Behavioral/Cognitive

Indirect Effect of Corticotropin-Releasing Hormone Receptor 1 Gene Variation on Negative Emotionality and Alcohol Use via Right Ventrolateral Prefrontal Cortex

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Variations in the corticotropin-releasing hormone receptor 1 (*CRHR1*) gene have been found to interact with stress in modulating excessive alcohol consumption. However, the neural mechanisms through which *CRHR1* influences this risk in humans is largely unknown. This study examined the influence of an intronic *CRHR1* gene variant, rs110402, on brain responses to negative emotional words, negative emotional traits, and alcohol use in adolescents and young adults at high risk for alcoholism. Childhood stress was investigated as a potential moderator. Using functional magnetic resonance imaging, we found that a region in the right ventrolateral prefrontal cortex (rVLPFC) was more engaged during negative emotional word processing in G homozygotes than in A allele carriers ($p_{\text{(FWE corrected)}} < 0.01$, $N = 77$). Moreover, an indirect effect of genotype on negative emotionality via rVLPFC activation ($p < 0.05$, $N = 69$) was observed, which was further moderated by childhood stress ($p < 0.05$, $N = 63$). Specifically, with low childhood stress, G homozygotes exhibited lower levels of negative emotionality associated with greater rVLPFC activation, suggesting that the rVLPFC is involved in reappraisal that neutralizes negative emotional responses. In addition, we found that genotype indirectly modulated excessive alcohol consumption ($p < 0.05$, $N = 69$). Specifically, G homozygotes showed greater rVLPFC activation and had lower levels of negative emotionality, which were associated with fewer binge-drinking days and fewer alcohol related problems. This work provides support for a model in which *CRHR1* gene variation modulates the risk of problem drinking via an internalizing/negative affect pathway involving rVLPFC and reappraisal of negative emotion.

Key words: alcohol consumption; childhood stress; *CRHR1*; genetics; negative emotionality; prefrontal cortex

Introduction

Corticotropin-releasing hormone (CRH) plays a critical role in modulating the neuroendocrine and behavioral responses to stress (Arborelius et al., 1999). CRH-containing neurons and CRH receptors are distributed throughout the brain, with a high density of CRH in the hypothalamus playing a critical role in regulating the hypothalamic-pituitary-adrenal axis (Swanson et al., 1983; Holsboer, 1999). Hyperactivity of the CRH system in the hypothalamus and other brain regions has been implicated in anxiety and mood disorders (Nemeroff et al., 1984; Heuser et al., 1994; Arborelius et al., 1999; Wong et al., 2000; de Kloet et al., 2005; Berton and Nestler, 2006).

Gene polymorphisms of the major CRH receptor (*CRHR1*) have been associated with stress-related phenotypes, including altered cortisol response (Heim et al., 2009), trait anxiety (Mahon et al., 2013), depression (Bradley et al., 2008; Polanczyk et al., 2009), and suicide (Ben-Efraim et al., 2011). Excessive alcohol use has also been found to be affected by *CRHR1* gene variations (Treutlein et al., 2006; Blomeyer et al., 2008; Schmid et al., 2010; Molander et al., 2012; Ray et al., 2013). Because negative affect and internalizing problems are risks for alcohol use disorder (AUD) (Kellam et al., 1980; Caspi et al., 1996; Hussong et al., 2011), it is hypothesized that the *CRHR1* gene may influence excessive alcohol use through its role in modulating negative emotion. This indirect effect hypothesis has not yet been tested.

In addition, studies have also revealed an interaction between *CRHR1* gene variations and stressful life experiences. In particular, childhood stress (Bradley et al., 2008; Heim et al., 2009; Polanczyk et al., 2009; Ben-Efraim et al., 2011; DeYoung et al., 2011) was demonstrated to modulate negative emotion and alcohol use. For example, the minor allele A of the intronic single nucleotide polymorphism (SNP) rs110402 showed a protective effect against depression and neuroticism, but only in individuals who experienced childhood stress (Bradley et al., 2008; Polanczyk et

Table 1. Subject characteristics for genotype groups

	AA/AG (n = 51)[a]	GG (n = 26)[a]	AA/AG vs GG χ^2 or t	p	df
Males	61%	65%	0.16	0.69	1
Age	19.39 (1.66)	19.24 (1.61)	0.56	0.58	75
Self-report race: Caucasian	96%	100%	1.05	0.31	1
Diagnosis of AUD	20%	15%	0.21	0.65	1
Family-history positive [b]	63%	58%	0.67	0.81	1
Ethnic factors					
1st	0.32 (0.21)	0.36 (0.15)	−0.92	0.36	75
2nd	0.15 (0.82)	0.16 (0.56)	−0.04	0.97	75
3rd	−0.00 (0.93)	−0.29 (1.15)	1.19	0.24	75
4th	0.15 (1.14)	−0.19 (1.04)	1.27	0.21	75
Valence	(n = 49)[c]	(n = 25)[c]			
Negative	2.42 (0.93)	2.87 (1.00)	−1.94	0.06	72
Neutral	5.07 (0.36)	5.15 (0.32)	−0.80	0.43	72
Arousal	(n = 48)[c]	(n = 25)[c]			
Negative	4.33 (1.68)	4.31 (1.53)	0.06	0.95	71
Neutral	4.22 (1.24)	4.19 (1.05)	0.11	0.91	71
Childhood stress	3.92 (2.53; n = 41)	4.57 (2.77; n = 21)	−0.92	0.36	60
Negative emotion outcomes					
Negative memory recognition bias (recognition performance: negative–neutral)	0.30 (0.77)	0.21 (0.31)	0.61	0.54	75
Positive memory recognition bias (recognition performance: positive–neutral)	0.21 (0.11; n = 51)	0.17 (0.05; n = 25)[d]	0.28	0.77	74
Negative emotionality	4.24 (1.07; n = 44)	3.99 (1.13; n = 25)	0.91	0.36	67
Neuroticism	76.75 (19.83; n = 37)	76.74 (22.14; n = 19)	−0.51	0.61	54
Depression facet	11.68 (5.62; n = 37)	11.75 (5.95; n = 20)	−0.05	0.96	55
Alcohol use measures[e]	(n = 44)	(n = 25)			
Past-year drinking volume	18.54 (12.23)	18.53 (13.74)	−0.004	0.99	67
Past-year alcohol-related problems	3.93 (4.72)	5.12 (5.50)	−0.94	0.35	67
Past-year binge-drinking days	4.99 (4.27)	6.36 (6.52)	−1.05	0.30	67

SDs are given in parentheses.

[a]Analyses on some variables were done with a smaller sample due to missing data in which case the actual sample sizes after removing outliers are given in parentheses. Three outliers were removed for negative emotionality; two outliers were removed for childhood stress, and one outlier was removed for neuroticism.

[b]At least one parent was diagnosed with an AUD (alcohol abuse or alcohol dependence) in the participant's lifetime.

[c]Three fMRI participants did not complete more than 80% of the valence ratings and four did not complete more than 80% of the arousal ratings.

[d]One participant did not have positive memory bias score.

[e]All alcohol use measures were square-root transformed.

al., 2009; DeYoung et al., 2011). An interaction between rs110402 and adulthood stress has also been found in predicting the risk for alcoholism, although stressors in adulthood seemed to function by different mechanisms than childhood stressors (Ray et al., 2013).

Only one study so far has examined how *CRHR1* gene variants influence human brain responses, and observed that brain activation during emotional word processing was modulated by rs110402 in healthy controls (Hsu et al., 2012). However, the implications of this finding as a potential risk for psychopathology are presently unknown. This study aimed to investigate the neural mechanism underlying the *CRHR1* gene effect on excessive alcohol use. We examined the effect of rs110402 on functional magnetic resonance imaging (fMRI) response during negative emotion processing in 16- to 22-year-olds at high risk for alcoholism. In addition, childhood stress, negative emotional traits, and alcohol use were measured. We tested the hypothesis that the *CRHR1* variant, rs110402, indirectly affects alcohol consumption by modulating neural response to negative emotional stimuli and the experience of negative affect. We also tested childhood stress as a potential moderator of these processes.

Materials and Methods

Participants. Participants were recruited from the Michigan Longitudinal Study (MLS). The MLS is an ongoing multiwave study of families with

alcoholic parents and control families drawn from the same neighborhoods (Zucker et al., 1996, 2000). Offspring in both types of families were contacted for fMRI study participation. Those with active medical illness, any current or recent treatment (within 6 months) with centrally active medications, or current or past psychiatric disorder in themselves or first-degree relatives were excluded except in the case of AUD (i.e., alcohol abuse or dependence). Participants with AUD were included so that the sample encompassed a wide range of alcohol consumption. Drug and pregnancy tests were conducted immediately before the scan. Seventy-seven participants (29 females and 48 males), aged 16–22 years, were included in the study (Table 1 shows detailed subject characteristics). Fourteen participants had a diagnosis of AUD at some point in their lifetime. Forty-seven participants had at least one parent diagnosed with an AUD in the participant's lifetime.

Written informed consent approved by the University of Michigan Medical School Institutional Review Board was obtained from each adult participant, or parent for participants under the age of 18 years, who also signed their assent to participate.

Genotyping. As part of the MLS protocol, all participants were genotyped using the Illumina Addiction biology SNP array (Hodgkinson et al., 2008) which includes SNPs from 130 candidate genes for alcoholism, other addictions, and disorders of mood and anxiety. These SNPs were genotyped using the Illumina Golden-Gate platform. Seventy-eight duplicates were included and no discrepancies were observed. Among these SNPs, rs110402 located in intron 2 of the *CRHR1* gene on chromosome 17 was selected as a SNP of interest considering its effects on brain activation and behavioral outcomes reported in previous studies (Bradley et al., 2008; Hsu et al., 2012). Genotypes were in Hardy Weinberg Equilibrium, $\chi^2(2) = 0.91$, $p > 0.50$. The minor allele (A) frequency is 40%

(AA = 10, AG = 41, GG = 26). Given the low number of A homozygotes, these participants and AG heterozygotes were combined into one group (i.e., A carriers).

All MLS participants were also genotyped for 178 ancestry informative markers (AIMs). 28 SNPs had >10% missing values. A principal component analysis on the remaining 150 SNPs was performed based on AIMs from 1139 MLS participants using SAS 9.3 (SAS Institute). The first four components explaining the highest variance (19.5, 2.5, 1.5, and 1.3%) were included as covariates to control for population stratification.

Measures

fMRI task. An emotion-arousal word task was used to probe negative emotion processing in the scanner (Heitzeg et al., 2008; Hsu et al., 2010, 2012; Mickey et al., 2011). Words presented in the task were selected from the Affective Norms for English Words (Bradley and Lang, 1999) which provides norm ratings for emotional valence and arousal on a scale of 1 (negative valence; low arousal) to 9 (positive valence; high arousal). Thirty-six words were selected for each of the following conditions: negative words (valence rating <3), neutral words (4.5 < valence rating < 5.5), and positive words (valence rating >7). Negative and positive words had an arousal rating >5. Neutral words' arousal ratings were >2.

Words were presented one at a time in a block design. Each block consisted of six trials. Each trial started with a 3 s word presentation followed by a fixation lasting 1 s. Participants were asked to press a switch once they understood the word. Following each task block, participants were instructed to relax and continue looking at a blank screen for 18 s. Six task blocks and six rest blocks were included in each run in which each condition was presented twice. The order of presented conditions was counterbalanced using a Latin Squares design. The entire experiment consisted of three runs, which in total lasted 12 min and 36 s.

Following the scan, participants completed a questionnaire on 54 words of which 36 words had been presented in the scanner. Equal numbers of words were included across conditions (negative, neutral, and positive). For each word, they were asked to identify whether they remembered seeing it in the scanner, rate its emotional valence and arousal respectively on a nine-point scale similar to that of the Affective Norms for English Words. Recognition performance for each word type was calculated by adjusting hit rate (p) with false alarm rate (fp) using the following formula: $p - fp/1 - fp$ (Epstein et al., 2006). A greater memory sensitivity to negative emotional stimuli has been found to characterize major depressive disorder (Leppänen, 2006; Hamilton and Gotlib, 2008). We calculated the negative memory bias by subtracting recognition performance for neutral words from that for negative words. In addition, the positive memory bias was calculated by subtracting recognition performance for neutral words from that for positive words. Three participants did not complete >80% of the valence ratings and four did not complete >80% of the arousal ratings.

Negative emotion. Three measures of negative emotional traits were selected from the MLS protocol. A negative emotionality scale was constructed with 10 items from the California Q-sort, an examiner-rated personality assessment measure (Block, 1961, 1971). The measure was assessed repeatedly at 3 year intervals beginning at age 15 until age 29. Scores collected closest to the scan (within 1 year prior and 3 years after) were analyzed. Due to missing data, 72 participants had available scores on negative emotionality within the allowable 4 year period.

An additional measure of negative emotion traits was the neuroticism dimension of the Revised NEO Personality Inventory (NEO PI-R; Costa and McRae, 1985). High neuroticism has been associated with increased risk for depression (Boyce et al., 1991; Kendler et al., 1993). In addition, the depression facet of neuroticism in the NEO PI-R was also examined. The NEO PI-R was measured for the first time at participants' age 18–20 and then again at age 24–26. Fifty-seven participants had scores collected within the 4-year period of 1 year before and 3 years after the scan.

Childhood stress. Childhood stress was measured using the Coddington Social Readjustment Rating Scale for Children (Coddington, 1972a,b) collected as part of the MLS protocol. This scale lists life events that might have had a positive or negative impact on the child. Twelve items describing the most negative and stressful events were selected.

These events included parental separation/divorce, death of family members/friends/pets, self and parent illness/accidents, parental absence, parental jail sentence, parental job lost, discovery of adoption, and family move. The primary caregiver (generally mothers) completed this questionnaire for each child at 3 year intervals beginning at child's age 3. Each time, they reported whether any of these events took place, (1) in the past 6 months, (2) between 6 months to a year ago, or (3) between 1 and 3 years ago. We summed reports across all time points before the age of 18 (or by the age of scan if scanned before the age of 18) for events that occurred during any of these periods. Among the participants who had negative emotionality scores, 65 had available data on this measure.

Drinking. Past-year drinking volume, number of past-year alcohol-related problems, and number of past-year binge-drinking days were measured using the self-report Drinking and Drug History Form (DDHx; Zucker et al., 1990; Zucker and Fitzgerald, 1994), which was collected annually beginning at age 11 as part of the MLS protocol. To examine prospective effects of fMRI response and negative emotion on alcohol consumption, the first -DDHx completed at least 1 year after the scan, or the collection of negative emotionality scores (whichever occurred later) was used in the analyses. Sixty-nine participants who had negative emotionality scores also had measures of alcohol use. Due to non-normal distribution, all drinking variables were square-root transformed in subsequent analyses.

fMRI data acquisition. Whole-brain functional images were acquired on a 3.0 tesla GE Signa scanner (GE Healthcare) using a T2*-weighted single-shot combined spiral in/out sequence (Glover and Law, 2001; repetition time (TR) = 2000 ms; echo time (TE) = 30 ms; flip angle, 90°; field-of-view (FOV), 200 mm; 64 ×64 matrix; in-plane resolution = 3.12 × 3.12 mm; slice thickness = 4 mm). A high-resolution anatomical T1 scan was obtained to provide three-dimensional spoiled gradient recalled echo (3-DSPGR; TR = 25 ms; minimum TE; FOV = 25 cm; 256 × 256 matrix; slice thickness = 1.4 mm). Participant motion was minimized using foam pads placed around the head along with a forehead strap. In addition, the importance of keeping as still as possible was emphasized during the informed consent-process, at scanner entry and between runs.

fMRI data preprocessing. Functional images were reconstructed using an iterative algorithm (Fessler et al., 2005). Eight runs (i.e., 3%) of all collected data were removed from the analyses due to image distortion. In addition, 10 runs (i.e., 4%) were removed due to head motion exceeding 2 mm translation or 2° rotation in any direction. For the remaining data, head motion correction was conducted using the FSL 5.0.2.2 analysis tools library (Analysis Group, Functional MRI of the Brain, Oxford, UK; Jenkinson et al., 2002). Slice timing corrections, normalization, and smoothing processing were conducted using SPM8 r4667 package (Wellcome Institute of Cognitive Neurology, London, UK). Functional images were spatially normalized to the MNI space and then smoothed with a 6 mm full-width half-maximum Gaussian spatial smoothing kernel to improve signal-to-noise ratio.

Data analysis

Individual subject statistical maps. Individual subject analysis was completed using a general linear model. Three regressors of interest (negative, neutral, positive condition) were convolved with the canonical hemodynamic response function. Six motion parameters and a white matter parameter were included as nuisance regressors. A 128 s high-pass threshold was used to filter out scanner drift and other low-frequency noise. Two contrasts, negative words versus neutral words (NEG–NEU) and positive words versus neutral words (POS–NEU), were examined.

fMRI group analyses. A whole-brain voxelwise analysis was conducted in SPM8 using an independent sample t test to identify brain regions exhibiting different responses between the two genotype groups. Participants' age at the scan was included as a covariate. Statistical significance was established at $p_{(FWE\ corrected)} < 0.01$ (cluster size > 45 voxel at $p_{threshold} < 0.001$) based on simulation results generated by the 3dClustSim program in the AFNI analysis package (Cox, 1996). The average activation difference between negative and neutral words and between positive and neutral words was extracted for significant clusters using
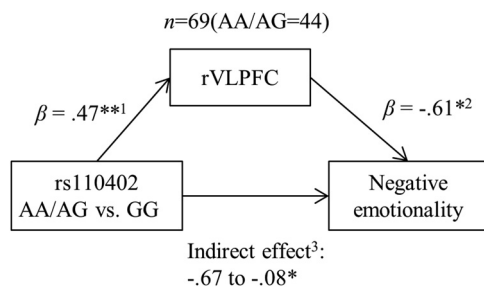
Figure 1. The results of the single mediation indirect effect model. Standards for a significant effect: $*p < 0.05$, $**p < 0.01$; [1]$p = 0.001$; [2]$p = 0.02$; direct effect of rs110402 on negative emotionality: $\beta = -0.05$, [3]$p = 0.87$.
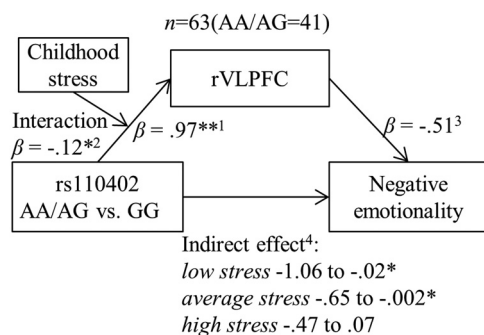

Figure 2. The results of the moderated mediation indirect effect model. Standards for a significant effect: $*p < 0.05$, $**p < 0.01$; [1]$p < 0.001$; [2]$p = 0.02$; [3]$p = 0.09$; direct effect of rs110402 on negative emotionality: $\beta = -0.09$, [4]$p = 0.76$.

Drinking behavior	β (negative emotionality ~ drinking behavior)	Indirect effect
1. Drinking volume	3.08[3]	-3.44 to -.03[6]
2. Binge drinking	1.34*[4]	-1.40 to -.02[7]
3. Alcohol problem	1.55*[5]	-1.53 to -.05[8]

Figure 3. The results of the multiple mediation indirect effect model. Standards for a significant effect at: $*p < 0.05$, $**p < 0.01$; [1]$p = 0.002$, [2]$p = 0.09$, [3]$p = 0.07$, [4]$p = 0.05$, [5]$p = 0.02$; direct effect of rs110402 on drinking volume: $\beta = 1.41$, [6]$p = 0.70$; direct effect of rs110402 on number of alcohol related problems: $\beta = 1.64$, [7]$p = 0.25$; direct effect of rs110402 on number of binge drinking days: $\beta = 2.15$, [8]$p = 0.14$.

MarsBaR (Brett et al., 2002) for further analyses. The extracted signals were compared again between genotype groups via ANCOVAs controlling for family history (i.e., positive if at least one parent diagnosed with AUD in the participant's lifetime), sex, age, age-squared, and four ethnic factors. This step was to verify that the genotype effect in the identified regions was not confounded by the control variables. The same covariates were included in all subsequent regression and indirect effect analyses.

Brain regions involved in emotion processing are expected to show different activation patterns between genotype groups. For example, regions involved in emotion regulation, such as VLPFC (Cunningham and Zelazo, 2007; Lieberman et al., 2007; Wager et al., 2008) should show greater activation in A allele carriers if its protective effect derived from a better emotion regulatory function.

Regression and indirect effect analyses. Three mediation indirect effect models (see Figs. 1–3) were tested using the PROCESS macro in SPSS (Hayes, 2013). The PROCESS macro tests indirect effects using a bootstrap approach developed by Preacher and Hayes (2004). This nonparametric approach can avoid the power problem introduced by nonnormality and is less restricted by sample size.

The first indirect effect model tested the hypothesis that *CRHR1* gene variant indirectly affects negative emotionality via its influence on fMRI response to negative emotional words (i.e., a single indirect effect model illustrated in Fig. 1). The second model further tested whether the indirect effect in the first model was modulated by number of reported childhood stressful events by adding childhood stress as a moderator (i.e., a moderated mediation model illustrated in Fig. 2). The third model (Fig. 3) was a multiple-mediation indirect effect model testing whether rs110402 indirectly influenced alcohol consumption by modulating brain response (the first mediator), which in turn, affected negative emotional traits (the second mediator).

Before conducting the PROCESS analyses, associations between brain response and measures related to negative emotion (i.e., negative emotionality, neuroticism, the depression facet, and negative/positive memory bias) were examined in separate linear regressions. Significance was
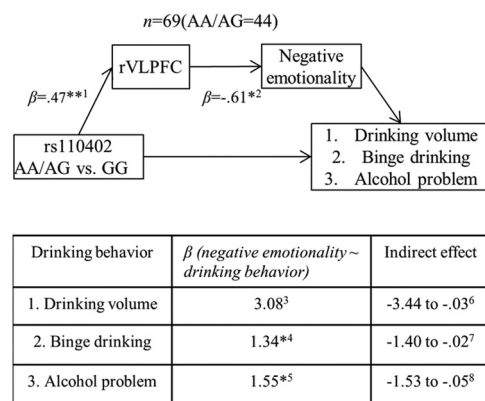
established at $p < 0.012$, correcting for multiple comparisons (0.05/4). Only the negative emotion variables that had a significant relationship with rVLPFC activation were tested in the indirect effect analyses. Similarly, only drinking variables that could be significantly predicted by negative emotional traits in regression analyses were tested in the multiple mediation indirect effect model.

In all regression and indirect effect analyses, G homozygotes were coded as 1. In addition, family history, sex, age, age-squared, and four ethnic factors were included as covariates. The number of bootstrap samples was 1000. Outliers with much larger Cook's *D* values or leverage values than the rest of the observations were removed based on regression outputs only if the removal substantially changed the results (Miyake et al., 2000). No more than three outliers were removed in any analysis.

It should be noted that due to missing data on questionnaire and scale measures, regression and indirect effect analyses involving these variables were conducted on different subsamples. To examine whether sampling bias was introduced, analyses were conducted to determine whether variables of interest, including negative emotionality and rVLPFC activation, differed between subjects that were included and excluded for each analysis. There were no significant differences between included and excluded subjects ($p > 0.13$) for all comparisons, suggesting that excluding subjects with missing data did not introduce sampling bias. In addition, there was no difference in genotype frequencies between each subsample and the whole sample ($p \geq 0.25$).

Results
Direct genotype effect on valence, arousal, and negative emotion-related measures
Based on a mixed effect ANOVA analysis, participants rated the negative words significantly lower on valence than the neutral words, $F_{(1,72)} = 395.36$, $p < 0.001$. In addition, G homozygotes rated words higher on valence compared with A allele carriers, $F_{(1,72)} = 4.36$, $p = 0.04$. This effect was greater for negative words than neutral words, although this interaction was not significant, $F_{(1,72)} = 2.42$, $p = 0.12$. There was no significant main effect of word type or genotype, or interaction on arousal ratings (all $p > 0.25$). No difference between genotype groups was found on negative memory bias, $F_{(1,67)} = 0.56$, $p = 0.46$ or positive memory bias $F_{(1,66)} = 0.29$, $p = 0.59$. No direct genotype effect was found on negative emotionality, $F_{(1,60)} = 1.77$, $p = 0.19$, neuroticism, $F_{(1,47)} = 0.05$, $p = 0.82$, or the depression facet, $F_{(1,48)} = 0.07$, $p = 0.79$.
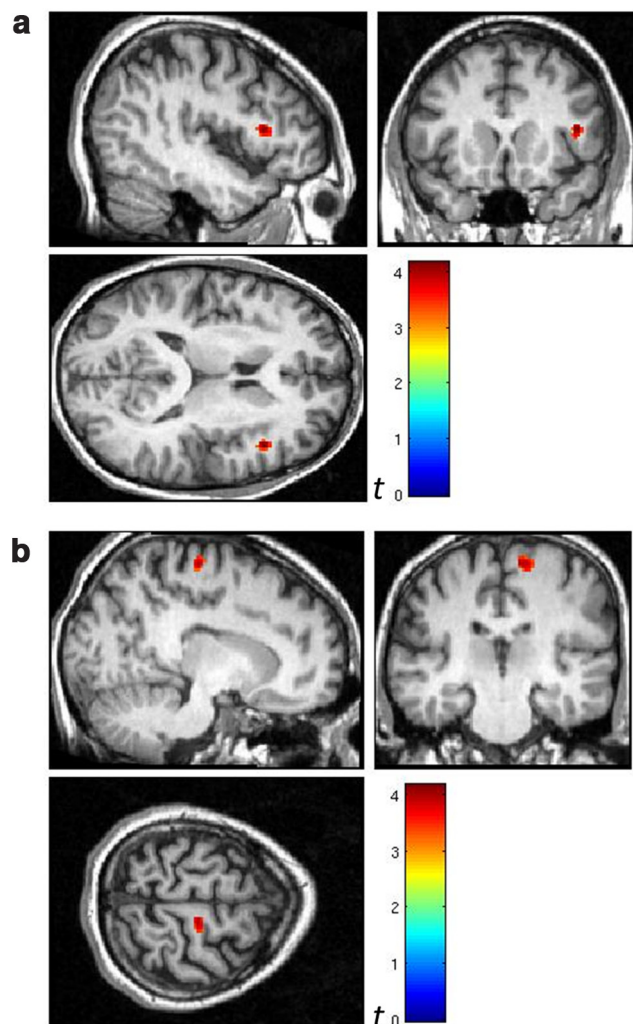
Figure 4. BOLD activation (negative–neutral words) in the contrast of A allele carriers minus G homozygotes, $p_{(FWE\ corrected)} < 0.01$. ***a***, The rVLPFC (centered at 40, 20, 10). ***b***, The right precentral cortex (centered at 12, −20, 66).

Figure 5. Correlation between rVLPFC activation and negative emotionality.

Genotype effect on fMRI response

For the NEG–NEU contrast, two regions showed greater activation in G homozygotes than A allele carriers. One region was in the rVLPFC (peak coordinate: 44, 20, 10; BA 44/45; voxel size: 53; Fig. 4*a*), and the other region was in the right precentral cortex (peak coordinate: 12, −20, 66; BA 4/6; voxel size: 53; Fig. 4*b*). No region showed greater activation in A allele carriers than G homozygotes. ANCOVAs on extracted fMRI signals showed that the genotype effects in these regions were not confounded by sex, age, age-squared, the four ethnic factors or family history (genotype effect in the rVLPFC for NEG–NEU contrast: $F_{(1,67)} = 14.97$, $p < 0.001$, AA −0.34, AG −0.20, GG 0.09; in the right precentral cortex for NEG–NEU contrast: $F_{(1,67)} = 16.62$, $p < 0.001$, AA −0.43, AG −0.16, GG 0.11). All genotype effects still held when excluding participants with diagnosis of alcohol dependence or abuse (genotype effect in the rVLPFC for NEG–NEU contrast: $F_{(1,53)} = 12.87$, $p = 0.001$; in the right precentral cortex for NEG–NEU contrast: $F_{(1,67)} = 9.03$, $p = 0.004$).

For the POS–NEU contrast, again a region in the right precentral cortex showed greater activation in G homozygotes than A allele carriers (peak coordinate: 26, −18, 52; BA 4/6; voxel size: 61), although no region in the rVLPFC passed the significance threshold. No region showed the opposite pattern either. ANCO-
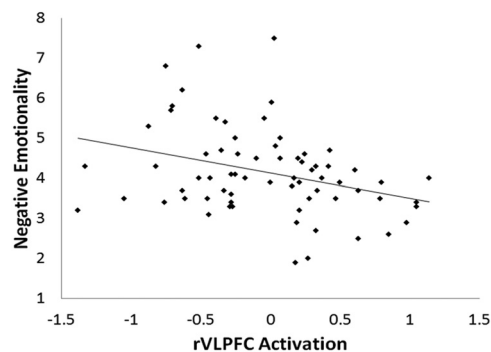
VAs on extracted signals in the right precentral cortex showed that the genotype effect in this region was still significant when controlling for all covariates ($F_{(1,67)} = 20.76$, $p < 0.001$, AA −0.37, AG −0.15, GG 0.21) and when excluding participants with alcohol dependence or abuse ($F_{(1,67)} = 14.31$, $p < 0.001$).

fMRI response predicts negative emotion

The sample size for each of the following analyses is listed in Table 1. For the two regions identified in the NEG–NEU contrast, neither rVLPFC nor precentral cortex activations were correlated with negative memory bias ($\beta = -0.04$, $p = 0.80$; $\beta = 0.01$, $p = 0.93$, respectively). The rVLPFC activation, however, was negatively correlated with negative emotionality ($\beta = -0.63$, $p = 0.01$, corrected for multiple comparisons $p < 0.012$; Fig. 5). In contrast, the precentral cortex activation extracted from neither the NEG–NEU nor the POS–NEU contrast was correlated with negative emotionality (NEG–NEU: negative emotionality: $\beta = -0.11$, $p = 0.63$). In addition, neither region showed activations that were correlated with neuroticism or the depression facet (rVLPFC neuroticism, $\beta = -2.16$, $p = 0.64$, the depression facet, $\beta = -1.76$, $p = 0.16$; precentral cortex neuroticism: $\beta = 5.56$, $p = 0.18$, the depression facet: $\beta = 1.31$, $p = 0.25$). Therefore, only the association between rVLPFC activation and negative emotionality was examined in the subsequent indirect effect analyses.

Single-mediation indirect effect on negative emotionality

The indirect effect of genotype on negative emotionality via rVLPFC activation was significant (Fig. 1). Specifically, G homozygotes exhibited greater rVLPFC response to negative emotional words, which in turn was associated with lower levels of negative emotionality.

Moderated-mediation indirect effect on negative emotionality

An initial regression analysis investigating the effect of genotype, childhood-life stress, and their interaction on rVLPFC activation revealed a significant interaction effect between childhood-life stress and *CRHR1* genotype ($\beta = -0.12$, $p = 0.02$; Fig. 2). Further regression analyses of rVLPFC activation on childhood stress in each genotype group revealed a trend of negative correlation between rVLPFC activation and number of stressful childhood life events for G homozygotes ($\beta = -0.09$, $p = 0.06$), whereas there was no such relationship for A allele carriers ($\beta = 0.04$, $p = 0.23$).

The moderated-mediation indirect effect analysis (Fig. 2) showed that the indirect effect of genotype on negative emotionality via rVLPFC was significantly moderated by childhood stress. Specifically, G homozygotes had greater rVLPFC activation,

Table 2. *CRHR1* SNPs implicated in HapMap and published articles on depression and alcohol consumption

Location	SNP	r^2	Outcomes	Effects	References
Intron 1	rs7209436	>0.90[a]	Depression	TT protective	(Bradley et al., 2008)
Intron 1	rs110402		Depression; SLE	A protective	(Bradley et al., 2008; Heim et al., 2009; Polanczyk et al., 2009; Ressler et al., 2010; Kranzler et al., 2011)
			Depression, neuroticism; SLE	A risk	(Grabe et al., 2010; DeYoung et al., 2011; Laucht et al., 2013)
			AD; trauma	G protective	(Ray et al., 2013)
Intron 2	rs242924	1.00	Risk AD; trauma	C protective	(Ray et al., 2013)
Intron 3	rs242938	0.03[b]	Binge drinking; SLE	AA risk	(Treutlein et al., 2006; Schmid et al., 2010)
Intron 4	rs173365	0.33	AD; trauma	T risk; T protective × trauma[c]	(Ray et al., 2013)
Intron 5	rs17689882	0.19	Depression; SLE	G risk	(Grabe et al., 2010; Laucht et al., 2013)
Intron 7	rs1876831	0.19[b]	Alcohol drinking; SLE	C risk	(Treutlein et al., 2006; Blomeyer et al., 2008; Nelson et al., 2010; Schmid et al., 2010)
Intron 9	rs17689966	0.37	AD; trauma	G risk; G protective × trauma[c]	(Ray et al., 2013)

LD (European Americans r^2) values in relation to rs110402. AD, Alcohol dependence; SLE, stressful life events.

[a]Bradley et al., 2008.

[b]Nelson et al., 2010.

[c]×, interaction.

which led to lower negative emotionality, but only at lower levels of childhood stress but not at higher levels.

Multiple-mediation indirect effects on drinking
Three indirect effect analyses of genotype effect were conducted on drinking volume, number of binge-drinking days, and number of alcohol-related problems with rVLPFC activation as the first mediator and negative emotionality as the second mediator (Fig. 3). All indirect effects were significant. In particular, compared with A allele carriers, G homozygotes had greater rVLPFC activation which led to lower levels of negative emotionality that was associated with less drinking volume, fewer alcohol related problems and fewer binge drinking days.

Discussion
This study examined the effect of the *CRHR1* gene variant rs110402 on fMRI response to negative emotional words, and also examined whether there was an indirect effect of the *CRHR1* gene on negative emotionality and alcohol consumption via the fMRI response.

The rVLPFC and right precentral cortex exhibited greater activation during negative emotional word processing in G homozygotes than in A allele carriers. In addition, rVLPFC activation in the NEG–NEU contrast was negatively correlated with negative emotionality at the behavioral level. This result is in accordance with previous findings that the rVLPFC plays a role in emotional reappraisal; a process that reevaluates an emotional situation to decrease its emotional impact (Gross, 2001; Ochsner et al., 2002; Gross and John, 2003). For example, reappraisal has consistently activated the VLPFC, especially the right VLPFC (Cunningham and Zelazo, 2007; Lieberman et al., 2007; Wager et al., 2008). In addition, greater engagement of the VLPFC in response to negative stimuli has been reported in bipolar and depressive disorders (Drevets et al., 1992; Lawrence et al., 2004), suggesting a greater demand on regulating elevated emotional responses in these patients. However, no rVLPFC activation was found in the POS–NEU contrast. A possible explanation for this effect is that reappraisal, as a protective mechanism, is more engaged for negative stimuli to avoid negative emotional responses, whereas this process is less needed for positive stimuli.

It is noted that in this study a posterior region in the rVLPFC (i.e., BA 44/45) demonstrated the genotype effect. Previous studies have suggested a functional dissociation between the posterior and anterior rVLPFC in emotion regulation. For example, Beer et al. (2006) proposed that the anterior rVLPFC is specifically involved in monitoring influences of emotion on decision making

whereas the other areas of rVLPFC are engaged in general emotion regulation. On the other hand, studies that have reported activation of the anterior rVLPFC (e.g., BA 47) often used tasks requiring simple suppression of emotional responses in contrast to reappraisal of emotional stimuli in which the posterior rVLPFC was involved (Lévesque et al., 2004; Ochsner et al., 2004; Phan et al., 2005). The present findings are in line with the hypothesis that the posterior rVLPFC plays a critical role in updating emotional state and responses (Levy and Wagner, 2011), rather than just providing a simple suppression of current emotional responses.

The other region that exhibited the *CRHR1* genotype effect, the precentral cortex, has also been repeatedly found to be activated in emotion regulation processes (Goldin et al., 2008; Roalf et al., 2011; Fusar-Poli et al., 2009). One possible function of this region is to generate an opposite expression to override prepotent facial responses to emotional stimuli (Goldin et al., 2008). However, studies have found that the suppression strategy did not necessarily lead to attenuated emotional responses or better emotional well being (Gross and John, 2003). Consistently, precentral cortex activation was not correlated with negative emotion.

Based on the correlation between rVLPFC activation and negative emotionality, we conducted an indirect effect analysis using a bootstrapping approach that provides a formal statistical test of indirect effects with many advantages over traditional methods (Preacher and Hayes, 2004). A significant indirect effect showed that G homozygotes exhibited lower levels of negative emotionality than A allele carriers by engaging the reappraisal process involving the rVLPFC. However, the advantage of G homozygotes in engaging reappraisal is divergent from previous studies that have reported a protective effect of the A allele, but a vulnerability to stress in GG carriers. To reconcile these findings, we propose that developing a better reappraisal function is a compensatory mechanism for G homozygotes to neutralize their sensitivity to stress. Consistent with this account, Refojo et al. (2011) reported a function of *CRHR1* in the VTA and the prefrontal cortex that was antagonistic to *CRHR1*'s function in the amygdala and hippocampus, suggesting a balance between these two CRH systems. In addition, positive effects of sensitivity to stress have been reported in previous studies (Ellis and Boyce, 2008). For example, children who were highly reactive to acute stress were healthier and had fewer injury incidences than children who showed lower-stress reactivity (Boyce et al., 1995; Boyce, 1996) in moderately stressful settings. It is possible that because these highly reac-

tive individuals are more sensitive to the environment, they are more likely to detect stress and develop regulatory functions accordingly, compared with less-sensitive individuals.

However, the advantage of stress sensitivity in G homozygotes in developing regulatory functions may not exist in a very low-stress environment, because emotion regulation is not needed. Consistent with this hypothesis, healthy adults primarily recruited on a college-campus setting did not exhibit greater activation in reappraisal related regions in rs110402 G homozygotes compared with A allele carriers (Hsu et al., 2012). In contrast, our sample was drawn from the MLS, a high-risk study with high levels of parental alcohol involvement and low socioeconomic status families; these individuals undoubtedly have experienced at least some stressful events, although these events may not have been serious enough to be captured by the stress measure we used. Hence, these individuals may be more likely to develop a compensatory regulatory function compared with samples drawn from high socioeconomic status populations in low stress environments.

On the other hand, the advantage of G homozygotes diminishes when the environment is highly stressful. We found an interaction between the *CRHR1* gene variant and childhood stress on rVLPFC activation. In particular, the rVLPFC activation showed a declining trend as the number of stressful childhood life events increased in G homozygotes, suggesting that the reappraisal function in these individuals was hindered as a result of childhood stressors. In contrast, there was no such trend in A allele carriers. In addition, the moderated indirect effect analysis revealed that the indirect effect of rs110402 on negative emotionality was absent in individuals who experienced a larger number of stressful childhood life events, suggesting that in highly stressful settings, G homozygotes no longer possessed the advantage in emotion regulation. Thus, in highly stressful settings, GG carriers should demonstrate vulnerability due to their specific sensitivity to stress in contrast to a protective effect of GG genotype in moderate stress situations. Finely distinguished differences in measured stress levels across studies may be the reason for incongruent findings. As shown in Table 2, five studies (Bradley et al., 2008; Heim et al., 2009; Polanczyk et al., 2009; Ressler et al., 2010; Kranzler et al., 2011) reported the A allele as protective from depression in adverse circumstances. In contrast, aligned with our results, three studies (Grabe et al., 2010; DeYoung et al., 2011; Laucht et al., 2013) found the A allele conferring risk for depression and neuroticism (i.e., GG protective).

Finally, we examined the underlying indirect effect of the *CRHR1* gene variant on drinking behavior. The multiple-mediation indirect effect analysis revealed a significant indirect path underlying the *CRHR1* genotype effect on alcohol consumption. Specifically, G homozygotes who exhibited greater rVLPFC response to negative words showed reduced negative emotionality that was associated with less drinking volume, fewer alcohol-related problems, and fewer binge-drinking days. This finding is especially valuable, given the heterogeneity of alcohol use disorder (Zucker, 2006, 2008). The *CRHR1* gene is involved in determining the particular pathway to problem drinking, above and beyond a simple demonstration that a *CRHR1* gene variant has an effect on excessive drinking. In addition, this study identifies the brain region: the rVLPFC that plays a critical role in bridging the association between *CRHR1* gene variant and negative emotionality/drinking behavior.

Prior studies of the impact of *CRHR1* on depression, negative emotionality and alcohol consumption have reported associations with SNPs in linkage disequilibrium (LD) with the SNP we report on here (rs110402). For example, studies on alcohol-related measures have found associations with SNPs distal and in low LD to rs110402 (Table 2). One study (Ray et al., 2013) reported the major alleles of rs110402 (G) and rs242924 (C) as protective against risk of AD in the presence of trauma, same direction as our results. They also reported significant associations with two distal SNPs in low LD with rs110402. Further studies are necessary to address effects of different *CRHR1* SNPs on neural responses and mechanisms through which they influence behavioral outcomes.

There are limitations that need to be acknowledged in this study. First, we did not have the power to test whether this multiple mediation indirect effect was also moderated by childhood stress. Nevertheless, given the findings that childhood stress moderated negative emotionality and rs110402 affects alcohol consumption through negative emotionality, we hypothesize that the indirect effect of rs110402 on alcohol consumption may also be moderated by childhood stress. Further studies are needed to test this moderated multiple-mediation model in a larger sample. Second, studies are needed to examine the effect of rs110402 on CRHR1 expression to test our hypothesis on functional balance of CRHR1 in different brain regions.

To conclude, this study found that in an adolescent/early adult sample at high risk for alcoholism, the *CRHR1* gene variant, rs110402, influenced fMRI response to negative emotional words in the rVLPFC. Moreover, there was an indirect effect of rs110402 on negative emotionality via rVLPFC activation, and this indirect effect was moderated by childhood stress. In addition, rs110402 played a role in the internalizing problem/negative affect pathway to problem drinking by modulating rVLPFC activation during negative emotional stimulus processing.

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