

The Human Ortholog of Acid-Sensing Ion Channel Gene ASIC1a Is Associated With Panic Disorder and Amygdala Structure and Function

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Background: Individuals with panic disorder (PD) exhibit a hypersensitivity to inhaled carbon dioxide, possibly reflecting a lowered threshold for sensing signals of suffocation. Animal studies have shown that carbon dioxide-mediated fear behavior depends on chemosensing of acidosis in the amygdala via the acid-sensing ion channel ASIC1a. We examined whether the human ortholog of the ASIC1a gene, *ACCN2*, is associated with the presence of PD and with amygdala structure and function.

Methods: We conducted a case-control analysis ($n = 414$ PD cases and 846 healthy controls) of *ACCN2* single nucleotide polymorphisms and PD. We then tested whether variants showing significant association with PD are also associated with amygdala volume ($n = 1048$) or task-evoked reactivity to emotional stimuli ($n = 103$) in healthy individuals.

Results: Two single nucleotide polymorphisms at the *ACCN2* locus showed evidence of association with PD: rs685012 (odds ratio = 1.32, gene-wise corrected $p = .011$) and rs10875995 (odds ratio = 1.26, gene-wise corrected $p = .046$). The association appeared to be stronger when early-onset (age ≤ 20 years) PD cases and when PD cases with prominent respiratory symptoms were compared with controls. The PD risk allele at rs10875995 was associated with increased amygdala volume ($p = .035$) as well as task-evoked amygdala reactivity to fearful and angry faces ($p = .0048$).

Conclusions: Genetic variation at *ACCN2* appears to be associated with PD and with amygdala phenotypes that have been linked to proneness to anxiety. These results support the possibility that modulation of acid-sensing ion channels may have therapeutic potential for PD.

Key Words: *ACCN2*, amygdala, ASIC1a, association, genetic, panic disorder

Panic disorder (PD) is a common psychiatric illness with highly stereotyped symptoms including a sense of shortness of breath or feelings of suffocation. Heightened sensitivity to carbon dioxide (CO₂) is an established biological correlate of PD. Inhaled CO₂ triggers panic attacks in most individuals with PD but only a minority of unaffected controls (1).

The prevalence of panic attacks is elevated among patients with hypercapnia-associated respiratory diseases (2). A molecular basis for these phenomena has been proposed more recently. The amygdala, which is known to play a prominent role in fear circuitry, has been shown to be a chemosensor for the detection of hypercarbia, a function mediated by the acid sensing ion channel-1a subunit (ASIC1a) (3).

Although ASIC1a is expressed throughout the nervous system, particularly high levels are expressed in the amygdala (4,5). In rodents, CO₂ inhalation reduces amygdala pH, inducing acidosis and fear behaviors (3–5). Conversely, disrupting *asic1a* in mice

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decreases acidosis-induced fear behavior, which can be restored through transgenic expression of *ASIC1a* in the amygdala (3). However, CO₂ inhalation was found to induce panic attacks in three individuals with bilateral amygdala damage, suggesting that amygdala chemosensing is not required for the expression of CO₂-triggered panic (6).

Nevertheless, the amiloride-sensitive cation channel 2 gene (*ACCN2*, the human ortholog of *ASIC1a*) remains a compelling candidate for involvement in the etiology of PD. We reported modest evidence of linkage between PD and a region of chromosome 12q encompassing *ACCN2* in a large densely affected pedigree (7). The only published study examining association between *ACCN2* variants and anxiety disorders (8) found a nominally significant association in a discovery sample that failed to replicate in a second cohort. However, the cases for this study included anxiety spectrum disorders, only a subset of which had PD.

We report here, in the largest analysis to date of *ACCN2* variation and PD, an association between *ACCN2* polymorphisms and PD risk. Given prior evidence that the amygdala is the key site for the effect of *ASIC1a* on fear behavior and CO₂-induced anxiety (3) and that proneness to anxiety is associated with amygdala hypertrophy (9) and enhanced reactivity to emotional threat (10), we examined the relationship of *ACCN2* variants with neuroimaging measures of amygdala structure and function. We hypothesized that individuals carrying *ACCN2* risk alleles would exhibit increased susceptibility to threat-induced amygdala activation on the theory that amygdala reactivity to threatening stimuli may trigger fear circuitry in part through increased metabolic activity resulting in locally reduced pH and activation of *ASIC1a* (11).

Methods and Materials

Participants

PD Case-Control Analysis. To maximize power, we pooled samples from cohorts derived from genetic studies of anxiety, mood disorders, attention-deficit/hyperactivity disorder, treatment response, and healthy controls (Table 1). A description of each cohort is provided in Supplement 1. Inclusion and exclusion criteria for the genetic analyses are described subsequently and summarized in Figure 1.

Inclusion Criteria. The PD case-control analyses were restricted to individuals with self-reported European American (or in the case of the Brazilian sample, European-Brazilian) ancestry to minimize confounding by population stratification. For studies with family-based recruitment, genetically related individuals were excluded. All participants consented to participate in genetic studies of anxiety or a broader class of mental health conditions. The Partners Human Research Committee approved all aspects of the current study.

Cases met DSM-IV criteria for PD, with or without agoraphobia. Cases were recruited for studies of anxiety, with the exception of a subset of individuals recruited for the Massachusetts General Hospital Genetic Determinants of Behavioral Inhibition and Disinhibition Study, in which some parental probands were ascertained based on a history of PD, mood disorder, or attention-deficit/hyperactivity disorder. For families not ascertained based on PD in the parent-proband, we selected non-ascertained family members who met criteria for PD to minimize the risk of biased ascertainment.

Controls were drawn from individuals without a history of PD, including volunteers from a large-scale study of brain imaging

and genetics (the Brain Genomics Superstruct Project; see Supplement 1 for details). When ascertainment was not based on anxiety disorder diagnosis (e.g., the Massachusetts General Hospital genetic studies of attention-deficit/hyperactivity disorder), controls were restricted to family members without a history of PD to minimize the risk of biased ascertainment. Controls were required to be at least age 26 (to increase the likelihood that they had passed through the window of risk for PD onset) and did not meet full DSM-IV criteria for any anxiety disorder, depression, or dysthymia.

Individuals with PD whose age of onset was ≤ 20 years ($n = 179$) were compared with controls as a secondary analysis. We selected age 20 as the cut point based on prior genetic epidemiologic evidence and because this was the median value observed in our data. Specifically, a prior large-interview family study documented a 17-fold familial risk of panic disorder using this cut point (12). The early-onset cases had a mean age of onset of 14.6 years (± 4.3 years) and a mean age at assessment of 30.9 years (± 11.5 years).

Given prior preclinical evidence associating *ASIC1a* with CO₂-induced fear behavior and the hypothesis that CO₂ hypersensitivity in PD may reflect an altered suffocation alarm system, we examined associations between *ACCN2* variants and a putative respiratory subtype of PD. Although definitions of the respiratory subtype vary (13), most require a predominance of the following symptoms during panic attacks: 1) chest pain or discomfort, 2) shortness of breath, 3) feelings of choking, 4) paresthesias, and 5) fear of dying. We defined respiratory subtype cases as individuals identifying four or more of these panic symptoms on diagnostic interview. These data were available for only a subset of PD cases. Nonrespiratory subtype panic was defined as three or more of these symptoms and no endorsement of shortness of breath or choking feelings.

Exclusion Criteria. Individuals with a diagnosis of schizophrenia, psychosis, or bipolar disorder were excluded.

Genetic Methods

DNA from blood or saliva samples was extracted at the Massachusetts General Hospital Center for Human Genetic Research. Single nucleotide polymorphisms (SNPs) were selected to pairwise tag all common SNPs in the *ACCN2* gene ± 10 kb flanking regions using the Tagger module in Haploview (14) and based on the linkage disequilibrium (LD) structure of the HapMap Centre d'Etude du Polymorphisme Humain sample. An r^2 threshold of .8 was used for tagging. Six SNPs captured 100% of the common variation ($\geq 5\%$ minor allele frequency) in the HapMap Centre d'Etude du Polymorphisme Humain sample. Two tag SNPs could not be designed into the primer group and were replaced with proxy SNPs ($r^2 \geq .9$).

We used iPLEX Gold chemistry and the MassARRAY System (Sequenom Bioscience, San Diego, California) to perform SNP genotyping. Genotyping of the six *ACCN2* SNPs was conducted as part of a larger study of multiple anxiety disorders and related phenotypes that comprised 139 SNPs (including 40 ancestry informative markers that differentiate individuals into continental populations) in 2976 samples (including 38 duplicate sample pairs). We made use of this larger dataset to examine genotyping performance and remove poor-quality SNPs and samples. Samples were excluded based on missingness $>.1$. SNPs were excluded based on at least one of the following: missingness $>.1$, minor allele frequency $<.01$, or Hardy Weinberg p value $< 10^{-4}$ as calculated in PLINK (15). Using these thresholds, 66 samples and 12 markers were excluded. The concordance rate for

Table 1. Case-Control Samples Included in the Panic Disorder Analysis: Participant Characteristics

Samples	Cases (n)	Controls (n)	Age (Mean, SD)	Sex (% Female)	Diagnostic Instrument	Ascertainment	Recruitment Sites
McLean Hospital PD Study	130	0	32.7, 11.9	72	SCID or MINI	Anxiety disorders (cases only)	Belmont, MA
MGH Genetic Determinants of Behavioral Inhibition and Disinhibition	50	107	40.0, 9.6	57	SCID or K-SADS	Parental PD, MDD, ADHD or none (cases and controls)	Boston, MA
HCPA Genetic Association Study of PD	76	91	39.0, 9.6	72	MINI	PD (cases and controls)	Porto Alegre, Brazil
Randomized Trial of D-Cycloserine Augmentation of CBT for PD	107	0	36.5, 12.5	56	SCID	PD treatment study (cases only)	Boston, MA; Hartford, CT; Chicago, IL
MGH Predictors of Response and/or Relapse in Anxiety Disorders	13	7	39.7, 10.2	40	SCID or MINI	Anxiety disorders (cases and controls)	Boston, MA
Improving Outcomes in Pharmacotherapy of Social Phobia	38	0	37.7, 14.5	37	MINI	Social phobia (cases)	Boston, MA; San Diego, CA; Hamilton, Ontario
MGH Genetic Studies of ADHD	0	315	43.6, 6.8	49	SCID or K-SADS	Family studies of ADHD (cases and controls)	Cambridge, MA
MGH Brain Genomic Superstruct Project	0	326	50.4, 21.6	52	Self-Report	Healthy controls	Cambridge, MA
Total	414	846	42.4, 14.8	56			

ADHD, attention-deficit/hyperactivity disorder; CBT, cognitive-behavioral therapy; HCPA, Hospital de Clínicas de Porto Alegre; K-SADS, Schedule for Affective Disorders and Schizophrenia for School-Age Children; MDD, major depressive disorder; MGH, Massachusetts General Hospital; MINI, Mini International Neuropsychiatric Interview; PD, panic disorder; SCID, Structured Clinical Interview for DSM-IV.

the 37 duplicate pairs was .99. After quality control, 2873 individuals and 127 markers remained, including five of the six ACCN2 SNPs (rs706793 failed because of missingness and deviations from Hardy Weinberg equilibrium) (Figure 2).

To control for ancestry, we restricted analyses to European American non-Hispanic individuals. To define this subset of our sample, we used multidimensional scaling analysis of data from the 36 ancestry informative markers (AIMs) SNPs that passed

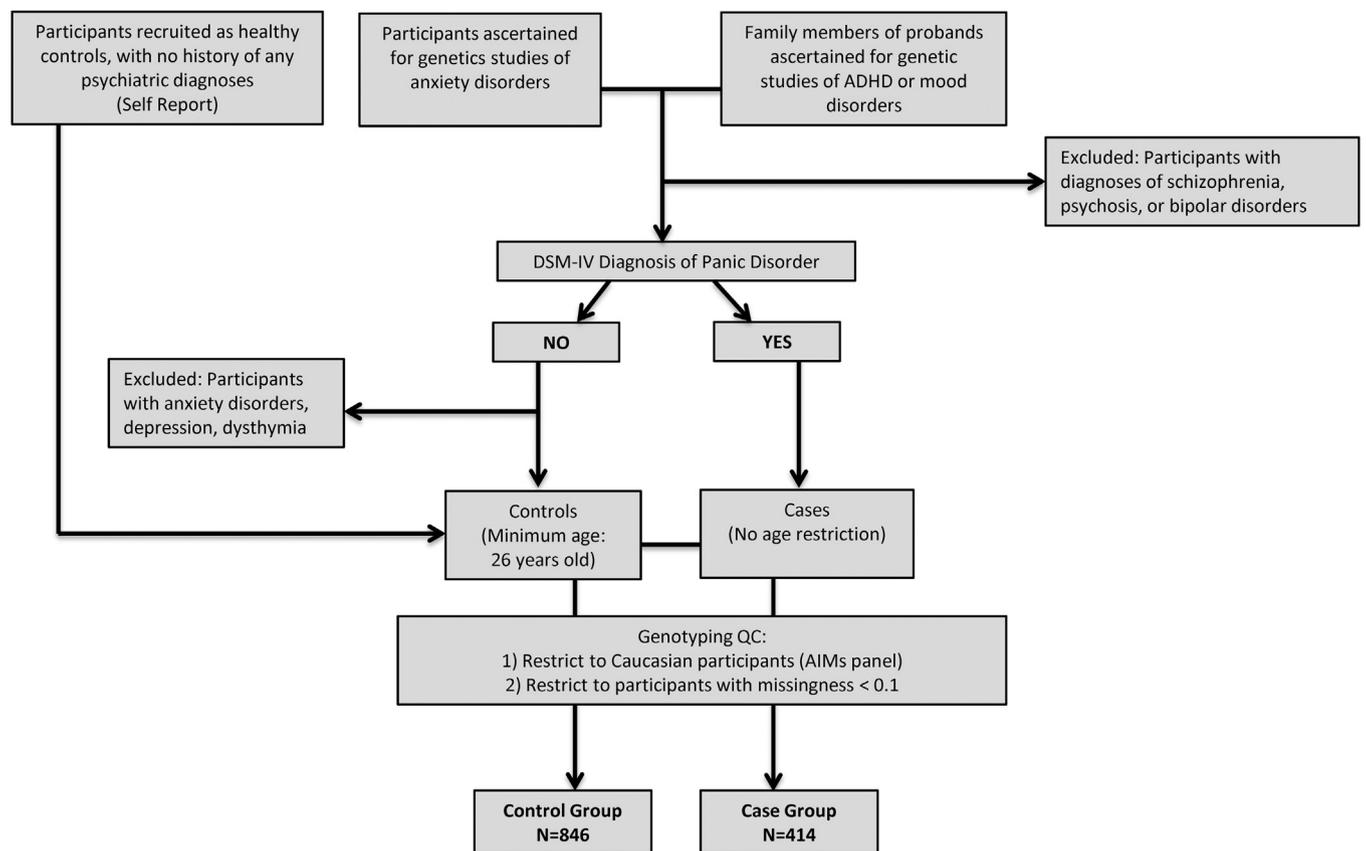


Figure 1. Case-control Consolidated Standards of Reporting Trials diagram. Ascertainment of cases and controls for the association analysis of ACCN2 variants and panic disorder. ADHD, attention-deficit/hyperactivity disorder; QC, quality control.

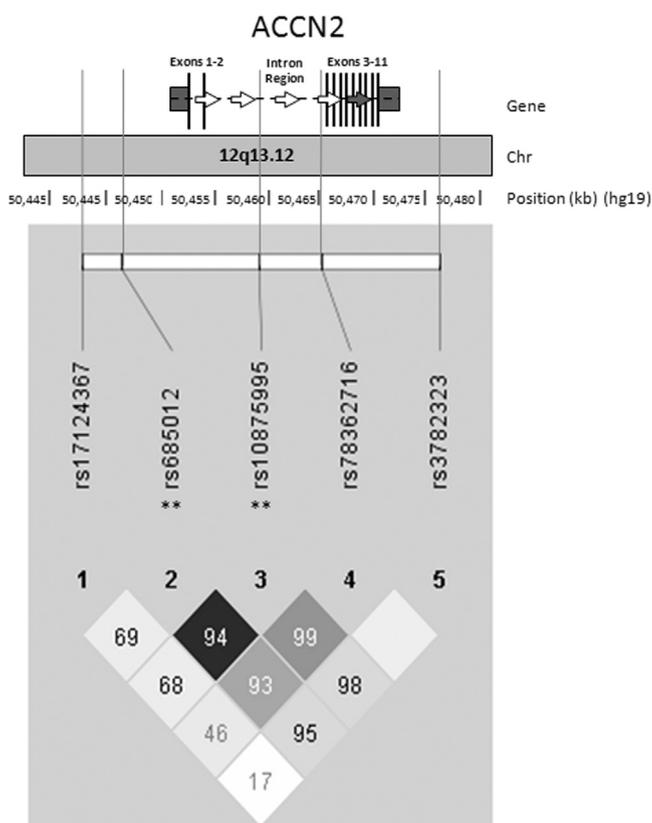


Figure 2. Genomic position and linkage disequilibrium (LD) relationships among *ACCN2* single nucleotide polymorphism positions relative to *ACCN2* gene with LD plot and significance denoted. *** $p_{\text{corrected}} < .05$. Numerical values within the blocks represent D' . The color scale within the blocks represent r^2 values: white corresponds to $r^2 = 0$, shades of gray correspond to $0 < r^2 < 1$, and black corresponds to $r^2 = 1$. The r^2 between rs685012 and rs10875995 is .828. Chr, chromosome.

quality control, along with self-reported race/ethnicity. Using the first two dimensions of variation and self-reported race/ethnicity for all 2873 individuals, we created a scatterplot that allowed us to visualize the population structure of our sample. A clear clustering of European American and non-Hispanic individuals was revealed (Figure 3). We defined the European American non-Hispanic sample set as individuals who 1) fell within or near the main European American non-Hispanic cluster and 2) self-reported race/ethnicity as European American (with ethnicity unreported), European American non-Hispanic, missing, unknown, or non-Hispanic. Of 2693 individuals who met these criteria, a subset of 1260 met the panic case or control criteria as outlined previously.

PD Case-Control Genetic Analysis

We tested an additive genetic model on case or control status using the logistic regression function in PLINK, adjusting for sex. Correction for multiple testing was performed using the PLINK max(T) procedure with 10,000 permutations. This procedure reassigns case or control labels for subjects, maintaining patterns of LD between SNPs. Each observed SNP test statistic is compared with the maximum of all permuted statistics over all SNPs to calculate the corrected p value.

Neuroimaging Methods

Participants. Native English-speaking young adults from the Brain Genomics Superstruct Project (18–30 years old) with normal

or corrected-to-normal vision were included for structural magnetic resonance imaging (MRI) analyses ($n = 1048$; age, 21.09 ± 2.59 years old; female, 54.80%; right-handed, 91.70%; years of education, 14.59 ± 1.90). A subset of participants ($n = 103$) performed task-based functional MRI allowing examination of the relationship between *ACCN2* genotype and task-evoked amygdala reactivity (age, 20.37 ± 2.45 years old; female, 52.40%; right-handed, 100%; years of education, 14.07 ± 1.75). Participants were genotyped concurrently with the case-control sample and underwent identical quality control procedures. Estimated slice-based signal-to-noise ratio and maximum absolute motion did not differ by *ACCN2* genotype ($t_s < .84$; $p_s > .41$).

MRI Data Acquisition. Imaging data were acquired on matched 3-tesla Tim Trio scanners (Siemens Medical Solutions, Erlangen, Germany) at Harvard University and Massachusetts General Hospital using the vendor-supplied 12-channel phased-array head coil. Structural data included a high-resolution multi-echo T1-weighted magnetization-prepared gradient echo image using the following parameters: repetition time = 2200 msec, inversion time = 1100 msec, echo time = 1.54 msec for image 1–7.01 msec for image 4, flip angle = 7° , $1.2 \times 1.2 \times 1.2$ mm, and field of view = 230. Task-evoked functional MRI data using a gradient echo sequence were collected using the following parameters: time points = 68, repetition time = 3000 msec, echo time = 30 msec, flip angle = 85° , $3 \times 3 \times 3$ mm voxels, and field of view = 216. There were 47 anterior commissure–posterior commissure aligned slices that employed interleaved acquisition and no gap between slices. Variance associated with site and software upgrades that occurred during data acquisition was partialled out of analyses.

Functional Task. A matching task previously shown robustly to activate the amygdala was used (16). Four blocks of emotional face matching were interleaved with five sensorimotor control blocks. During emotion blocks, participants were presented with trios of faces (angry, afraid) derived from a standard set of pictures of facial affect (17) and were instructed to select one of two faces (bottom) that matched the target face (top). During sensorimotor control blocks, participants viewed trios of geometric shapes (circles, ellipses) and were instructed to select one of two shapes (bottom) identical to the target shape (top). Each block consisted of six images, presented sequentially for 3000 msec.

MRI Data Analyses. Structural data were analyzed using FreeSurfer version 4.5.0 software (<http://surfer.nmr.mgh.harvard.edu>). FreeSurfer provides automated algorithms for the volumetric segmentation of subcortical structures and estimation of cortical thickness (18,19). Cortical thickness was calculated as the closest distance from the gray matter/white matter boundary to the gray matter/cerebrospinal fluid boundary at each vertex on the tessellated surface (19). After surface based registration, but before the analysis of cortical thickness, a 22-mm full width at half maximum smoothing kernel was applied to each participant's data. Follow-up surface-based analyses assessed the specificity of the observed effects. Surface effects were thresholded at $p < .001$ uncorrected for multiple comparisons. Estimated intracranial volume was calculated using the approach of Buckner *et al.* (20) as implemented in FreeSurfer.

Task data preprocessing included 1) removing the first four volumes to allow for T1 equilibration; 2) compensation of systematic, slice-dependent time shifts; and 3) motion correction. Data were spatially normalized to the Montreal Neurological Institute standard anatomic atlas space (2-mm isotropic voxels) using a T2-weighted echo planar image blood oxygen level–

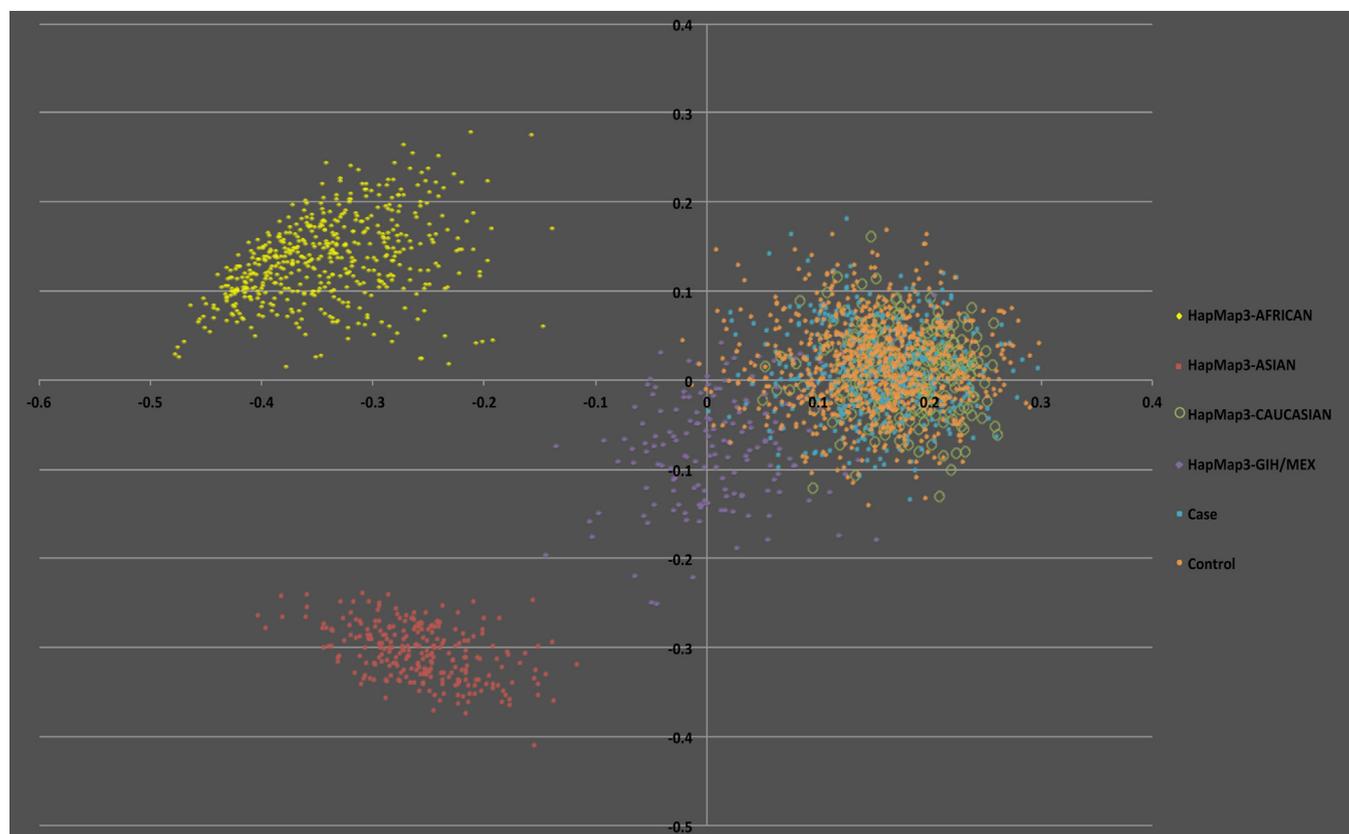


Figure 3. Multidimensional scaling (MDS) plot of ancestry informative markers for cases and controls. The first two dimensions of variation generated by the MDS analysis of the ancestry informative markers single nucleotide polymorphisms (for each individual) are plotted on the x and y coordinates of the figure. The cases (blue dots) and controls (orange dots) included in the primary case and control analyses for this project are shown in comparison with the HapMap phase 3 reference populations. The panic case and control clusters overlap with one another and the HapMap Caucasian reference sample (green circles). MEX, Mexican ancestry in Los Angeles, California.

dependent (BOLD) contrast atlas in SPM2 (21). A 6-mm full width at half maximum smoothing kernel was applied to each participant's data. After preprocessing, a general linear model was conducted in SPM8, modeling each condition as a boxcar regressor convolved with a hemodynamic response function and a high-pass (1/128 Hz) filter for linear drift. Group-level analyses were conducted, selecting contrasts of interest for each participant (faces > shapes) and entering them in second-level random-effects *t* tests.

Analysis of the task-evoked data revealed significant bilateral amygdala response during the emotion task ($p < .0001$ family-wise error-corrected) (Figure 4B). Hypothesis-driven region of interest analyses were performed by defining a 6-mm-radius region of interest centered on the maximally increased BOLD response in the left and right amygdalae ($x = -22$, $y = -4$, $z = -22$ and $x = 22$, $y = -4$, $z = -22$). Average response was extracted from each region of interest, and follow-up analysis of variance (ANOVA) was conducted in SPSS (IBM Corp., Armonk, New York).

Imaging Genetic Analysis. Analyses of subcortical anatomic variability and task-evoked reactivity targeted the amygdala. We examined associations between these anatomic and functional phenotypes and the two SNPs associated with PD from the case-control analysis (rs10875995 and rs685012). For morphometric analyses, a repeated-measures ANOVA was conducted partialing out variance associated with site, console software version, age, sex, handedness, and intracranial volume and examining relations

between amygdala volumes and *ACCN2* genotype. To examine specificity of the observed effects, follow-up analyses were conducted on the remaining subcortical structures and the surface-based estimates of cortical thickness. For analyses of task-evoked reactivity, repeated-measures ANOVA was conducted examining relations between genotype and amygdala reactivity, partialing out variance associated with console software version, handedness, age, and sex. For the sake of brevity, only effects involving genotype are presented (other findings are available on request).

Results

Association with PD

Three of the five SNPs tested demonstrated nominally significant association with PD. Two survived gene-wise correction for multiple testing: rs685012 (odds ratio [OR] for C allele = 1.32, 95% confidence interval [CI] = 1.1–1.57, $p_{\text{corrected}} = .011$) and rs10875995 (OR for C allele = 1.26, 95% CI = 1.06–1.51, $p_{\text{corrected}} = .046$). These models were adjusted for sex, but results were virtually unchanged when models were not adjusted for sex (Table 2).

Effect sizes and statistical significance increased in the secondary analysis of early age-of-onset cases versus controls. Three SNPs were significant in sex-adjusted models after correcting for the number of SNPs tested: rs10875995 (OR for C allele = 1.58, 95% CI = 1.24–2.00, $p_{\text{uncorrected}} = .0002$, $p_{\text{corrected}} = .0006$),

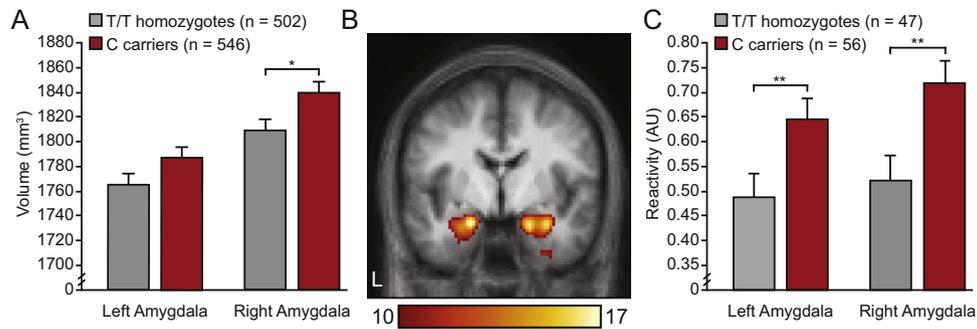


Figure 4. Increased amygdala volume and heightened reactivity in rs10875995 C allele carriers relative to T/T homozygotes. **(A)** Mean (and SE) amygdala volumes after partialing out variance associated with site, console software version, age, sex, handedness, and intracranial volume. **(B)** Coronal slice ($y = -4$) showing task-evoked amygdala reactivity (faces > shapes). Color bar reflects T scores ($p < .0001$ family-wise error-corrected). **(C)** Mean (and SE) amygdala reactivity after partialing out variance associated with site, console software version, age, and sex. * $p < .05$, ** $p < .01$. AU, arbitrary units.

rs685012 (OR for C allele = 1.49, 95% CI = 1.17–1.90, $p_{\text{uncorrected}} = .0009$, $p_{\text{corrected}} = .0042$), and rs17124367 (OR for T allele = 1.80, 95% CI = 1.22–2.66, $p_{\text{uncorrected}} = .0034$, $p_{\text{corrected}} = .013$) (Table S1 in Supplement 1 for genotype frequencies).

In a second exploratory analysis, we identified 43 respiratory-subtype PD cases and compared them with controls. The SNPs most strongly associated with PD overall were also associated with respiratory-subtype PD: rs685012 (OR for C allele = 1.84, 95% CI = 1.17–2.89, $p_{\text{uncorrected}} = .008$, $p_{\text{corrected}} = .027$) and rs10875995 (OR for C allele = 1.90, 95% CI = 1.22–2.96, $p_{\text{uncorrected}} = .0046$, $p_{\text{corrected}} = .017$). Neither rs685012 nor rs10875995 was associated with PD when non-respiratory-subtype cases ($n = 87$) were compared with controls (both $p > .70$).

Association of *ACCN2* Variants with Brain Structure and Function

The ANOVA examining the relation between rs10875995 genotype and amygdala volume revealed a main effect of genotype [$F_{1,1040} = 4.46$, $p = .04$, $\eta_p^2 = .01$], driven by bilaterally greater amygdala volumes in the C allele (PD associated) carriers ($1808.81 \pm 7.11 \text{ mm}^3$) relative to T/T homozygotes ($1787.10 \pm 7.41 \text{ mm}^3$) (Figure 4A). As suggested by the absence of an rs10875995 genotype-by-hemisphere interaction [$F_{1,1040} = .81$, $p = .37$, $\eta_p^2 < .01$], this effect was present when separately considering the right amygdala [$F_{1,1040} = 5.12$, $p = .02$, $\eta_p^2 = .01$] and in the same direction, although not significant, when considering the left amygdala [$F_{1,1040} = 2.56$, $p = .11$, $\eta_p^2 < .01$]. Follow-up tests examined the specificity of the relations between rs10875995 and amygdala volume across each subcortical structure. The volumes of the remaining subcortical structures (caudate, globus pallidus, nucleus accumbens, putamen, and thalamus) did not display relations with genotype [all $F < 3.39$, all $p > .07$]. No significant relations emerged between rs685012 genotype and amygdala volume [all $F < 1.04$, all $p > .30$]. Cortical thickness estimates did not significantly differ by *ACCN2* genotype.

The ANOVA examining the relation between rs10875995 genotype and amygdala reactivity revealed a main effect of genotype [$F_{1,98} = 8.31$, $p = .0048$, $\eta_p^2 = .08$], driven by heightened bilateral amygdala response in C allele carriers ($.68 \pm .04$) relative to T/T homozygotes ($.51 \pm .05$) (Figure 4C). As suggested by the absence of a genotype by hemisphere interaction [$F_{1,98} = .61$, $p = .44$, $\eta_p^2 = .006$], this effect was present when considering the right amygdala [$F_{1,98} = 7.91$, $p = .006$, $\eta_p^2 = .08$] and left amygdala [$F_{1,98} = 6.31$, $p = .01$, $\eta_p^2 = .06$]. After partialing out left and right amygdala volume (corrected for intracranial volume), the main effect of rs10875995

genotype on amygdala reactivity remained [$F_{1,95} = 6.93$, $p = .01$, $\eta_p^2 = .07$]. Follow-up analyses restricted to the voxels displaying maximally increased BOLD response confirmed the observed bilateral increase in the amygdala response of C allele carriers, relative to T/T homozygotes ($x = -22$, $y = -4$, $z = -22$ and $x = 22$, $y = -4$, $z = -22$) [$F_{1,98} = 7.96$, $p = .0058$, $\eta_p^2 = .08$].

The ANOVA examining the relation between rs685012 genotype and amygdala reactivity revealed a trend toward a main effect of genotype [$F_{1,97} = 2.90$, $p = .09$, $\eta_p^2 = .03$], driven by heightened bilateral amygdala response in C allele carriers ($.65 \pm .04$) relative to T/T homozygotes ($.54 \pm .05$). After partialing out left and right amygdala volume (corrected for intracranial volume), there was no significant association between rs685012 genotype and amygdala reactivity [$F_{1,94} = 2.13$, $p = .14$, $\eta_p^2 = .02$].

Discussion

Although PD is heritable, success in identifying susceptibility genes has been limited relative to progress in other domains of psychiatric genetics (22). Nevertheless, genetic studies of anxiety disorder phenotypes have the advantage that animal models and neuroimaging research have established molecular and neuro-anatomic underpinnings of panic and related anxiety phenotypes. Among psychiatric disorders, PD is one of the few disorders in which symptoms can be reliably provoked by a biological challenge (CO_2 inhalation) (23). In the present study, we capitalize on this evidence, focusing on variation in *ACCN2*, a gene previously associated with amygdala-mediated anxiety and implicated in anxiety-related hypersensitivity to CO_2 (3). Our results suggest that variants in *ACCN2* are associated both with PD and with alterations in amygdala structure and function.

In case-control analyses, the minor alleles of 2 SNPs—rs685012, located in the 5' putative promoter region of *ACCN2*, and rs10875995, located in intron 3—were associated with PD after correcting for the number of markers tested. We are unable to determine whether either of these association signals directly reflects a causal variant or LD with such a variant. We observed a stronger effect of *ACCN2* alleles on early-onset PD, a subtype that has been shown to be associated with increased familial risk of PD (12), and among cases of PD exhibiting a respiratory subtype, which has been associated with increased sensitivity to inhaled CO_2 (13).

Wemmie *et al.* (4,5) demonstrated that ASIC1a is required for normal conditioned and unconditioned fear behavior and that

Table 2. Association Results for the Panic Disorder Case-Control Analysis: Genotype Data and Association Statistics

AS1C1a SNPs	Alleles: Minor/ Major	Group	Genotypes ^a			Case-Control Analysis			Sex-Adjusted Case-Control Analysis		
			A1/ A1	A1/ A2	A2/ A2	OR (CI)	<i>p</i> Value	<i>p</i> Value (Corrected)	OR (CI)	<i>p</i> Value	<i>p</i> Value (Corrected)
rs17124367	T/A	Cases	5	59	350	1.35 (.98–1.85)	.064	.219	1.36 (.99–1.87)	.060	.21
		Controls	1	105	740						
rs685012	C/T	Cases	60	192	162	1.31 (1.10–1.57)	.0024	.0093	1.32 (1.10–1.57)	.0026	.011
		Controls	78	378	388						
rs10875995	C/T	Cases	51	192	171	1.25 (1.05–1.50)	.012	.047	1.26 (1.06–1.51)	.011	.046
		Controls	78	361	407						
rs78362716	G/A	Cases	8	131	275	1.13 (.91–1.41)	.28	.707	1.15 (.92–1.44)	.23	.62
		Controls	26	219	600						
rs3782323	A/G	Cases	23	152	239	.97 (.80–1.17)	.75	1.0	.97 (.80–1.18)	.75	1.0
		Controls	59	296	490						

CI, confidence interval; OR, odds ratio; SNPs, single nucleotide polymorphisms.

^aA1/A1 = homozygote minor allele; A1/A2 = heterozygote; A2/A2 = homozygote major allele.

this effect is mediated by ASIC1a sensing of reduced pH (3). Mirroring human studies of PD, inhalation of CO₂ evokes fear behavior in mice, an effect that is reduced by deletion or blockade of ASIC1a within the amygdala (3). Overexpression of ASIC1a in the amygdala was sufficient to trigger CO₂-induced fear behavior. In light of this evidence and several prior studies showing that increased proneness to anxiety is associated with increased amygdala volume in healthy volunteers (9,24,25), we examined the association between amygdala volume and variation in *ACCN2*. Consistent with our observation that the C allele of rs10875995 SNP contributes to PD risk, we found evidence of association between this variant and both amygdala volume and reactivity to emotional faces. The association with brain structure appeared to be specific to amygdala volume in that no significant association was seen with other subcortical structures or with cortical thickness. We reported more recently an association between increased amygdala volume and measures of trait anxiety in the same sample used in the present report (*N* = 1050) (9). The present results indicate that the increased amygdala volume phenotype is related in part to *ACCN2* alleles that are also associated with risk for PD. Although MRI studies of PD have more often found reduced amygdala volume (26), our results suggest that *ACCN2* may contribute to the relative enlargement of the amygdala. Although this finding might appear to be discrepant with prior MRI studies that find reduced amygdala volume among individuals with PD, our findings do not imply that risk allele carriers with PD should have large amygdalae. In addition, it is possible that the reduction in amygdala volume previously reported with PD itself is a consequence of the illness—we would not have seen this because our imaging sample was free of Axis I pathology. In healthy volunteers, subsyndromal levels of anxiety may be associated with increased amygdala volume, as we found in our prior work (9). The mechanism by which *ACCN2* alleles could increase amygdala volume is unclear and warrants further study. However, disruption of ASIC1a attenuates hippocampal brain-derived neurotrophic factor (BDNF) suppression in response to stress (27). Stress has been shown to increase BDNF in the amygdala (28), an effect opposite to that seen in the hippocampus (29), and BDNF increases amygdala dendritic and axonal growth (30). One hypothesis that warrants further investigation is that enhanced ASIC1a activity increases amygdala volume by promoting stress-induced amygdala BDNF secretion.

We also found that the same *ACCN2* allele associated with both PD and amygdala volume is associated with increased amygdala reactivity to emotional faces, a phenotype linked to PD in previous studies (26). Wemmie *et al.* (31) suggested that amygdala activation by fear-inducing stimuli may trigger fear responses by lowering local pH (e.g., by synaptic release of H⁺ and metabolic effects of neural activity). If this is so, the observed association between allelic variants and amygdala reactivity may reflect enhanced sensitivity to reduced pH secondary to neuronal activity that mediates the processing of emotional stimuli. Such activation would clearly be less direct (and less intense) than that induced by direct CO₂ inhalation.

We previously reported modest evidence of linkage (NPL = 4.96) between the region on chromosome 12q encompassing the *ACCN2* locus and PD in a large pedigree densely affected with the disorder (7). In a prior association study between *ACCN2* SNPs and anxiety disorders, Hettema *et al.* (8) reported a two-stage analysis comprising twin pairs. They used structural equation modeling to identify a latent genetic factor underlying susceptibility to neuroticism, major depression, generalized anxiety disorder, PD, agoraphobia, and social phobia. Twin pairs scoring at the extremes on this factor were selected for case-control analyses. In the first stage (*n* = 188 cases, 188 controls), the investigators found modest evidence of association for the C allele of rs685012 (*p* = .077) and the T allele of rs1108923 (*p* = .023) but no association in a second-stage sample (*n* = 401 cases, 351 controls) or in the subset of cases with PD (*n* = 122). The relatively small sample size limits the interpretability of this null finding with respect to PD. Additional evidence for a role of acid-sensing ion channels in PD comes from a study in the Faroe Islands population isolate that observed nominal association with variants in *ACCN1* (the gene encoding ASIC2a) (32). Prior research suggests that ASIC2a may interact with ASIC1a and may facilitate the synaptic function of ASIC1a (31).

In conjunction with preclinical studies of ASIC1a, our results support the possibility that variants in *ACCN2* contribute to PD risk by lowering the threshold for amygdala sensing of acidosis. The association between *ACCN2* and PD would be consistent with a leading biological model of PD pathogenesis. Klein (33) and others proposed that panic attacks and the heightened sensitivity to CO₂ seen in PD represent the abnormal triggering of an evolved suffocation alarm system. Alternatively, CO₂ hypersensitivity might trigger panic attacks by inducing somatic symptoms that individuals with PD are more likely to fear. Individuals with

heightened amygdala acid sensing might be more prone to experience interoceptive cues in response to internal (e.g., metabolic) or external (e.g., CO₂) stimuli, which might further trigger panic responses among individuals with increased anxiety sensitivity. This mechanism provides a potential link between our findings and cognitive-behavioral models of the pathogenesis of panic. The more recent observation of rare individuals with amygdala damage who nevertheless experienced panic in response to CO₂ inhalation suggests that amygdala ASIC1a chemosensors are not required for panic (6).

Our results should be interpreted in light of the limitations of this study. First, although our sample is large relative to most prior genetic studies of PD, we had >80% power to detect moderate allelic effects (e.g., genotypic risk ratio >1.32 for allele frequency $\geq .20$ at $\alpha < .01$) but not smaller effects. Second, to obtain adequate sample sizes, the case-control samples were drawn from multiple studies that had varying ascertainment procedures. However, phenotypic heterogeneity would be expected to obscure true effects rather than increase the risk of spurious association. Third, the markers we tested are not known to have functional effects, and the association signals might reflect LD with the causal variation. Replication of our findings is needed to establish an association between ACCN2 and PD.

Taken together, our results suggest that variants of ACCN2 contribute to PD risk as well as influence amygdala structure and function. These findings build on prior preclinical research that has strongly implicated this gene in amygdala-mediated fear behavior. They also support the possibility that inhibitors of ASIC1a might have therapeutic potential in the treatment of PD and related disorders involving dysregulation of amygdala-mediated fear responses. Preclinical studies suggest that acid-sensing ion channel inhibitors produce anxiolytic effects comparable to a benzodiazepine (34). Given this, proof-of-concept studies of ASIC1a inhibitors may be warranted.

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1. Battaglia M, Ogliaeri A (2005): Anxiety and panic: From human studies to animal research and back. *Neurosci Biobehav Rev* 29:169–179.
2. Smoller JW, Pollack MH, Otto MW, Rosenbaum JF, Kradin RL (1996): Panic anxiety, dyspnea, and respiratory disease. Theoretical and clinical considerations. *Am J Respir Crit Care Med* 154:6–17.
3. Ziemann AE, Allen JE, Dahdaleh NS, Drebot II, Coryell MW, Wunsch AM, *et al.* (2009): The amygdala is a chemosensor that detects carbon dioxide and acidosis to elicit fear behavior. *Cell* 139:1012–1021.
4. Coryell MW, Ziemann AE, Westmoreland PJ, Haenfler JM, Kurjakovic Z, Zha XM, *et al.* (2007): Targeting ASIC1a reduces innate fear and alters neuronal activity in the fear circuit. *Biol Psychiatry* 62:1140–1148.
5. Wemmie JA, Askwith CC, Lamani E, Cassell MD, Freeman JH Jr, Welsh MJ (2003): Acid-sensing ion channel 1 is localized in brain regions with high synaptic density and contributes to fear conditioning. *J Neurosci* 23:5496–5502.
6. Feinstein JS, Buzza C, Hurlmann R, Follmer RL, Dahdaleh NS, Coryell WH, *et al.* (2013): Fear and panic in humans with bilateral amygdala damage. *Nat Neurosci* 16:270–272.
7. Smoller JW, Acierno JS Jr, Rosenbaum JF, Biederman J, Pollack MH, Meminger S, *et al.* (2001): Targeted genome screen of panic disorder and anxiety disorder proneness using homology to murine QTL regions. *Am J Med Genet* 105:195–206.
8. Hettema JM, An SS, Neale MC, van den Oord EJ, Kendler KS, Chen X (2008): Lack of association between the amiloride-sensitive cation

- channel 2 (ACCN2) gene and anxiety spectrum disorders. *Psychiatr Genet* 18:73–79.
9. Holmes AJ, Lee PH, Hollinshead MO, Bakst L, Roffman JL, Smoller JW, *et al.* (2012): Individual differences in amygdala-medial prefrontal anatomy link negative affect, impaired social functioning, and polygenic depression risk. *J Neurosci* 32:18087–18100.
 10. Shin LM, Liberzon I (2010): The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology* 35:169–191.
 11. Wemmie JA (2011): Neurobiology of panic and pH chemosensation in the brain. *Dialogues Clin Neurosci* 13:475–483.
 12. Goldstein R, Wickramaratne P, Horwath E, Weissman M (1997): Familial aggregation and phenomenology of “early”-onset (at or before age 20 years) panic disorder. *Arch Gen Psychiatry* 54:271–278.
 13. Freire RC, Perna G, Nardi AE (2010): Panic disorder respiratory subtype: psychopathology, laboratory challenge tests, and response to treatment. *Harv Rev Psychiatry* 18:220–229.
 14. Barrett JC, Fry B, Maller J, Daly MJ (2005): Haploview: Analysis and visualization of LD and haplotype maps. *Bioinformatics* 21:263–265.
 15. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, *et al.* (2007): PLINK: A tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81:559–575.
 16. Hariri AR, Mattay V, Tessitore A, Kolachana B, Fera F, Goldman D, *et al.* (2002): Serotonin transporter genetic variation and the response of the human amygdala. *Science* 297:400.
 17. Ekman P, Friesen W (1976): *Pictures of Facial Affect*. Palo Alto, CA: Consulting Psychologists Press.
 18. Fischl B, Dale AM (2000): Measuring the thickness of the human cerebral cortex from magnetic resonance images. *Proc Natl Acad Sci U S A* 97:11050–11055.
 19. Fischl B, Salat DH, Busa E, Albert M, Dieterich M, Haselgrove C, *et al.* (2002): Whole brain segmentation: Automated labeling of neuroanatomical structures in the human brain. *Neuron* 33:341–355.
 20. Buckner RL, Head D, Parker J, Fotenos AF, Marcus D, Morris JC, *et al.* (2004): A unified approach for morphometric and functional data analysis in young, old, and demented adults using automated atlas-based head size normalization: Reliability and validation against manual measurement of total intracranial volume. *Neuroimage* 23:724–738.
 21. Friston KJ, Ashburner J, Frith CD, Poline JB, Heather JD, Frackowiak RSJ (1995): Spatial registration and normalization of images. *Hum Brain Mapp* 2:165–189.
 22. Smoller JW (2011): Who’s afraid of anxiety genetics? *Biol Psychiatry* 69: 506–507.
 23. Perna G, Gabriele A, Caldirola D, Bellodi L (1995): Hypersensitivity to inhalation of carbon dioxide and panic attacks. *Psychiatry Res* 57:267–273.
 24. Baur V, Hanggi J, Jancke L (2012): Volumetric associations between uncinate fasciculus, amygdala, and trait anxiety. *BMC Neurosci* 13:4.
 25. van der Plas EA, Boes AD, Wemmie JA, Tranel D, Nopoulos P (2010): Amygdala volume correlates positively with fearfulness in normal healthy girls. *Soc Cogn Affect Neurosci* 5:424–431.
 26. Kim JE, Dager SR, Lyoo IK (2012): The role of the amygdala in the pathophysiology of panic disorder: Evidence from neuroimaging studies. *Biol Mood Anxiety Disord* 2:20.
 27. Coryell MW, Wunsch AM, Haenfler JM, Allen JE, Schnizler M, Ziemann AE, *et al.* (2009): Acid-sensing ion channel-1a in the amygdala, a novel therapeutic target in depression-related behavior. *J Neurosci* 29: 5381–5388.
 28. Aguilar-Valles A, Sanchez E, de Gortari P, Balderas I, Ramirez-Amaya V, Bermudez-Rattoni F, *et al.* (2005): Analysis of the stress response in rats trained in the water-maze: Differential expression of corticotropin-releasing hormone, CRH-R1, glucocorticoid receptors and brain-derived neurotrophic factor in limbic regions. *Neuroendocrinology* 82: 306–319.
 29. Lakshminarasimhan H, Chattarji S (2012): Stress leads to contrasting effects on the levels of brain derived neurotrophic factor in the hippocampus and amygdala. *PLoS One* 7:e30481.
 30. Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002): Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* 22:6810–6818.
 31. Wemmie JA, Taugher RJ, Kreple CJ (2013): Acid-sensing ion channels in pain and disease. *Nat Rev Neurosci* 14:461–471.
 32. Gregersen N, Dahl HA, Buttenschon HN, Nyegaard M, Hedemand A, Als TD, *et al.* (2012): A genome-wide study of panic disorder suggests the amiloride-sensitive cation channel 1 as a candidate gene. *Eur J Hum Genet* 20:84–90.
 33. Klein DF (1993): False suffocation alarms, spontaneous panics, and related conditions: An integrative hypothesis. *Arch Gen Psychiatry* 50: 306–317.
 34. Dwyer JM, Rizzo SJ, Neal SJ, Lin Q, Jow F, Arias RL, *et al.* (2009): Acid sensing ion channel (ASIC) inhibitors exhibit anxiolytic-like activity in preclinical pharmacological models. *Psychopharmacology* 203:41–52.