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Validation of Candidate Anxiety Disorder Genes Using a Carbon Dioxide Challenge Task

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Abstract

Few replicable genetic variants have been identified in the etiology of heritable anxiety disorders such as panic disorder. Endophenotypic measures that have reduced heterogeneity may provide more powerful targets for gene identification. We assessed hypersensitivity to carbon dioxide (a reliable endophenotype of panic and anxiety) in 174 Caucasian college students, who were genotyped on 26 polymorphic markers from 11 genes previously associated with panic/anxiety. Individual trajectories of respiratory and subjective anxiety response to carbon dioxide were measured and tested for association with these genetic markers. One marker in the acid-sensing ion channel 1 (*ASIC1*) gene, rs1108923, had a significant association with respiratory rate. No genes had a significant association with subjective anxiety response. Our findings support previously reported associations between *ASIC1* and panic/anxiety, but not other genes previously associated with anxiety disorders. The use of endophenotypic markers is a promising avenue for gene identification in anxiety and other complex disorders.

Keywords

carbon dioxide hypersensitivity; *ASIC1*; acid-sensing ion channel 1; panic disorder; anxiety disorders; endophenotype

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Introduction

Research has begun to achieve some success in identifying genetic variants underlying heritable psychiatric disorders, yet molecular genetic studies of anxiety disorders lag behind. Well-powered genome-wide association studies in this area are still rare, thus most of the existing knowledge is based on candidate gene studies, which have largely been inconsistent and unreplicated (Maron, Hettema, & Shlik, 2010). One reason for this difficulty is that anxiety disorders may be genetically heterogeneous (Smoller & Tsuang, 1998), with genetic determinants that cross diagnostic boundaries (Hettema, Neale, Myers, Prescott, & Kendler, 2006). A possible solution is to utilize endophenotypes, intermediate phenotypes that sit in the biological pathway between genes and psychological outcomes and may be more powerful targets for gene discovery (Gottesman & Gould, 2003; Meyer-Lindenberg & Weinberger, 2006).

The goal of the present study was to replicate previously reported candidate gene associations with anxiety disorders using hypersensitivity to carbon dioxide (CO₂), a well-validated endophenotype of panic disorder (PD) (Coryell, 1997) and other anxiety disorders (Caldirola, Perna, Arancio, Bertani, & Bellodi, 1997; Telch, Rosenfield, & Pai, 2012). We examined the association between CO₂ hypersensitivity and 11 genes using both subjective and physiologic measures. Subjective anxiety post-CO₂ inhalation has strong support as a heritable, panic-relevant trait marker (Battaglia et al., 2007; Coryell, Fyer, Pine, Martinez, & Arndt, 2001; Schmidt & Szolensky, 2007; Vickers, Jafarpour, Mofidi, Rafat, & Woznica, 2012), and respiratory rate appears to be moderately heritable ($h^2=.51-.62$; Snieder, Boomsma, Van Doornen, & De Geus, 1997) and differentiates anxiety disorder patients and at-risk persons from controls (Griez, de Loof, Pols, Zandbergen, & Lousberg, 1990; Perna, Barbini, Cocchi, Bertani, & Gasperini, 1995; Rassovsky & Kushner, 2003).

Methods

Sample

Participants were 174 Caucasian students from two large, urban, public universities in Virginia (53.4% female, age: $M = 19.8$, $SD = 2.6$, range = 18-38). Participants were not screened for anxiety disorders, but completed the Anxiety Sensitivity Index (ASI; Reiss, Peterson, Gursky, & McNally, 1986), and were selected to form approximately equal numbers across all quartiles of the score distribution. The university Institutional Review Boards approved this study and participants provided informed consent. Genetic analyses were limited to Caucasian students to control against spurious results from population stratification (Cardon & Palmer, 2003).

Measures

We used a sustained carbon dioxide (CO₂) breathing task that included three phases: a five-minute baseline of breathing room air, eight minutes of breathing 7.5% CO₂ enriched air, and a five minute recovery period. Breath-to-breath respiratory rate (RR), averaged across 60-second intervals, was measured continuously throughout the task via a transducer around the abdomen/chest and was recorded with a Biopac system with Acqknowledge software

(Biopac Systems, Inc, Goleta, CA). Subjective anxiety was recorded at two-minute intervals throughout the task using the Subjective Units of Distress Scale (SUDS; Wolpe, 1973), which indexes perceived anxiety on a 0-100 scale.

Saliva was collected using Oragene•DISCOVER (OGR-250) Sampling Kits (DNA Genotek, Ontario, Canada). DNA was extracted per the manufacturer's protocol. Samples were genotyped on the 7900HT real-time PCR instrument using Taqman genotyping assays (Life Technologies, Grand Island, New York). Allele discrimination analysis and genotype calls were made with the ABI 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, California). To verify genotyping accuracy, 10% of the samples were randomly selected for re-genotyping, with 100% concordance rates.

The 26 genotypic markers were chosen based on previously reported empirical associations with panic/anxiety disorders or related traits (see Table 1). Individual SNPs were selected because those SNPs demonstrated a significant phenotypic association, or tagged associated regions or genes. Markers were excluded for Hardy-Weinberg equilibrium p -values below 10^{-4} ($n=0$) or genotyping success below 95% ($n=2$). Individuals were excluded for genotyping success below 95% ($n=2$).

Data Analysis

We assessed individual trajectories of physiological (RR) and subjective (SUDS) response to the CO₂ task using latent growth curve analysis in Mplus version 6 (Muthén & Muthén, 2010). This method gives participants a score on each of the latent growth factors (intercept, slope, quadratic) that characterize the best-fitting line of their RR/SUDS level throughout the task. Linear regression (SPSS version 21) was then conducted with each SNP (minor allele count) to determine how these genetic markers predicted initial RR/SUDS (intercept), linear increase/decrease (slope), and non-linear increase/decrease (quadratic) in RR/SUDS in response to CO₂ inhalation.

Because co-localized SNPs were not independent ($r=0.08$ to $r=0.81$), and factor scores were also correlated ($r=.174$ to $r=.995$), we calculated the effective number of tests to correct for multiple testing (Han & Eskin, 2010; Li & Ji, 2005; Nyholt, 2004). This resulted in an adjusted alpha level for our two phenotypic association tests of 0.0024 for RR and 0.0017 for SUDS.

Results

The trajectories of RR and SUDS response followed a quadratic pattern, with most participants experiencing a sharp increase in RR and SUDS during CO₂ administration, followed by a return to near-baseline levels in the recovery period.

Table 2 presents results from the SNP regression analyses, which indicate the direction and magnitude of the association between each marker and the latent growth factors. One SNP, rs1108923, located within an intronic region of the *ASIC1* gene, had a significant association with the intercept of respiratory rate. The association between the number of minor (T) alleles at this marker and RR intercept was negative (standardized $\beta=-0.27$, $p=.001$), such

that carriers of the T allele had lower initial RR, as illustrated in Figure 1. No markers met the significance threshold for association with subjective anxiety (SUDS). Additionally, female gender was predictive of higher baseline RR but was not associated with other outcome measures.

To follow up on this result, we conducted a linear regression analysis with rs1108923 predicting ASI scores. There was suggestive evidence for a negative association between the T allele and trait anxiety sensitivity (standardized $\beta=-0.18$, $p=.027$). rs1108923 was also negatively, but not significantly, associated with experiencing panic during the task, including quitting the task early ($n=29$, 17%; odds ratio=0.74, $p=.582$) and panic symptoms assessed by the Diagnostic Symptoms Questionnaire (Sanderson, Rapee, & Barlow, 1989) during the baseline period (standardized $\beta=-0.13$, $p=.117$).

Conclusions

In an attempt to validate several previously reported genetic associations with panic/anxiety using the CO₂ hypersensitivity endophenotype, we found that only one marker in the acid-sensing ion channel gene *ASIC1* was associated with respiratory rate, and none with subjective anxiety response. The *ASIC1* gene was negatively associated with the intercept, or initial respiratory rate, with some additional evidence for a negative association with self-reported anxiety sensitivity. Further, trait anxiety sensitivity (ASI) scores were positively correlated with baseline RR ($r=.16$, $p=.045$), indicating an interrelationship between the *ASIC1* gene, anxiety sensitivity, and basal physiological anxiety, especially with respect to respiration.

Acid-sensing ion channels are responsible for detecting changes in pH in the brain (including those evoked by CO₂), and their abundance in the amygdala suggests a central role in interpreting chemical signals and eliciting fear behavior (Wemmie, 2011; Ziemann et al., 2009). The orthologous *Asic1a* gene directly influences the fear response in mice during a CO₂ inhalation task, due to CO₂-induced decreases in pH (Coryell et al., 2007; Price et al., 2014; Wemmie et al., 2004; Ziemann et al., 2009). Recent human genetic studies also found evidence for an association of *ASIC1* (Smoller et al., 2014) and the related gene *ASIC2* (Gegersen et al., 2012) with panic disorder.

Although *ASIC1* predicted basal RR rather than CO₂ response, the physiological interrelations between respiration, CO₂, and panic suggest further investigation of this gene's role in CO₂ response/hypersensitivity. In particular, dysfunction of the pH regulation mechanisms has been hypothesized to underlie respiratory dysregulation and the suffocation false alarm theory of PD (Maddock, 2001), meaning that individuals with a liability to PD could have genetic differences in genes such as *ASIC1* that affect pH regulation, in turn leading to basal differences in their respiratory functioning and/or anxiety levels. Our results could indicate a direct effect of *ASIC1* on respiration (rather than basal physiological anxiety levels), but rodent research implies that *ASIC1* does not directly affect ventilation (Ziemann et al., 2009), at least under resting conditions (Song et al., 2012). That we did not see a simultaneous effect on subjective anxiety levels could be due to overall low variance in

anxiety levels in our non-clinical sample, or due to the global SUDS measure's failure to capture specific relevant aspects of subjective anxiety.

Our results provided limited support for a role of any other candidate genes in the physiological or subjective anxiety response to CO₂. Our sample was relatively small; however, power analyses indicated that we had 63-72% power to detect genetic effects accounting for 5% of the variance in the latent intercept or slope for RR/SUDS trajectories (the *ASIC1* marker accounted for 7.1%) and over 80% power for the quadratic. Given the expectation of reduced phenotypic variability for well-defined endophenotypes assessed in a controlled environment, we can be more confident that our failure to detect significant effects was unlikely due to lack of power. Given the heterogeneity of, and genetic overlap between, anxiety disorders, the use of endophenotypes is a promising approach to address the current challenges in unraveling the molecular genetic basis of anxiety disorders.

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Figure 1. Mean respiratory rate (breaths per minute) across the carbon dioxide inhalation task for participants with the G/G, T/G, and T/T genotypes of the polymorphic marker rs1108923.

Table 1

Genetic markers included in the present study.

Gene	Marker/Haplotype	Alleles	MAF	Reason for inclusion
Cholecystokinin B Receptor (<i>CCKBR</i>)	rs906895	T < C	0.50	tagging SNPs in the gene; gene associated with PD (Kennedy et al., 1999); gene related to stress-induced fear memory and anxiety in mice (Chen et al., 2010); cholecystokinin agonist is a panicogen (Singh et al., 1991)
	rs2941025	A < G	0.29	
	rs1396860	C < T	0.20	
	rs2880898 ^a	G < A	0.18	
Solute Carrier Family 6, Member 4 (<i>SLC6A4</i>)	rs3813034	A < C	0.5	associated with PD (Gyawali et al., 2010) and fear extinction memory (Hartley et al., 2012)
	rs140701	T < C	0.49	associated with PD and PD/social anxiety disorder (Strug et al., 2010)
	rs6354	G < T	0.18	associated with anxious depression (Wray et al., 2009)
	rs2020936	G < A	0.21	associated with anxious depression (Wray et al., 2009)
	rs4251417-rs2020934	C-A	-	haplotype tags the short version of the serotonin transporter promoter (5HTTLPR; Wray et al., 2009), which is associated with fear acquisition (reviewed by Lonsdorf and Kalisch, 2011)
Glutamate Decarboxylase 1 (<i>GAD1</i>)	rs2241165-rs769407-rs3791851-rs3791850	A-C-G-C	-	haplotype associated with an anxiety-neuroticism factor in females (Hettema, An, et al., 2006); rs2241165 associated with PD in females (Weber et al., 2012)
Catechol-O-methyltransferase (<i>COMT</i>)	rs4680	A < G	0.44	associated with an anxiety-depression factor score in females (Hettema et al., 2008); increased risk for PD (Rothe et al., 2006); resistance to fear extinction (Lonsdorf et al., 2009)
	rs165599	G < A	0.43	associated with an anxiety-depression factor score in females (Hettema et al., 2008)
	rs4680-rs165599	G-A	-	haplotype strongly associated with an anxiety-depression factor score in females (Hettema et al., 2008)
Brain-Derived Neurotrophic Factor (<i>BDNF</i>)	rs6265	T < C	0.18	associated with anxious depression in men (Middledorp et al., 2010); neuroticism (Frustaci et al., 2008); fear extinction (Soliman et al., 2010)
Transmembrane Protein 132D (<i>TMEM132D</i>)	rs7309727	T < C	0.28	replicated association in a GWAS meta-analysis of PD (Erhardt et al., 2012)

Gene	Marker/Haplotype	Alleles	MAF	Reason for inclusion
	rs11060369	C < A	0.39	replicated association in a GWAS meta-analysis of PD (Erhardt et al., 2012)
Acid-Sensing (Proton-Gated) Ion Channel 1 (<i>ASIC1</i>)	rs1108923	T < G	0.11	gene associated with fear acquisition/conditioning in mice (Wemmie et al., 2004) and fear behavior in response to CO ₂ inhalation in mice (Ziemann et al., 2009)
	rs685012	C < T	0.31	
	rs10875995	C < T	0.25	
Acid-Sensing (Proton-Gated) Ion Channel 1 (<i>ASIC2</i>)	rs9915774 ^a	A < G	0.16	significant association in a GWAS of PD (Gegersen et al., 2012)
Corticotropin Releasing Hormone Receptor 1 (<i>CRHR1</i>)	rs878886	G < C	0.12	associated with PD (Keck et al., 2008); fear acquisition (Heitland et al., 2013)
Adenylate Cyclase Activating Polypeptide 1 (Pituitary) Receptor Type 1 (<i>ADCYAP1R1</i>)	rs2267735	G < C	0.47	associated with PTSD and impaired startle discrimination (Ressler et al., 2011) and dark-enhanced startle response (Jovanovic et al., 2013)
FK506 Binding Protein 5 (<i>FKBP5</i>)	rs1360780	T < C	0.30	related to attention bias towards threat (Fani et al., 2013) and threat-related amygdala reactivity (White et al., 2012)

MAF = minor allele frequency, PD = panic disorder, PTSD = post-traumatic stress disorder.

^aSNP did not pass quality control in our sample.

Table 2

Standardized results from linear regression analyses measuring the effect of candidate genetic markers/haplotypes on latent growth factors (intercept, slope, and quadratic) underlying trajectories of response to carbon dioxide inhalation, indicated by respiratory rate (left-hand column) and subjective anxiety (right-hand column).

Gene	Marker/Haplotype	Respiratory Rate						Subjective Units of Distress					
		Intercept			Slope			Intercept			Slope		
		β	<i>p</i>		β	<i>p</i>		β	<i>p</i>		β	<i>p</i>	
<i>CCKBR</i>	rs906895	0.03	0.755	0.07	0.348	0.01	0.480	-0.06	0.414	-0.14	0.078	0.00	0.598
	rs2941025	0.07	0.399	0.06	0.448	-0.02	0.084	0.07	0.354	0.12	0.133	0.00	0.958
	rs1396860	-0.02	0.776	-0.13	0.088	0.00	0.694	-0.02	0.759	0.02	0.763	0.00	0.620
<i>SLC6A4</i>	rs3813034	0.03	0.689	-0.05	0.533	-0.01	0.234	0.02	0.815	0.07	0.360	0.00	0.646
	rs140701	-0.07	0.417	-0.03	0.678	0.01	0.174	-0.03	0.709	-0.08	0.290	0.00	0.637
	rs6354	0.11	0.193	-0.14	0.047	-0.01	0.552	-0.07	0.362	0.05	0.545	0.00	0.801
<i>GAD1</i>	rs2020936	0.10	0.216	-0.14	0.055	-0.01	0.528	-0.06	0.442	0.06	0.477	0.00	0.690
	rs4251417-rs2020934	0.00	0.978	0.04	0.588	0.03	0.006	-0.01	0.908	-0.10	0.185	0.00	0.850
	rs2241165-rs769407-rs3791851-rs3791850	-0.09	0.277	-0.03	0.664	0.00	0.758	0.00	0.968	-0.07	0.382	0.00	0.740
<i>COMT</i>	rs4680	-0.09	0.306	-0.11	0.136	0.02	0.080	-0.02	0.740	-0.01	0.927	0.00	0.701
	rs165599	-0.06	0.487	0.05	0.536	-0.01	0.455	-0.05	0.368	0.01	0.901	-0.01	0.080
	rs4680-rs165599	0.18	0.032	0.09	0.253	-0.01	0.119	0.00	0.991	0.06	0.413	0.02	0.065
<i>BDNF</i>	rs6265	0.08	0.315	-0.10	0.160	0.00	0.647	0.02	0.782	0.03	0.732	0.01	0.451
	rs7309727	0.08	0.373	0.01	0.899	0.00	0.722	-0.10	0.216	-0.05	0.539	0.01	0.441
	rs11060369	0.03	0.738	-0.02	0.803	0.00	0.966	0.01	0.918	0.04	0.622	0.01	0.540
<i>AS1C1</i>	rs1108923	-0.27	0.001	-0.03	0.657	0.01	0.518	-0.06	0.463	-0.05	0.559	0.00	0.827
	rs685012	-0.09	0.286	0.01	0.852	0.00	0.968	0.05	0.547	0.01	0.859	-0.01	0.250
	rs10875995	-0.16	0.051	-0.04	0.583	0.00	0.923	-0.06	0.476	0.04	0.621	-0.01	0.258
<i>CRHR1</i>	rs878886	0.11	0.194	-0.13	0.067	-0.01	0.113	0.05	0.534	-0.10	0.211	-0.01	0.333
	rs2267735	0.06	0.474	-0.06	0.396	-0.01	0.374	0.11	0.143	0.12	0.128	0.00	0.788
	rs1360780	0.01	0.954	-0.02	0.786	-0.01	0.289	0.01	0.854	-0.17	0.025	0.01	0.096