hallmark symptoms of CFS, which overlaps with psychiatric illness, including mood and anxiety disorders [31], suggests that while these conditions can be clinically distinct, they may share neurobiological underpinnings. In support of this, we observed associations between CFS and genetic polymorphisms in 2 glutamate receptors, *GRIK2* and *GRIN2B*. We also observed associations between CFS and mRNA expression of *GRIK2*, *GRM1* and *GRM4*. Polymorphisms in glutamate receptors *(GRIK2, GRIN2B)* have been associated with a number of neuropsychiatric disorders such as autism [32], Huntington's disease [33], attention deficit/hyperactive disorder [34], obsessive compulsive disorder [35], antidepressant treatment-emergent suicidal ideation [36] and antipsychotic-induced weight gain [37] but have never been investigated in CFS. Among these polymorphisms, SNPs associated with suicidal ideation in patients being actively treated for depression (rs2518224) [36] reside in the same intron of *GRIK2* that harbors the polymorphisms associated with CFS in this study (rs2247218 and rs2247215). Interestingly, the *GRIN2B* polymorphism (rs10505778) associated with CFS was also associated with antipsychotic-induced weight gain [37]. Since glutamate receptors mediate most excitatory neurotransmission in the brain and play an important role in learning, memory and mood regulation, the association of genetic variants in glutamate receptors as well as previous results implicating an association between chronic pain and mental fatigue and reduced glutamate uptake [38] support a shared role for the glutamatergic system in several cellular and cognitive processes involved in CFS, depression and autism.

Altered functioning of the endogenous circadian clock can lead to neurobiological, behavioral and psychiatric deteriorations. Multiple polymorphisms in genes involved in regulation of the circadian clock have been associated with sleep disorders and psychiatric diseases [39, 40]. In this study, we observed an association between CFS and a polymorphism (rs356653) in *NPAS2,* a member of the basic helix-loop-helix PAS family of transcription factors that is a central component of the molecular circadian oscillator. NPAS2 heterodimerizes with BMAL1 and functions as a positive element of the circadian system to drive the transcription of clock-controlled genes [39, 41]. NPAS2 may function primarily as part of a molecular clock operating in the forebrain, but being a paralog of *CLOCK,* it can also substitute for *CLOCK* in the master clock in the hypothalamic suprachiasmatic nuclei to regulate circadian rhythmicity [41, 42]. NPAS2 has been associated with cued and contextual memory [43] and may function as a transcriptional regulator of non-rapid eye movement sleep in mice [44, 45]. Polymorphisms in *NPAS2* have been associated with multiple psychiatric disorders. For example, variants have been associated with autism (rs1811399) [46], winter depression (rs11541353) [47], mood disorders and schizophrenia (rs13025524 and rs11123857) [48, 49] as well as breast cancer and non-Hodgkin's lymphoma (NHL) [50]. The associations between *NPAS2* variants and CFS and NHL are interesting in the context of anecdotal reports suggesting that CFS may predispose subjects to NHL [51]. While none of these studies report an association with a common *NPAS2* polymorphism, these associations suggest an overlapping role for *NPAS2* in CFS and psychiatric disorders, potentially via common symptomatology such as sleep architecture or metabolic imbalances [52].

Associated polymorphisms in *GRIK2* (rs2247218 and rs2247215) and *NPAS2* (rs356653) are located in the first and second introns of their respective genes. Since these SNPs are in introns, their impact on gene function and ultimately the associated phenotype is not immediately clear. These markers may be in linkage disequilibrium with other causative markers or they may play a direct role in gene expression by affecting transcription factor binding, alternative splicing or microRNA production. Of the 2 *GRIK2* SNPs, rs2247215 showed in silico evidence for alteration of consensus binding sites for 2 overlapping transcription factors, GATA1 and EVI1. The G allele of rs2247215 creates a consensus binding site for GATA1, suggesting that *GRIK2*, like other GATA1-repressed genes, may be repressed through the participation of polycomb repressive complex 2 [53]. This potential role of

rs2247215 is consistent with the association of its G allele with CFS and decreased expression of *GRIK2* in CFS subjects. In silico analysis also identified a binding site for RFX1 transcription factor at rs356653 *(NPAS2)*. The T allele of rs356653 is likely to disrupt RFX1 binding. RFX1 may be a transcriptional repressor of *NPAS2* [54], and if so the T allele would lead to increased *NPAS2* expression by disrupting RFX1 binding. This interpretation is consistent with our findings that the T allele is associated with CFS and increased expression of *NPAS2* in CFS. However, experimental validation is required to support the role of rs2247215 and rs356653 variants in gene expression.

Associations between CFS and polymorphisms in *LILRB4, NLRP11* and *NLRP13* were observed, but we observed no difference in mRNA expression for these genes. However, only probes for *LILRB4* were available on the expression array that was used, so it remains unclear whether or not *NLRP11* and *NLRP13* are differentially expressed in subjects with CFS. Interestingly, *KIR2DL4* and *KIR2DL5,* which are located between *LILRB4* and *NLRP13,* exhibited decreased expression in subjects with CFS. These two genes have been associated with Gulf War illness [55], whose symptoms resemble that of CFS in terms of fatigue, musculoskeletal discomfort and cognitive dysfunction. All of these genes are encoded within the leukocyte receptor complex on human chromosome 19q13. Studies suggest that postinfective fatigue persists in a subset of patients following infection by viral or nonviral microorganisms [56]. It is possible that individuals with a genetic susceptibility in the leukocyte receptor complex may develop CFS in response to a serious immune challenge.

Gene expression analysis identified two genes potentially replicating earlier discoveries of pathways or genes associated with CFS or postinfective fatigue syndrome in other populations. This study identified an orexin/hypocretin receptor, *HCRTR1*, as one of the highly upregulated genes (nearly 20-fold) in CFS subjects. A functionally related gene, hypocretin receptor 2 *(HCRTR2),* was found to be positively correlated with sleep in subjects with postinfective fatigue in Australia [57]. *HIF1A,* which was downregulated 6-fold in CFS subjects in this study, was also reported to be downregulated in CFS subjects in the UK [7]. Based on these replicated gene expression array results and the reported interaction between orexin signaling and HIF1 activity [58], functional studies on the role of these orexin receptors and *HIF1A* in the pathogenesis of CFS are warranted.

While the present study has considerable strengths, including recruitment of subjects from the general population and rigorous clinical evaluations of both cases and controls, the study is limited by the small sample size and the fact that the statistical thresholds for both our genetic association and gene expression results are not stringent enough to achieve experiment-wide significance on their own. However, the statistical thresholds utilized in this study are comparable to or more stringent than those in recent studies that undertook a convergent functional genomics approach to identify viable candidate genes for further study from multiple lines of evidence [17–19]. Another limitation is the use of SNP and expression arrays that are not comprehensive of the genome, thus limiting coverage and making it less likely to identify polymorphisms that directly influence gene expression. However, it should be noted that the polymorphisms reported in this study achieved a greater level of statistical significance than those of any candidate gene study of CFS published to date. Further, *GRIK2* and *NPAS2* are differentially expressed, providing proof of principle support for the use of convergent functional genomics approaches for studies that would otherwise be underpowered, as well as support for further investigations of these genes for their potential contribution to the pathophysiology of CFS. Nonetheless, false-positive (type I error) results remain a possibility because of population stratification, multiple comparisons, the complexity and heterogeneity of CFS and difficulties with reproducible case ascertainment. Using a genomic control method, we found no evidence of population stratification. While increasing the sample size and determining Bonferroni-corrected p values can be helpful to address multiple comparisons, these approaches alone may not be enough to address the complexity and heterogeneity of an illness like CFS [20]. Validating these findings will require replication of SNP and gene expression analysis in a replication study in a larger independent population. Further, examining genetic associations at measurable narrow subphenotypic levels may minimize false-positive findings across multiple populations.

The expression analysis in the present study was based on PBMCs, which may not be directly involved in CFS pathogenesis. However, given the bidirectional communication between the central nervous and peripheral systems [59], changes in PBMC expression are relevant as a reflection of that communication [22]. In addition, previous studies indicate that PBMCs express approximately 60% of the genes expressed in brain tissue [12, 60]. The most compelling genes identified in this study are expressed in both the blood and brain tissues, but direct investigations of the influence of genetic variations on expression of these genes in multiple brain regions and blood cells remain to be performed.

In conclusion, this study provides the first evidence for a role of genes involved with glutamatergic neurotransmission (*GRIK2*) and circadian rhythm (*NPAS2*) in CFS. Since *GRIK2* and *NPAS2* were identified from convergent results from complementary SNP and gene expression analyses, additional investigation in independent populations of CFS subjects is warranted. Replication of these findings will open novel avenues for the study of CFS pathogenesis. This study also underscores the utility of leveraging both SNP association and gene expression approaches to identify candidate genes from studies that otherwise would be underpowered.

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