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5-HTTLPR, *HTR1A*, and *HTR2A* cumulative genetic score interacts with mood reactivity to predict mood-congruent gaze bias

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Abstract

Genetic variation within the serotonin system has been associated with biased attention for affective stimuli and, less consistently, with vulnerability for Major Depressive Disorder. In particular, 5-HTTLPR, *HTR1A* (rs6295), and *HTR2A* (rs6311) polymorphisms have been linked with biased cognition. The current study developed a serotonergic cumulative genetic score (CGS) that quantified the number of risk alleles associated with these candidate polymorphisms to yield a single CGS. The CGS was then used to model genetic influence on the relationship between reactivity to a negative mood induction and negatively biased cognition. A passive viewing eye tracking task was administered to 170 healthy volunteers to assess sustained attention for positive, dysphoric, neutral, and threatening scenes. Participants were then induced into a sad mood and readministered the passive viewing task. Change in gaze bias, as a function of reactivity to mood induction, was the primary measure of cognitive vulnerability. Results suggest that, although none of the individual genes interacted with mood reactivity to predict change in gaze bias, individuals with higher serotonin CGS were significantly more likely to look towards dysphoric images and away from positive images as mood reactivity increased. These findings suggest that a CGS approach may better capture genetic influences on cognitive vulnerability, and reaffirms the need to examine multilocus approaches in genomic research.

Keywords

serotonin; sad mood induction; mood reactivity; attention bias; cumulative genetic risk; cognitive vulnerability

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Introduction

Maladaptive cognitive processes are a common characteristic of affective psychopathologies, including Major Depressive Disorder (MDD) (Beck, 1967; 1987). The failure to properly allocate cognitive resources has been linked to dysregulated experience of emotion, and has been identified as putative risk factor for the onset and maintenance of mood disorders (Gotlib & Joormann, 2010; Gross, 2001). Specifically, attention biased towards mood-congruent stimuli has been consistently observed in depressed individuals relative to healthy controls (Koster, De Raedt, Goeleven, Franck, & Crombez, 2005). Sustained attention for negative, mood-congruent stimuli is believed to contribute to depression by increasing the emotional impact of negative information in the environment and thereby exacerbating the effects of stressful life events (Beck, 1967; Clark, Beck, & Alford, 1999). Furthermore, attention biased towards mood-congruent stimuli is thought to detract from more adaptive cognitive mechanisms, such as cognitive reappraisal and the integration of contextual information (Beck, 2008; Clark et al., 1999).

Increased attentional bias has been observed following negative environmental experiences (Bar-Haim et al., 2010; Disner et al., 2013), and is believed to be an intermediary endophenotype linking life stress to mental illness (Beevers & Carver, 2003; De Raedt & Koster, 2010). In a laboratory environment, negative environmental factors can be manipulated in a controlled fashion by inducing a sad mood state in research participants. Sad mood inductions increase negative affect and serve as a laboratory analog for an individual's reactivity to external stressors that create sad mood (Blaney, 1986; Segal, Gemar, & Williams, 1999).

Importantly, individuals who are more reactive to environmental stressors and display stronger cognitive biases following mood provocation, often referred to as cognitive reactivity, are considered particularly vulnerable to the onset or recurrence of affective disorders such as MDD (De Raedt & Koster, 2010; Pine et al., 2005; Scher, Ingram, & Segal, 2005; Segal et al., 2006). Understanding the factors that influence cognitive reactivity to sad moods is critical for better understanding the etiology of cognitive vulnerability to depression.

One such etiologic factor involves the monoamine neurotransmitter serotonin (5-hydroxytryptamine). Significant research has linked serotonin with behaviors associated with affective psychopathology, including cognition and emotion. Abnormal serotonin function has been observed in mood and anxiety disorders (Owens & Nemeroff, 1994), and numerous studies have shown that experimentally decreased serotonin, elicited via acute tryptophan depletion, can lead to the relapse of dysphoric symptoms (Booij, Van der Does, & Riedel, 2003; Booij, Van der Does, Haffmans, Spinhoven, & McNally, 2005). Emotion dysregulation and biased cognition have also been linked with limbic hyperactivity and frontal hypoactivity (Canli & Lesch, 2007; Cools, Roberts, & Robbins, 2008; Disner, Beevers, Haigh, & Beck, 2011; Gutknecht et al., 2007; Hariri & Holmes, 2006; Phillips, Drevets, Rauch, & Lane, 2003), a circuit that is densely innervated by serotonin neurons and receptors (H. R. Smith, Daunais, Nader, & Porrino, 1999). In particular, the 5-HT_{1A} receptor is commonly found in limbic regions, including the hippocampus, parahippocampal gyrus,

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and amygdala, while the 5-HT_{2A} receptor is commonly found in regions associated with top-down mood regulation, such as the middle frontal gyrus, anterior cingulate cortex, and claustrum (Hawrylycz, Lein, & Guillozet-Bongaarts, 2012). These two receptors are charged with inhibiting the limbic regions and exciting the frontal regions respectively (Buhot, 1997).

Considering the prominent role of 5-HT_{1A} and 5-HT_{2A} receptors in the corticolimbic circuit, variation in genes that code for these receptors have been commonly identified as candidate markers for genetic vulnerability to affective pathology. In particular, the *HTR1A* (rs6295) and *HTR2A* (rs6311) variants moderate neurotransmission associated with these receptors (Illi et al., 2009). The C allele of rs6295, commonly referred to as the C(-1019)G polymorphism, is a part of a 26 bp palindrome that impacts the expression of the presynaptic 5-HT_{1A} somatodendritic autoreceptor through repressive transcription factors, such as HES1, HES5, and DEAF1 (Albert, 2012; Jacobsen, Vanderluit, Slack, & Albert, 2008; Kishi et al., 2009; Le François, Czesak, Steubl, & Albert, 2008; Lemonde et al., 2003). In addition, postsynaptic 5-HT_{1A} heteroreceptor expression is modulated by *HTR1A* genotype, particularly in limbic areas, which may be augmented by glucocorticoid-mediated downregulation (Albert, 2012). Taken together, the impact of these factors has been shown to greatly reduce expression and binding of 5-HT_{1A} receptors relative to the GG genotype (Kishi et al., 2009; Le François et al., 2008; Lemonde et al., 2003), which has been associated with greater amygdala reactivity (Fakra et al., 2009) and increased risk for MDD amongst Asian populations (Kishi et al., 2009).

The C allele of rs6311, commonly known as the A(-1438)G polymorphism, lies just upstream from the promoter region of *HTR2A* and is theorized to influence gene expression and protein levels by modulating promoter function (Parsons, D'Souza, Arranz, Kerwin, & Makoff, 2004; Polesskaya & Sokolov, 2002; Turecki, Brière, & Dewar, 1999). However, the moderation of promoter function that differentiates the C and T alleles appears to only be observed in the presence of specific transcription factors, such as pGL3 and IMR-32, suggesting that the functional influence of the C allele may only be present during induced promoter activity (Parsons et al., 2004). This relationship between the C allele and specific transcription factors may partially explain some of the inconsistent reports in the *HTR2A* literature (e.g. R. M. Smith et al., 2013). Behaviorally, *HTR2A* variation has been linked to greater angry, aggressive, and suicidal behaviors (Du, Bakish, Lapierre, Ravindran, & Hrdina, 2000; Giegling, Hartmann, Möller, & Rujescu, 2006; Wrzosek et al., 2011), and is frequently investigated along with *HTR1A* to explore the genetic substrates of mental illness such as MDD, panic disorder, and alcohol dependence (Blaya et al., 2010; Wrzosek et al., 2011; Xu et al., 2012). However, since the influence of the *HTR2A* polymorphism appears to only occur in specific contexts (e.g. situations that induce endogenous promoter activity), factors that moderate context sensitivity are likely to play a significant role in the relationship between serotonin receptor function, emotion dysregulation, and biased cognition.

One prominent factor that can moderate context sensitivity is the serotonin transporter protein (5-HTT), which is primarily responsible for clearing serotonin from the synaptic cleft. 5-HTT is encoded by the *SLC6A4* gene, the expression of which is modulated by key

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variations in the *SLC6A4*-linked polymorphic region (5-HTTLPR; Heils et al., 1996). 5-HTTLPR has two common variants: the long (L) and short (S) allele. The long allele can be further categorized into L_A and L_G variants, with L_G treated as an approximate functional equivalent to S (Hu et al., 2005; Zalsman et al., 2006).

Carriers of the S or L_G variants of the 5-HTTLPR gene have demonstrated various cognitive and physiological characteristics consistent with maladaptive emotional processing (Canli & Lesch, 2007; Hariri & Holmes, 2006). Specifically, dynamic causal modeling has linked the S and L_G variants with reduced prefrontal inhibitory regulation (Volman et al., 2013), which likely contributes to the established connection between 5-HTTLPR and attentional bias towards emotion stimuli (Beevers, Wells, Ellis, & McGeary, 2009; Fox, Ridgewell, & Ashwin, 2009; Pergamin-Hight, Bakermans-Kranenburg, van IJzendoorn, & Bar-Haim, 2012), amygdalar hyperactivity during processing of emotional faces (Dannlowski et al., 2007; Hariri et al., 2002; Munafò, Brown, & Hariri, 2008), and greater depressive symptoms following life stress (Caspi et al., 2003; Karg, Burmeister, Shedden, & Sen, 2011).

However, the interaction between 5-HTTLPR genotype and life stress has been subject to debate in recent years, with several meta-analyses providing conflicting perspectives. Two prominent meta-analyses showed no significant association between 5-HTTLPR and responsivity to life stress (Munafò, Durrant, Lewis, & Flint, 2009; Risch et al., 2009), while subsequent meta-analyses have refuted the null findings (Karg et al., 2011). This controversy underscores the challenge associated with gene-by-environment interactions, particularly when targeting single polymorphisms (Duncan & Keller, 2011). Modeling cumulative genetic influences on serotonin raises new challenges, but may be a more robust technique for identifying gene-by-environment interactions.

A cumulative genetic score (CGS) combines the risk conferred by two or more polymorphisms into a single score that can be used to describe cumulative risk. A CGS can be defined by identifying alleles associated with altered serotonergic function across several candidate genes. The total number of putative risk alleles is then used to form the CGS. Use of a CGS provides several notable advantages, including increased power compared to the small effect sizes of individual loci and the ability to model more variability in the serotonin system. The CGS technique has been used for diverse purposes, including modeling dopamine functioning (Nikolova, Ferrell, Manuck, & Hariri, 2011), predicting relapse from bupropion treatments for smoking cessation (McGeary et al., 2012), assessing risk for type 2 diabetes (Meigs et al., 2008), and predicting cardiovascular disease in women (Paynter et al., 2010).

For the current study, we are using a CGS approach to determine whether the cummulative genetic score of *HTR1A*, *HTR2A*, and 5-HTTLPR influences the relationship between mood reactivity and attention bias. These three genes, and the serotonergic functions that they influence, appear to interact in key areas of the brain. 5-HTT binding is heavily colocalized with 5-HT_{1A} and 5-HT_{2A} receptors in limbic and prefrontal regions respectively (Hawrylycz et al., 2012). Carriers of the S allele of 5-HTTLPR have been found to have decreased 5-HT_{1A} receptor binding (David et al., 2005). The *HTR2A* gene appears to moderate 5-HTT binding in key limbic regions, such as the thalamus (Laje et al., 2010). The concentration of

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5-HTT and 5-HT_{1A} binding sites in the dorsal raphe nuclei (a region central to serotonin innervation) was significantly lower in the brains of suicide victims compared to controls (Arango et al., 2001). In addition, the combined influence of *HTR1A* and 5-HTTLPR has been found to moderate the relationship between life stress and MDD (Zhang et al., 2009). These findings suggest that the 5-HTT, 5-HT_{1A}, and 5-HT_{2A} receptor subtypes may be biologically and behaviorally linked.

Considering that the relationship between mood reactivity and attentional bias has been closely associated with serotonergic receptor and transporter functioning, the present study sets out to identify how putatively functional variants in *HTR1A*, *HTR2A*, and 5-HTTLPR moderate this critical interaction. Using sad mood induction as a proxy for environmental influences in healthy individuals, we observed the relationship between genetic variation, mood reactivity, and attentional bias for mood-congruent stimuli.

We first examined whether each individual polymorphism interacts with mood reactivity to predict change in attentional bias. The genotypes were then combined into a CGS to determine whether this multilocus approach provided greater explanatory power when predicting gene-by-environment interactions compared to a single polymorphism approach.

We hypothesized that individuals at greater genetic risk (i.e., higher CGS) will spend more time attending to negative stimuli and less time attending to positive stimuli as a function of their mood reactivity. That is, those at highest genetic risk who experience a strong increase in negative mood should also display the strongest mood congruent attentional bias. This approach would advance our knowledge of the relationship between genes coding for serotonin receptors and 5-HTT, with a particular emphasis on multilocus approaches to understanding cognitive vulnerability to depression.

Methods

Participants

Participants were selected from a sample of 224 adults from the Austin community with no current or past history of major depressive disorder and no current history of any other Axis I disorder, as determined using the *Structured Clinical Interview for DSM-IV Diagnoses, Research Version* (SCID; First et al 2002). Because we wanted a homogenous group of healthy control participants, we also excluded individuals who had received psychiatric treatment. From the full sample, 27 were excluded for history of psychotherapy, 7 were excluded for current or past antidepressant medication use, and 3 withdrew from the study. Of the remaining participants, we were able to successfully genotype 173 for *HTR1A*, 171 for *HTR2A*, and 175 for 5-HTTLPR. There were 170 who were successfully genotyped for all three variants, and were therefore included in the CGS. The participants who were excluded did not significantly differ from the study sample in age, gender, race, education level, or household income. The 170 participants eligible for the CGS (age: mean= 28.32, SD= 8.24; 61% female) were 54% Caucasian, 21% Asian, 8% African American, 6% mixed race, 1% American Indian, and 1% Hawaiian/Pacific Islander, with 9% not endorsing an ethnicity. All genetic association studies are at possible risk of population stratification. All of the models presented below were highly consistent in a sub-sample made up entirely of

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Caucasian participants, reducing the risk that population stratification was driving the results. In addition, including race, gender, and age as covariates did not alter the outcomes of any of the models. As such, the impact of demographic factors on the present findings will not be discussed further.

Genetic sample—Genomic DNA was isolated from buccal cells and saliva using a modification of published methods (Freeman et al., 1997; Lench, Stanier, & Williamson, 1988; Meulenbelt, Droog, Trommelen, Boomsma, & Slagboom, 1995; Spitz et al., 1996). The cheeks and gums were rubbed for 20 s with three sterile, cotton-tipped wooden swabs. The swabs were placed in a 50- ml capped polypropylene tube containing lysis buffer (500 μl of 1 MTris–HCl; pH 8.0; 500 μl of 10% sodium docecyl sulfate; and 100 μl of 5 M sodium chloride). The participants then rinsed out the mouth vigorously with 10 ml of distilled water for 20 s and this was added to the 50-ml tube. Samples were stored at 4 °C until the DNA was extracted.

SNPs were genotyped using Taqman assays: *HTR1A* (rs6295) with C_ _11904666_10 and *HTR2A* (rs6311) with C_ _ 11696922_10 (both from Applied Biosystems) using an ABI 7300 Real time PCR system. The rs6295 assay genotypes the forward strand. The assay for 5-HTTLPR was a modification of that used by Lesch and colleagues (Lesch et al., 1996). The primer sequences are: forward, 5′-GGCGTTGCCGCTCTGAATGC-3′ (fluorescently labeled), and reverse, 5′-GAGGGACTGAGCTGGACAACCAC-3′ with yield products of 484 or 528 bp. To distinguish between the S, L_A, and L_G fragments, the PCR fragment was digested with MspI by methods described in Wigg et al (Wigg et al., 2006). Consistent with standard convention, the L_G fragment was treated as equivalent to S in all subsequent analyses (Hu et al., 2005; Zalsman et al., 2006). Allele sizes are scored by two investigators independently and inconsistencies were reviewed and rerun when necessary.

All three polymorphisms were in Hardy-Weinberg equilibrium (HWE), both for the total sample and also when stratified by race. Pearson chi-square tests and exact tests (i.e., for the multiallelic 5-HTTLPR) showed no significant difference from the population for 5-HTTLPR (total sample: p=0.596, Caucasian sub-sample: p=0.860, African-American sub-sample: p=0.620, Asian sub-sample: p=0.376), *HTR1A* (total sample: p=0.970, Caucasian sub-sample: p=0.760, African-American sub-sample: p=0.279, Asian sub-sample: p=0.620), or *HTR2A* (total sample: p=0.051, Caucasian sub-sample: p=0.224, African-American sub-sample: p=0.638, Asian sub-sample: p=0.38). Multiracial participants and participants who did not provide racial information were only included in total sample HWE analyses.

Profile of Mood States (POMS): Sad mood was measured using four descriptors taken from the POMS (McNair, Lorr, & Droppleman, 1992). These descriptors included items with the best factor loadings for the depression mood scale: “sad”, “worthless”, “blue”, and “hopeless”. Participants used a 5-point Likert scale to determine how well each descriptor could be used to describe their current mood (ranging from “0 = not at all” to “4 = very much”). These values were summed to yield an index of sad mood at each time point. The current study used POMS data collected before and immediately after the sad mood induction. Internal consistency of POMS scores before and after the mood induction ranged from good to excellent (Cronbach’s alpha pre = 0.93, post = 0.82). Mood reactivity was

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operationalized as the difference in sad mood across the two time points (i.e. the change in mood as a function of the sad mood induction).

Sad mood induction: Participants were randomized to undergo a sad mood induction using either a film clip or a combination of sad music and autobiographical memories. The film induction was a brief, standardized clip that has been shown to specifically elicit mild and transient sadness (Gross & Levenson, 1995). The clip is a 170 second long scene from the film *The Champ*, in which a young boy learns that his father has died following a severe beating in a boxing match. The clip was presented digitally on a 20-inch LCD computer monitor. For the music/memory induction, participants were asked to imagine a time in their life when they were very sad, while simultaneously listening to Samuel Barber's *Adagio for Strings* through headphones. This technique has also been shown to elicit mild and transient sadness (W. Van der Does, 2002). Multiple sad mood induction techniques were used to ensure that any effects were the result of sad mood, and not simply a response to specific induction procedures. For participants whose mood did not return to baseline levels after study participation, a positive mood induction was administered. All participants were offered the opportunity to speak with a doctoral level clinician following the positive mood induction, and community treatment referrals were made available to all participants.

Passive Viewing / Eye tracking paradigm: Before and after the sad mood induction, participants completed a passive viewing paradigm using images selected from the International Affective Picture System (IAPS; Lang, Bradley, & Cuthbert, 2005). The IAPS consists of well standardized pictures that are frequently used for research on psychopathology. IAPS pictures are systematically rated for valence, and are given a score ranging from 1 (unpleasant) to 9 (pleasant). Forty eight images were divided equally into four emotion categories: dysphoric, threat, positive, and neutral[1]. Positive images were rated from 6 to 8, neutral images were rated approximately 5, and dysphoric and threat images were rated from 2 to 4.

In order to differentiate dysphoric from threat images, a pilot study was conducted using 90 undergraduate students at the University of Texas at Austin. Participants were presented with one of the 24 images previously identified as dysphoric or threat in random order and were asked to rate how "sad" and how "threatening" each image was. As expected, the dysphoric images were rated as significantly more sad than threat images, $t(89)=13.4$, $p<0.001$, and the threat images were rated as significantly more threatening than the dysphoric images, $t(89)=-21.6$, $p<0.001$.

Four IAPS images (one from each category) were displayed simultaneously in four quadrants of a 20 inch LCD computer monitor (see Figure 1). Emotion valences were randomly assigned to each quadrant with equal frequency. Gaze location and duration was assessed using a remote optics eye tracking system model R6 from Applied Science Laboratories (Bedford, Massachusetts, USA). Gaze coordinates were sampled every 16.7

[1]The following IAPS images were used: dysphoric – 2141, 2205, 2276, 2455, 2700, 2703, 2799, 2900, 3230, 9220, 9421, and 9530; threat – 1120, 1300, 2811, 3500, 6260, 6312, 6313, 6350, 6510, 6560, 6562, and 6821; positive – 1340, 2091, 2165, 2208, 2224, 2299, 2339, 2340, 2501, 4599, 4700, and 8461; neutral – 2038, 2102, 2393, 2397, 2745, 2850, 5500, 5731, 7009, 7041, 7080, and 7185.

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milliseconds (60Hz), producing 1,796 gaze location measurements for each 30-second trial. Fixations were defined as any period of 100ms or longer where eye movements were stable within 1° of visual angle. To calculate each participant's total fixation duration, the duration of time spent fixating on each valence was summed for each trial and averaged across trials within each session. Using this technique, each participant generated four total fixation duration scores, reflecting the total time spent fixating on each of the four stimulus valences averaged across all trials. Total fixation duration has been reliably used to assess sustained attention (e.g. (Disner et al., 2013; Green, Williams, & Davidson, 2003; Horley, Williams, Gonsalvez, & Gordon, 2004)).

The use of eye tracking to assess attentional bias provides several advantages over alternative attention measurements that rely on reaction time or self-report. Eye tracking using the passive viewing task captures more sustained and effortful viewing patterns without relying on behavioral response time, which can be influenced by factors besides attention (Hermans, Vansteenwegen, & Eelen, 2013). Sustained attention for affective stimuli has been identified as an important marker of emotion regulation (Todd, Cunningham, Anderson, & Thompson, 2012), suggesting that eye tracking with the passive viewing task would be a natural choice to assess the influences of the sad mood induction.

Procedures: Eligible participants underwent all study procedures at the Mood Disorders Laboratory located at the University of Texas at Austin. After providing informed consent, participants completed the baseline assessment, which included collection of the genetic sample, the POMS, and the passive viewing eye tracking task. Participants were than randomized to either the film or music sad mood induction, which was immediately followed by completing the POMS and the passive viewing task for the second time.

Statistical analysis: Statistical analyses were conducted using Stata 12 (StataCorp, College Station, TX, USA). Preliminary analyses were broken down into two phases. The first phase modeled *HTR1A*, *HTR2A*, and 5-HTTLPR in separate analyses using a Bonferroni correction, a conservative approach to accounting for multiple comparisons. The second phase examined the influence of the serotonin CGS. For all analyses, the dependent variable was total fixation duration following mood induction and covariates were total fixation duration prior to mood induction and type of mood induction. Independent variables were stimulus emotion (sad, positive, threat, and neutral), mean-centered mood reactivity (i.e. change in POMS score following sad mood induction), and mean-centered genetic influence (i.e. total number of risk alleles). The main analyses used mixed effects regression (with random intercepts) to explore the three-way interaction between genetic influence (i.e., genotype group or CGS), mood reactivity, and stimuli valence (i.e. positive, negative, threat, or neutral). Mixed effects regression is ideally suited to analyzing autocorrelated, within-person data in combination with between-person data, as it takes into account the nested data structure and resulting dependencies (Bryk & Raudenbush, 1987).

Statistically significant three-way interactions were followed-up using non-parametric robust regression techniques to observe the two-way interaction of gene influence on mood reactivity across each of the four emotion valences. Robust regression was used to better account for highly influential participants. This technique eliminates any observation with

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Cook's D greater than 1 and then utilizes iterative Huber and biweight estimation to converge on the best model. Based on this technique, 6 participants were excluded from the final model.

Results

Mood induction

Participants were randomized to undergo a sad mood induction using either a short film clip or a combination of sad music and autobiographical memory prompt. Overall, participants' POMS scores increased by 1.65 points (SD = 1.96) following sad mood induction, representing a 36% increase in sad mood. For the participants who watched the short film clip, POMS scores increased by an average of 1.31 points (SD = 1.69), or 29.8% versus 1.99 points (SD = 2.15), or 41.6%, for participants who completed the sad music and autobiographical memory induction. Because the sad music and autobiographical memory prompt was significantly more effective at increasing sad mood than the short film clip, $t(168) = -2.28$, $p=0.01$, all subsequent analyses controlled for the type of mood induction by including induction type as a factor in all lower level interactions (Keller, 2013).

Gaze fixation for emotion stimuli

Individual polymorphisms—The 175 participants genotyped for 5-HTTLPR were broken down into the following groups: S/S (n=54), S/L (n=86), and L/L (n=35). The 173 participants genotyped for *HTR1A* were broken down into the following groups: C/C (n=48), C/G (n=87), and G/G (n=38). The 171 participants genotyped for *HTR2A* were broken down into the following groups: C/C (n=117), C/T (n=53), and T/T (n=1).

Three separate mixed effects regression models were conducted. For 5-HTTLPR and *HTR1A*, models estimated the relationship between mood reactivity, emotion valence, and the additive effect of each additional risk allele. For *HTR2A*, the same interaction was calculated comparing carriers of the T allele to the C/C group. All analyses used a Bonferonni correction to determine significance level (i.e., $\alpha = .05/3 = .0167$) to account for multiple tests across the three SNPs. The model including the *HTR2A* polymorphism showed a marginal three-way interaction between mood reactivity, emotion valence, and genotype, $F(3, 171) = 3.00$, $p = 0.0293$, Bonferroni-corrected $p = 0.0879$. None of the other three-way interactions approached statistical significance (See Table 1). In addition, reactivity to the sad mood induction did not vary as a function of 5-HTTLPR, $\beta = 0.218$, $SE = 0.168$, $t(172)$, $p = 0.198$, *HTR1A*, $\beta = 0.071$, $SE = 0.197$, $t(170)$, $p = 0.717$, or *HTR2A*, $\beta = 0.147$, $SE = 0.209$, $t(168)$, $p = 0.635$.

CGS—The CGS was constructed using *HTR1A*, *HTR2A*, and 5-HTTLPR risk alleles identified in previous research. The selection of risk alleles was corroborated by the directionality of the individual gene models. The number of risk alleles based on this CGS were normally distributed (Shapiro-Wilk $W > 0.99$) and are distributed as follows: 1 risk allele (N=1), 2 risk alleles (N=22), 3 risk alleles (N=40), 4 risk alleles (N=58), 5 risk alleles (N=36), and 6 risk alleles (N=13) (see Table 2 for CGS construction, including references pertaining to direction of risk).

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The mixed effect regression model estimating the three way interaction between mood reactivity, emotion valence, and CGS was significant, $F(3, 167) = 5.04$, $p = 0.0017$, Bonferroni-corrected $p = 0.0068$[2]. Non-parametric robust regression models were used to examine the interaction between CGS and mood reactivity across each valence. The CGS by mood reactivity interaction was significant for dysphoric stimuli, $\beta = 0.173$, $SE = 0.066$, $F(1, 164) = 6.78$, $p = 0.0101$, Bonferroni-corrected $p = 0.0404$. Further, estimated marginal effects indicated that the CGS significantly predicted post-induction fixation for dysphoric stimuli for participants whose POMS score increased by at least 1.91 points. For positive stimuli, the effect was inverted but also significant, $\beta = -0.208$, $SE = 0.075$, $F(1, 164) = 7.58$, $p = 0.0066$, Bonferroni-corrected $p = 0.0264$. CGS significantly predicted post-induction fixation for positive stimuli for participants whose POMS score either increased by at least 2.43 points or decreased by at least 1.89 points[3].

These preliminary results indicate that individuals with higher CGS who were more reactive to mood induction spent more time gazing at sad stimuli and less time gazing at happy stimuli compared to similarly reactive individuals with lower CGS. There was no CGS by mood reactivity interaction for either neutral stimuli ($p = 0.2223$) or threat stimuli ($p = 0.7572$) (see Figure 2), suggesting that genetic variation was not associated with attention for these stimuli[4]. CGS did not independently predict mood reactivity, $F(1, 168) = 1.31$, $p=0.255$; nor did CGS predict fixation change as a function of stimulus valence, $F(3, 166) = 1.08$, $p=0.355$. In addition, mood reactivity did not predict fixation change as a function of stimulus valence independently from genetic influence, $F(3, 172)=0.10$, $p=0.961$.

The robust regression models including the CGS explained a greater percentage of the variance towards dysphoric and positive images compared to the same models using individual gene variants. The model including CGS explained 36.0% of the variance for attention to dysphoric stimuli, which was 2.6% more variance explained than the model using 5-HTTLPR, 1.3% greater than *HTR1A*, and 1.6% greater than *HTR2A*. The model including CGS explained 37.1% of the variance for attention to positive stimuli, which was 1.7% more variance explained than the model using 5-HTTLPR, 1.7% greater than *HTR1A*, and 1.6% greater than *HTR2A*.

Discussion

This study uses a cumulative genetic approach to expand on the biological underpinnings of cognitive vulnerability for depression. In particular, 5-HTT, 5-HT_{1A} and 5-HT_{2A} receptors have been linked to sensitivity to the environment and cognitive biases (Canli & Lesch, 2007; Cools et al., 2008; Hariri & Holmes, 2006; Owens & Nemeroff, 1994). The

[2]This finding was replicated in a sub-sample containing only non-Hispanic Caucasian participants, $F(3, 88) = 3.85$, $p = 0.0091$, Bonferroni-corrected $p = 0.0364$.

[3]Although the region of significance test for the two-way interaction for positive stimuli was significant for reactivity values lower than −1.89, it should be interpreted with great caution as only one participant yielded a negative reactivity score (i.e. a decrease in sad mood following the sad mood induction).

[4]In addition to the three variants presented here, additional analyses were conducted using three additional candidate genes that were genotyped in this sample for other purposes: two polymorphisms coding for tryptophan hydroxylase 2 (*TPH2*: rs7305115 and rs1386494) and the 10 repeat variant in intron 2 of the serotonin transporter gene (STin). None of the three additional candidate genes independently interacted with valence and mood reactivity. A CGS constructed using the alleles from all six SNPs did not significantly interact with valence and mood reactivity, $F(3, 165) = 1.34$, $p= 0.26$.

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cumulative genetic approach allows us to expand on that literature by identifying a multilocus genetic profile that putatively influences serotonin signaling. We developed a CGS comprised of putative functional polymorphisms in *SLC6A4*, *HTR1A*, and *HTR2A* with a consistent direction of effects with regard to environmental reactivity that models the cumulative genetic influence on attention bias as a function of mood reactivity. Healthy individuals with a higher CGS (reflecting a greater number of risk alleles) spent more time attending to dysphoric images and less time looking at positive images following an environmental manipulation that induced a sad mood. Specifically, individuals at highest genetic risk induced into a sad mood were especially likely to display a sustained attentional bias for mood congruent stimuli and not attend to positive stimuli. Conversely, individuals with a lower CGS but comparably high mood reactivity spent less time attending to dysphoric images and more time looking at positive images, suggesting increased cognitive resilience.

This finding is among the first to identify a gene-by-environment interaction using CGS rather than individual candidate genes. These three polymorphisms combine to serve as a putative diathesis, jointly interacting with reactivity to a sad mood induction to predict mood-congruent attentional bias, a key endophenotype for the development of mood disorders. Importantly, this effect was observed in healthy controls without any history of depression or psychiatric treatment, a fairly restrictive sample. This suggests that the observed CGS-by-environment interaction likely precedes the development of a mood disorder, rather than stemming from one, reinforcing its putative role as a cognitive risk factor.

The current findings support the growing literature in favor of multilocus approaches in behavioral and psychiatric genetics. The CGS approach yielded a statistically significant interaction, whereas none of the individual polymorphisms approached significance following correction for multiple comparisons. In the case of the 5-HTTLPR polymorphism, these findings are inconsistent with prior work examining attention bias (Beevers et al., 2009; Beevers, Ellis, Wells, & McGeary, 2010). In addition, CGS models accounted for 1.3 – 2.6% more variance than individual variants alone (a comparatively large difference in genomic research). Even a relatively simple CGS, such as the one presented here, can account for some of the background genetic influence that may have contributed to inconsistent findings in individual gene analyses. Indeed, new techniques utilizing a polygenic approach for estimating genetic influence on phenotypes are emerging and could be utilized in future research (A. J. Holmes et al., 2012; Purcell et al., 2009). This finding could serve as a springboard for more a comprehensive, systems-level biological approach to modeling cognitive vulnerability, as is called for by the Research Domain Criteria (RDoC) of the National Institute of Mental Health (Insel et al., 2010).

Individuals with a greater serotonin CGS appear to have an attentional system that is more easily influenced by the environment. This is consistent with prior work, which has found that serotonin transporter and receptor variation are associated with environmental sensitivity. Specifically, 5-HTTLPR and *HTR2A* (rs6311) have been previously linked with the differential susceptibility hypothesis, which posits that genetic factors moderate the extent that an individual is influenced by his environment (Belsky & Pluess, 2009). The link

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between higher CGS and increased environmental influence on attention is consistent with existing examples of differential susceptibility, such as individual differences in response to attention bias modification associated with 5-HTTLPR (Fox, Zougkou, Ridgewell, & Garner, 2011).

To further examine the association between the serotonin CGS and differential susceptibility, future research could induce positive mood to see if the opposite results are observed (i.e. increased attention for positive stimuli and decreased attention for negative stimuli). Doing so would more clearly establish the serotonin CGS as an attentional plasticity factor, reflecting an individual's responsiveness to environmental influences. Identifying factors that predict greater response to positive influences, such as psychotherapy, is a useful and growing area of research (Beevers & McGeary, 2012; Eley et al., 2012; Pluess & Belsky, 2013). Modeling serotonin influence using a similar CGS approach could add to this field, and may help to identify potential biomarkers to predict improved outcomes from positive environmental interventions.

The additive risk conferred by combining 5-HTTLPR, *HTR1A*, and *HTR2A* into a single CGS merits further consideration about gene effects at the molecular level. Specifically, previous research has observed that 5-HTT, 5-HT_{1A}, and 5-HT_{2A} receptor bindings are colocalized in limbic and prefrontal areas (Arango et al., 2001; David et al., 2005; Laje et al., 2010). Changes in serotonergic functioning (and, by extension, changes to cognitive processes associated with mood reactivity) may be endophenotypes mediating the influence of the genetic factors observed in this study. Learning more about how genes interact at a molecular level will play a critical role into uncovering the combined genetic influence of risk alleles.

Relatively small sample size is arguably the most important study limitation. Replication of these preliminary findings with a significantly larger sample size is clearly warranted. In addition, measuring mood reactivity using only a sad mood induction leaves open the possibility that the present findings are driven by unspecific arousal effects. Including positive mood inductions in future studies would allow for a more comprehensive measure of mood reactivity. Finally, the polymorphisms included in the CGS were chosen to reflect the relationship between 5-HTT and 5-HT_{1A} and 5-HT_{2A} receptor function. However, the use of three candidate genes is not representative of the cumulative impact of serotonin-related genes. Future studies would benefit from more comprehensive genotyping, perhaps on a genome-wide scale.

The CGS approach is based on the assumption that all included variants interact equivalently with the environment, which is unlikely to be true. The CGS can help us understand the simplistic cumulative effect of genetic influence, but likely fails to capture the differential gene-by-environment interactions conferred by individual polymorphisms. Future research using separate samples would benefit from developing a weighted CGS, where the weights assigned to each variant used in the CGS are determined by the first sample (Purcell et al., 2009). This technique is likely to be more sensitive than the current approach. However, obtaining two large samples for phenotypes that are time intensive and difficult to obtain,

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such as eye-tracking, requires substantial resources and often multi-site collaborations. Nevertheless, this is an important future direction for this area of research.

The predictive ability of the multilocus CGS comprised of 5-HTTLPR, *HTR1A* (rs6295), and *HTR2A* (rs6311) will hopefully foster future attempts to identify cumulative genetic factors underlying cognitive vulnerability to depression. In addition to serotonin, MDD has been linked with abnormal expression of numerous neurotransmitters, including dopamine and norepinephrine (Nutt, 2008), suggesting that there may be many genes, or combinations of genes, that confer cognitive vulnerability. Still, the identification of three serotonergic polymorphisms that together can be used to assess vulnerability for maladaptive cognition in healthy participants is a critical step forward in the literature. By identifying biological factors that exacerbate the effects of the environment, we can facilitate a better understanding of the factors that contribute to the onset and maintenance of MDD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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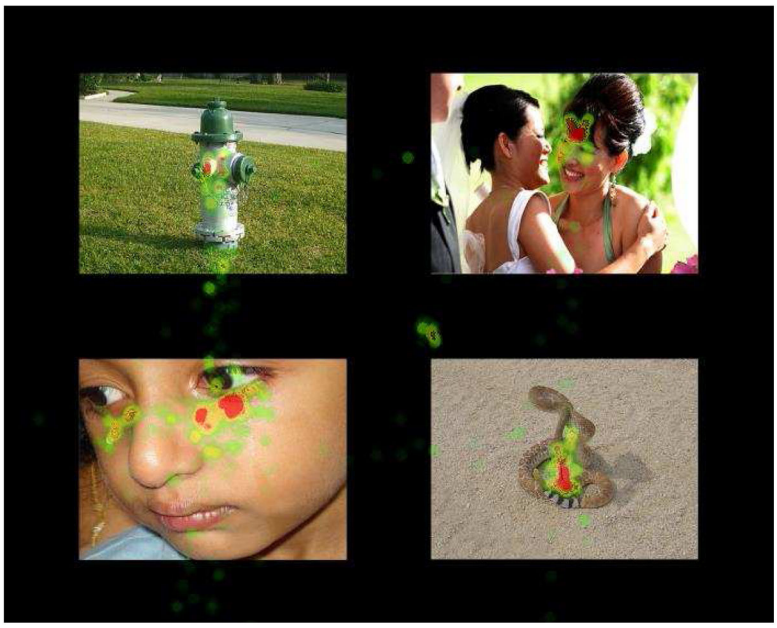

Figure 1.

An example of stimuli used in a trial of the passive viewing task. Images reflect (clockwise from top right) positive, threat, dysphoric, and neutral emotion categories. The images are overlaid with a heat map reflecting gaze fixation across a single trial, collected using eye tracking technology. Warmer colors in the heat map reflect locations of greater sustained attention across the trial. Note: the images displayed here are similar to those used in the study, but were not selected from the IAPS library due to copyright restrictions.

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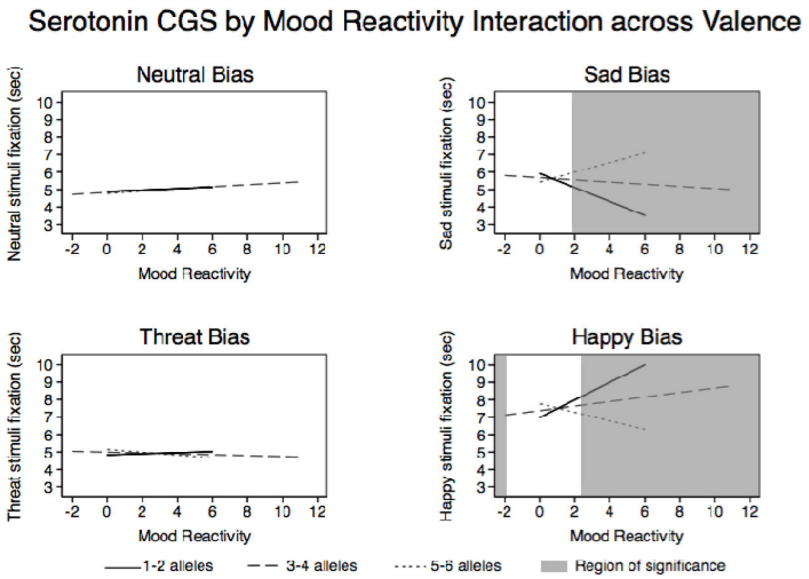

Figure 2.

The three-way interaction of CGS by mood reactivity by emotion category is broken down into four two-way interactions for each emotion valence. There was a significant interaction such that individuals with higher CGS who experience greater mood reactivity spent more time fixating on dysphoric images (Bonferroni corrected $p = 0.0404$) and less time fixating on positive images (Bonferroni corrected $p = 0.0264$). For dysphoric images, the influence of CGS on total fixation duration was significant for participants whose reactivity was 1.91 or greater. For positive images, the influence of CGS on total fixation duration was significant for participants whose reactivity was either greater than 2.43 or less than −1.89. There was no such gene by environment interaction for neutral or threat images. Note that CGS alleles were collapsed into three groups to facilitate interpretation of figure 2. However, CGS alleles were analyzed as a continuous variable in all models.

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Table 1

The three-way interaction between mood reactivity, emotion category, and each of the individual SNPs is listed below. Individual SNP models were Bonferroni corrected to account for multiple comparisons. None of the three corrected models reached statistical significance, though the *HTR2A* came closest. However, the CGS, which combines the genetic influence of 5-HTTLPR, *HTR1A*, and *HTR2A*, yielded a statistically significant three-way interaction (see Figure 2).

Genotype	rs	df	F	Uncorrected p	Bonferroni Corrected p
HTR1A	rs6295	3, 170	1.55	0.2000	0.6000
HTR2A	rs6311	3, 168	3.00	0.0293	0.0879
5-HTTLPR	-	3, 172	0.77	0.5092	>0.99
CGS	-	***3, 167***	***5.04***	***0.0017***	***0.0068***

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Table 2

The CGS was calculated by counting risk alleles from the 5-HTTLPR, *HTR1A*, and *HTR2A* polymorphisms. The alleles used to code for genetic risk, along with examples of the literature used to justify the risk designation, is presented below.

Genotype	Score = 2	Score = 1	Score = 0	References
5-HTTLPR	S/S	S/L	L/L	(Canli & Lesch, 2007; Caspi et al., 2003; Karg et al., 2011)
***HTR1A* (rs6295)**	C/C	C/G	G/G	(Wang et al., 2009; Zhang et al., 2009)
***HTR2A* (rs6311)**	C/C	C/T	T/T	(Du et al., 2000; Giegling et al., 2006)