Effects of Panic Symptoms and Problematic Alcohol Use on

Sensitivity to Unpredictable Threat

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DISSERTATION

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LIST OF ABBREVIATIONS

PD	Panic Disorder
U-threat	Unpredictable Threat
AD	Alcohol Dependence
RSA	Respiratory Sinus Arrhythmia
P-threat	Predictable Threat
EMG	Electromyography
DSM	Diagnostic and Statistical Manual of Mental Disorders
APA	American Psychiatric Association
AUD	Alcohol Use Disorder
BNST	Bed Nucleus of the Stria Terminalis
N	No-threat
NPU	No-Predictable-Unpredictable
PTSD	Post-Traumatic Stress Disorder
BAC	Blood Alcohol Concentration
PFC	Prefrontal Cortex
fMRI	Functional Magnetic Resonance Imaging
OFC	Orbitofrontal Cortex
HPA	Hypothalamic-Pituitary-Adrenal
SCID	Structured Clinical Interview for DSM
RDoC	Research Domain Criteria
EKG	Electrocardiogram
SD	Standard Deviation
SE	Standard Error
IDAS	Inventory of Depression and Anxiety Symptoms
CD	Countdown
ISI	Interstimulus Interval
EEG	Electroencephalography

SUMMARY

Individuals with panic disorder (PD) may engage in alcohol use because it effectively dampens their anticipatory anxiety about unpredictable, future panic attacks (i.e., threat). Heightened sensitivity to unpredictable threat (U-threat) may also contribute to risk for problematic drinking and differentiate individuals with PD and comorbid alcohol dependence (AD) from PD-only. To date, the independent effect of problematic alcohol use on reactivity to U-threat is unknown and it is unclear whether prior findings are specific to discrete diagnostic constructs or symptomatology more broadly. Moreover, there is evidence to suggest that resting respiratory sinus arrhythmia (RSA) may be a mediator underlying the association between PD and/or AD and responsiveness to unpredictable threat. The aims of the current study were therefore to examine the unique and interactive effects of panic symptoms and problematic alcohol use (i.e., binge drinking) on startle potentiation and startle responding over time during predictable (P-) and U-threat, and whether resting RSA mediates the associations between panic symptoms/alcohol binges and threat responding. A total of 134 individuals, recruited from the community, completed assessments of panic disorder symptoms and current binge drinking, and resting levels of RSA were collected. Aversive reactivity and responding was measured using a well-validated electromyography (EMG) startle potentiation threat-of-shock paradigm. Results indicated that binge drinking was associated with greater initial startle reactivity and average startle potentiation to U-threat, but not P-threat. Binge drinking also interacted with current panic symptoms such that for those who had no recent binges, elevated panic symptoms were associated with elevated average startle potentiation and less of a decline in startle responding over time during U-threat, and lower levels of resting RSA. In contrast, for those who had recent binge episodes, greater panic symptoms were associated with less initial reactivity to both forms of threat, less average startle potentiation and a typical (or adaptive) decline in startle responding

during U-threat. These results suggest that problematic alcohol use does exert important

independent effects on sensitivity to U-threat, and also significantly alters the association

between panic symptoms and threat responding. The present findings could also suggest that

anxious individuals that select to engage in problematic alcohol use are meaningfully different

from anxious individuals that do not engage in problematic alcohol use.

1. INTRODUCTION

Epidemiological data indicate that concurrent diagnoses of multiple mental disorders within the same individual occur more often than would be expected by chance (Clark, Watson, & Reynolds, 1995; Kessler et al., 1994; Kessler, Chiu, Demler, & Walters, 2005; Widiger & Sankis, 2000). For example, the National Comorbidity Survey (Kessler et al., 2005) reported that more than 40% of 12-month disorders defined by the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV; American Psychiatric Association [APA], 1994) were comorbid. Earlier reports using DSM-III defined disorders have reported similar rates (Kessler et al., 1994; Robins & Reiger, 1991). Comorbidity is therefore considered the norm, rather than the exception, and this has called into question the validity of the current psychiatric nosology (Insel et al., 2010; Krueger & Piasecki, 2002). Importantly, the clinical implications of comorbidity extend far beyond classification issues as evidence suggests that comorbid conditions are associated with a more chronic course, worse prognosis, and poorer treatment outcomes (Blanco et al., 2013; Brown & Barlow, 1992; Newman, Moffit, Caspi, Silva, 1998; Schneier et al., 2010). As such, comorbidity represents a serious public health issue and research examining potential explanations for mental disorder co-occurrence is critical for the development of targeted prevention and intervention techniques.

Two disorders that commonly co-occur are panic disorder (PD) and alcohol use disorders (AUDs) – particularly alcohol dependence (AD; Grant et al., 2004; Kessler et al., 2006; Kushner, Abrams, & Borchardt, 2000). Evidence indicates that approximately 4-12% of adults with AD have a lifetime diagnosis of PD (Kessler et al., 1997; Schneider et al., 2001), and 25-37% of adults with PD have a lifetime diagnosis of AD (Kessler et al., 2006; Otto et al., 1992). Each disorder in isolation produces deleterious consequences (Kessler et al., 2006; Mokdad, Marks, Stroup, & Gerberding, 2004); however, individuals with both PD and AD experience a more

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pernicious course including greater impairment and disability, high rates of service utilization and suicide, and poor treatment outcomes (Chambless, Cherney, Caputo, & Rheinstein, 1987; Kessler et al., 1997; Kushner et al., 2005; Kushner, Donahue, Frye, Book, & Randall, 2007; Regier et al., 1990). Surprisingly, very little is known about the processes that contribute to the co-occurrence of PD-AD despite the fact that the negative consequences of the two disorders have been well-documented. Consistent with the broader comorbidity literature, there is a need for research aimed at identifying potential factors that may lead to the co-occurrence of PD-AD (and AUDs more broadly) in an effort to better understand basic processes that could ultimately lead to the identification of treatment markers and the development of more effective interventions.

1.1 Heightened Reactivity to Unpredictable Threat as a Mechanism of Panic Disorder

PD is characterized by periods of intense fear (i.e., panic attacks) and anxiety (i.e., anxious apprehension between panic attacks; APA, 1994, 2013; Barlow, 2000). More specifically, individuals with PD experience recurrent, unexpected panic attacks - defined as discrete episodes of intense fear accompanied by cognitive (e.g., thoughts of loss of control) and physical symptoms (e.g., racing heart, shortness of breath). Panic attacks are followed by at least one month of persistent concern about having additional attacks, worry about the consequences of attacks, and/or by significant changes in behavior in response to the attacks (APA, 2013). One influential theory of PD posits that after experiencing an initial panic attack, individuals progress to full-blown PD via a process in which anticipatory anxiety regarding the uncertainty of the timing of the next attack increases the likelihood of additional attacks, precipitating disorder onset (Başoğlu et al., 1994; Bouton, Mineka, & Barlow, 2001). Exaggerated anticipatory anxiety

related to the unpredictability of future panic attacks is therefore considered a core dysfunction in PD.

The role of unpredictability in PD underscores an important distinction between predictable and unpredictable aversive events. A growing body of literature suggests that predictable and unpredictable threat elicit qualitatively distinct aversive states, such that predictable aversive stimuli elicit a phasic response to an identifiable stimulus (labeled *fear*), while unpredictable aversive stimuli elicit a generalized feeling of apprehension not associated with a clearly identifiable source (labeled *anxiety*; Davis, 1998; Barlow, 2000).

These responses have been shown to be pharmacologically distinct (Grillon et al., 2006; Grillon et al., 2011) and mediated by overlapping, but separable, neural circuits (Alvarez, Chen, Bodurka, Kaplan, & Grillon, 2011; Davis, 2006). For instance, the amygdala is implicated in both fear and anxiety responses; however, fear is more related to the central nucleus of the amygdala and anxiety is more related to the bed nucleus of the stria terminalis (BNST; Davis, 1998; Davis et al., 2010; Walker & Davis, 2008).

In order to distinguish between predictable and unpredictable threat in the laboratory, the no-predictable-unpredictable (NPU)-threat paradigm was developed by Grillon and colleagues (Grillon, Baas, Lissek, Smith, & Milstein, 2004; Grillon et al., 2008; Schmitz, & Grillon, 2012). The task consists of three within-subjects conditions: 1) no threat (N; subjects are safe from threat), 2) predictable threat (P; threat is signaled by a discrete cue), and 3) unpredictable threat (U; threat is not signaled). During the task, startle eyeblinks in response to probes (e.g., short bursts of white noise) are recorded as indices of aversive motivation/responding. Notably, the startle response has been shown to be reliability potentiated during aversive motivational states (Bradley, Cuthbert, & Lang, 1999; Lang, 1995) and is sensitive to changes in emotional valence

(Lang, Bradley, & Cuthbert, 1990). An additional novel aspect of the task is that it can be translated from human to animal studies and vice versa (Davis, 1998, 2006).

Using the NPU-threat paradigm, laboratory investigations have shown that relative to healthy controls, patients with PD display greater startle potentiation during unpredictable threat (Grillon et al., 2008; Shankman et al., 2013). A similar pattern of results has been found in individuals with posttraumatic stress disorder (PTSD; Grillon et al., 2009) – a disorder which is also characterized by sustained heighted arousal (APA, 1994). In addition, an efficacious treatment for PD (i.e., benzodiazepines) has been shown to reduce startle potentiation to unpredictable, but not predictable, threat (Grillon et al., 2006). Therefore, PD is associated with heightened responsiveness to unpredictable threat and this tendency can be accurately measured in the laboratory.

1.2 Heightened Reactivity to Unpredictable Threat as a Mechanism for Alcohol Use

Numerous theoretical models of AUDs suggest that the reduction or avoidance or anxiety is a major motive for excessive alcohol use. For instance, the negative reinforcement model of addiction (Baker, Piper, McCarthy, Majeskie, & Fiore, 2004), self-medication hypothesis (Khantzian, 1997), tension reduction hypothesis (Greeley & Oei, 1999), and stress-response-dampening model (Sher & Levenson, 1982) all theorize that alcohol intoxication provides relief from stress and anxiety, which negatively reinforces consumption. Over time, the reliance on alcohol as a means of avoidance-based coping is thought to contribute to the onset and worsening of AUDs (Kassel, Jackson, & Unrod, 2000; Robinson, Sareen, Cox, & Bolton, 2011; Schroder & Perrine, 2007).

Over the past several decades, empirical findings have provided support for the role of negative reinforcement processes in AUDs. Survey data indicate that individuals frequently use

alcohol to ameliorate negative affect (Bibb & Chambless, 1986; Robinson, Sareen, Cox, & Bolton, 2009) and report that alcohol effectively reduces anxiety (Cox, Swinson, Shulman, Kuch, & Reichman 1993). Anxiety symptoms have also been shown to increase the risk of AUD onset (Zimmermann et al., 2003) and predict relapse in abstinent drinkers (Kushner et al., 2005; Willinger et al., 2002). Even in rodents, anxiety promotes alcohol consumption (Le et al., 1998; Overstreet et al., 2007). It is important to highlight, however, that not all studies have found that alcohol has stress-dampening properties (e.g., Greeley & Oei, 1999; Sutker, Allain, Brantley, & Randall, 1982), suggesting that alcohol may only be anxiolytic for certain individuals under certain environmental conditions. For example, some studies have found that the relation between negative affect and alcohol consumption is stronger among men than women and among individuals with higher positive alcohol expectancies (Armeli et al., 2000; Cooper, Russell, Skinner, Frone, & Mudar, 1992).

Recently, Curtin and colleagues have conducted a series of studies clarifying the environmental conditions by which alcohol is anxiolytic by demonstrating that acute alcohol intoxication dampens startle reactivity to unpredictable, but not predictable, threat (Hachiya, Moberg, & Curtin, 2010; Hefner & Curtin, 2012; Hefner et al., 2010; Moberg & Curtin, 2009). For instance, in a sample of healthy adults, Moberg and Curtin (2009) found that alcohol intoxication (blood alcohol concentration [BAC] of approximately 0.08%) significantly reduced startle potentiation during cues signaling unpredictable shock, but not during cues that signaled predictable shock. Moreover, this pattern of results was mediated by vigilance, such that alcohol impaired attentional processes which in-turn dampened startle potentiation to unpredictable threat. Expanding on these findings, Hefner and Curtin (2012) found that alcohol intoxication resulted in significantly greater reductions in startle potentiation in response to electric shocks

that were 20% and 60% predictable (i.e., fairly unpredictable) relative to shocks that were 100% predictable. Thus, as threat uncertainty increased, startle potentiation decreased during alcohol intoxication. In an examination of alcohol's dose-dependent changes in startle potentiation during threat of low, moderate, or high intensity electric shock administration, Moberg and colleagues (2011) also confirmed that as blood alcohol concentration (BAC) increased, startle potentiation decreased. Moreover, this effect was moderated by threat intensity, such that alcohol dampened startle potentiation to the greatest extent during threat of high intensity shocks.

Taken together, alcohol appears to selectively ameliorate responses to unpredictable, intense aversive events. It has therefore been postulated that as threat cues become more ambiguous, anxiety is elicited and the motivation to effectively alleviate this aversive state with alcohol is instated. If an individual is particularly sensitive to unpredictable aversiveness they may be especially motivated to use alcohol to dampen their distress. Indeed, in support of this hypothesis, a recent study found that individual differences in intolerance of uncertainty were positively associated with coping-related drinking motives in a sample of undergraduate college students (Kraemer, McLeish, & O'Bryan, 2015).

To date, the precise mechanisms underlying alcohol's ability to dampen affective responding during unpredictable threat remain unclear. Given that unpredictable threat responding is mediated via activation of corticotropin releasing factor (CRF) and norepinephrine sensitive pathways in the extended amygdala (see Davis et al., 2010 for a review), it is thought that alcohol may target this circuit and subsequently reduce amygdala reactivity to threat (Sripada, Angstadt, McNamara, King, & Phan, 2011). Gorka and colleagues (2013a) expanded on these hypotheses by examining the neural effects of alcohol on functional interactions between the amygdala and regions of the prefrontal cortex (PFC) during the processing of threat

stimuli using pharmaco-functional magnetic resonance imaging (fMRI). Findings indicated that acute alcohol intoxication resulted in reduced functional connectively between the amygdala and right orbitofrontal cortex (OFC). These results are noteworthy given that the amygdala and OFC interact to interpret and evaluate affective stimuli (Murrary & Izquierdo, 2007), such that the amygdala detects affective valence and forwards this assessment to the OFC to make decisions regarding goal-directed behavior (Bechara, Damasio, & Damasio, 2000; Blair, 2007). Thus, reciprocal interactions between the amygdala and OFC are necessary to determine threat value and create an affective and behavioral reaction (Ghashghaei, Hilgetag, & Barbas, 2007; Price, 2003). Since alcohol may disrupt perception of threat salience via reductions in amygdala-OFC connectivity, it is possible that during acute intoxication, individuals are unable to decode ambiguous/uncertain threat cues resulting in reduced negative affect and anxious responding (e.g., scanning the environment for threatening cues, hypervigilance). In contrast, predictable/unambiguous threat cues do not require decoding, and thus, threat perception and subsequent fear responding are relatively unaffected by alcohol.

1.3 Reactivity to Unpredictable Threat in Comorbid PD and AD

Given that a major motivational basis for the engagement in alcohol use is the reduction or avoidance of aversive anxiety states, one could speculate that individuals with PD may engage in alcohol use to reduce their heightened anticipatory anxiety about the unpredictability of future panic attacks. However, not all individuals with PD use alcohol or develop AUDs. It is therefore possible that only individuals with PD who also have high reactivity (or sensitivity) to unpredictable threat engage in problematic alcohol use as a means to alleviate their heightened affective responding. As a preliminary test of this question, Gorka et al. (2013b) examined differences in startle potentiation to predictable and unpredictable threat-of-shock using the

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NPU-threat paradigm in three diagnostic groups: 1) current PD and remitted AD (PD-AD), 2) current PD but no lifetime diagnosis of AD (PD-only), and 3) no lifetime diagnosis of PD or AD (controls). Results indicated that relative to controls and individuals with PD-only, those with PD-AD displayed heightened startle potentiation to unpredictable, but not predictable, threat. These findings provide evidence to suggest that reactivity to unpredictable threat may promote excessive alcohol use among individuals with PD and thus contribute to PD-AD comorbidity. These results may also suggest that heightened reactivity to unpredictable threat is a risk factor for PD-AD onset.

An important alternative explanation for the findings from Gorka et al. (2013b) is that heightened reactivity to unpredictable threat is a consequence (rather than a risk factor) of problematic alcohol use. A large body of evidence indicates that chronic alcohol use leads to pervasive, cumulative neuroadaptations across several neurotransmission systems that mediate emotional responding (Clapp et al., 2008; Koob, 2003; Koob and LeMoal, 1997, 2001; Roberto et al., 2006), resulting in increased anxiety and altered functioning of the mesolimbic reward system (Breese et al., 2011; Sinha et al., 2009). More specifically, data suggest that alcohol dependent individuals exhibit dysfunctional hypothalamic-pituitary-adrenal (HPA) axis and mesocorticolimbic activity, high expression of CRF and glucocorticoids, and a chronic internal stress state (see Ward, 2008). It has also been suggested that the anxiety and negative affect during alcohol withdrawal states/sobriety is due to decreases in the function of the extended amygdala coupled with increased recruitment of stress neurocircuitry (Koob, 2006). Importantly, these effects persist beyond acute withdrawal phases, such that repeated alcohol exposure leads to chronic expression of alcohol withdrawal symptoms (Zhang et al., 2007).

As would be expected from these findings, data indicate that alcohol withdrawal can exacerbate panic symptoms (George et al., 1990; Kushner et al., 1990) and aversive affective responding (Cosci et al., 2007). Therefore, while acute alcohol consumption is anxiolytic (Abrams, Kushner, Medina, & Voight, 2001; Moberg & Curtin, 2009; Sripada et al., 2011), prolonged alcohol use is anxiogenic and consequently exacerbates anxiety psychopathology (Bonassoli, Milani, H., de Oliveira, 2011; Santucci, Cortes, Bettica, & Cortes, 2008). Anxiety and alcohol use are also thought be to mutually reinforcing as individuals with anxiety disorders are more sensitive to the physiological sensations of withdrawal, which could in-turn increase rates of drinking and cause a repetitive cycle between anxiety symptoms and alcohol intoxication (Johnston et al., 1991; Schuckit et al., 1995). Therefore, it is possible that the findings from Gorka et al. (2013b) are due to an interaction between comorbid individuals' AD symptoms and PD symptoms. In other words, regardless of whether onset of AD or PD came first, concurrent AD and PD symptoms may increase reactivity to unpredictable threat.

1.4 Key Questions Remaining in the Literature

Although the findings from Gorka et al. (2013b) suggest that heightened reactivity to unpredictable threat may be an important factor that differentiates comorbids from non-comorbids, several key questions remain. First, it is unclear whether heightened reactivity to unpredictable threat is a risk factor and/or an acquired factor of comorbidity. Both of these explanations would have significant, yet different, public health implications. For instance, if heightened reactivity to unpredictable threat is a risk factor, in-order to prevent onset of AUDs, existing PD treatments may benefit by targeting the mechanisms or neural structures underlying sensitivity to unpredictable threat (e.g., extended amygdala; Davis et al., 2010). Alternatively, if heightened reactivity to unpredictable threat is a consequence of prolonged alcohol use, targeting

sensitivity to unpredictable threat within the context of AUD treatments may disrupt the feed-forward cycle between alcohol withdrawal and anticipatory anxiety, which maintains alcohol use. Given these potentially valuable prevention and treatment implications, it is critical that research continue to elucidate the relationships between PD, alcohol use, and reactivity to unpredictable threat.

One of the first steps in addressing the gaps within this literature is to examine the effects of alcohol, in the absence of anxiety psychopathology (or at least low levels of anxiety), on reactivity to unpredictable threat. As Gorka et al. (2013b) did not include an AD group without PD (i.e., AD-only), the unique effect of problematic alcohol use on threat responding is currently unknown. It is possible that during periods of sobriety, repeated problematic alcohol use in-and-of-itself increases reactivity to unpredictable threat via mesocorticolimbic neuroadapations and dysregulation of affective systems (Koob, 2003; Koob et al., 2004). Problematic alcohol use may therefore have a main effect on startle potentiation to unpredictable threat that is greater than or equal to the main effect of PD. Although less likely, it is also possible that the effects of acute alcohol consumption may persist beyond intoxication states such that individuals that engage in heavy alcohol use display dampened startle potentiation to unpredictable threat during sobriety (e.g., Moberg & Curtin, 2009). This would indicate a main effect of problematic alcohol use, but in the opposite direction of the main effect of PD. Importantly, clarifying the unique effects of alcohol use would in-turn shed light on whether the observed effect of PD-AD comorbidity on startle potentiation to unpredictable threat is accounted for by the effects of an AD diagnosis, an additive effect of PD and frequent alcohol use, or is the result of an interaction between PD and AD psychopathology. This clarification is extremely important for interpreting the clinical implications of comorbid individuals' sensitivity to unpredictable threat.

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A second key question is whether heightened reactivity to unpredictable threat is specific to DSM-IV defined constructs (i.e., PD and/or AUDs). The current system for classifying mental disorders via the DSM-IV is categorical, such that disorders are considered discrete categories that are defined by unique criteria. Patients are typically interviewed using a semi-structured diagnostic instrument (e.g., the Structured Clinical Interview for DSM [SCID]; First, Spitzer, Gibbon, & Williams, 2002) and a trained clinician determines whether the symptoms of a disorder are present or absent. If the interviewer determines that the patient has a particular number of symptoms, within a specified time-frame, greater than or equal to the diagnostic criteria defined in the DSM-IV, the patient is considered to have the disorder. In-order to enhance reliability and prevent high rates of comorbidity, the system also includes an elaborate set of hierarchical exclusion and differential diagnosis rules. For example, an individual is not deemed to have two separate categorical diagnoses if one diagnosis is considered to be "due to" a co-occurring disorder. Importantly, these rules have successfully enhanced diagnostic reliability, as the DSM-IV has been shown to have high inter-rater reliability among clinicians (coefficients: PD: = .67, alcohol abuse/dependence = .65; Lobbestael, Leurgans, & Arntz, 2011; although see Regier et al., 2013).

As was previously discussed, however, the issue of comorbidity continues to be a major challenge to the validity of categorical diagnoses and sparking an ongoing debate in the literature on the advantages and disadvantages of a categorical diagnostic system for classifying psychopathology (Kessler, 2002; Pickles & Angold, 2003). The current categorical approach has several noteworthy benefits including high diagnostic reliability, simplified communication among researchers, clinicians and patients, precisely defined diagnostic instruments, and structured, universal teaching guidelines (see Kendell & Jablensky, 2003). There are also several

important drawbacks. First, symptoms of many disorders significantly overlap (e.g., depression and PTSD) which raises concern about the concept of categorically distinct syndromes. Second, the same mental disorder can often assume phenomenologically heterogeneous forms, such that two individuals receiving the same diagnosis can present with very different symptom profiles. Third, imposing categories on dimensional constructs leads to a loss of clinically important information (Widiger & Samuel, 2005). For instance, individuals that fail to meet the total symptom cut-off for a disorder are consider disorder-free, despite being symptomatic and clinically impaired. Relatedly, the statistical power available for hypothesis testing is significantly reduced when restricted to categorical constructs (Cohen, 1983), creating a need for large sample sizes to examine clinical relationships. Lastly, the diagnosis of mental disorders is based solely on overt clinical presentation and consequently, may not capture fundamental underlying mechanisms of dysfunction. Therefore, the categorical approach is considered to be purely descriptive, as it does not propose any underlying etiology or core dysfunction.

Given these limitations, it has been suggested that a dimensional approach to defining and diagnosing psychopathology may be more valid. Instead of determining the presence or absence of a diagnosis, patients would be rated on a quantitative dimension, whereby diagnosis-specific quantitative scores could be generated and standardized across patients. Within the anxiety and alcohol use fields, similar approaches already exist including the Hamilton Scale for Anxiety (Hamilton, 1959) and the Alcohol Use Disorders Identification Test (AUDIT; Babor et al., 2001). Consistent with this perspective, the National Institute of Mental Health (NIMH) recently put forth the Research Domain Criteria (RDoC) initiative, which seeks to be agnostic towards DSM defined categories and aims to identify transdiagnostic dimensional constructs that reflect core mechanisms of psychopathology (Insel et al., 2010; Sanislow et al., 2010). It has

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been suggested that defining groups based on dimensional domains would help address many of the limitations of the DSM-IV syndromal approach, while simultaneously enhancing understanding of the underlying neurobiology of psychopathology.

1.5 Primary Aim of the Current Study

Considering the limitations of categorical diagnoses and the enhanced regard for the RDoC initiative, the primary aim of the current study is to examine the unique and interactive effects of PD and AUD *symptomatology* (rather than diagnoses) on startle potentiation to predictable and unpredictable threat. Although sensitivity to unpredictable threat has been identified as a potential core dimensional construct underlying panic psychopathology (Shankman et al., 2013), critical questions remain as to whether such underlying dysfunction extends to AUDs and contributes to the high rates of co-occurrence between these disorders (e.g., endophenotype for PD-AUD comorbidity). Towards this end, the current study will ignore categorical cutoffs and examine: 1) the main effect of problematic alcohol use on startle potentiation to threat, 2) the main effect of PD symptoms on startle potentiation to threat, and 3) the interactive effect of PD symptoms and problematic alcohol use on startle potentiation to threat. Consistent with extant literature, it is hypothesized that greater problematic alcohol use and PD symptoms will be associated with greater startle potentiation to unpredictable, but not predictable, threat. However, these main effects will be qualified by a significant two-way interaction, such that at higher levels of problematic alcohol use, greater PD symptoms will be associated with greater startle potentiation to unpredictable threat.

1.6 Secondary Aim of Current Study

In addition to threat reactivity, PD and problematic alcohol use may impact the time course of responding during unpredictable threat. In the broader affective literature, research on

the time course of affective responding is scarce as studies typically only examine responding to a single event or collapse across events. However, emotional processes are rarely isolated incidents and deficits in the time course of affective responding may reflect different disease mechanisms (Gross, 1999; Werner & Gross, 2009). For instance, responding over time may reflect top-down inhibitory/regulatory influences whereas reactivity may reflect initial stimulus processing (Banks et al., 2007; Phan et al., 2005). Although very few studies have directly compared measures of reactivity versus responding over time, data suggest that at times, there are differences in findings across the two measures (e.g., Gorka et al., 2013c; Gorka et al., in press; Campbell et al., 2014).

Research aimed at examining both reactivity and the pattern of responding over time is needed to elucidate affective deficits associated with PD and AUD psychopathology. Our group recently demonstrated that individuals with PD display less of a reduction in startle responding over time during unpredictable threat relative to individuals without PD (Gorka et al., in press), suggesting that PD may indeed be associated with time course deficits in response to unpredictable threat. However, consistent with above, many questions remain including the effects of problematic alcohol use. The secondary aim of the current study is therefore to examine the unique and interactive effects of PD symptoms and problematic alcohol use on the time course of startle responding during predictable and unpredictable threat. Since individuals with PD and alcohol use problems have been shown to have emotion regulation deficits (Fox, Hong, & Sinha, 2008; Roth, Ehlers, Taylor, Margraf, & Agras, 1990; Tull, 2006), it is hypothesized that greater PD symptoms and problematic alcohol use will be associated with less of a reduction in startle responding over time (i.e., lack of habituation) during unpredictable, but not predictable, threat. Moreover, these main effects will be qualified by a significant time by PD

symptoms by problematic alcohol use interaction (i.e., three-way interaction), such that at higher levels of problematic alcohol use, greater PD symptoms will be associated with less of a reduction in startle responding over time during unpredictable threat.

1.7 Tertiary Aim of Current Study

The final aim of the current study is to examine a potential mechanism underlying the aforementioned hypotheses – specifically, individual differences in respiratory sinus arrhythmia (RSA). RSA is the rhythmic fluctuation of heart rate during the respiratory cycle and is considered a non-invasive measure of the extent to which the vagus nerve mediates parasympathetic influences on the heart (Porges, 1995, 1997, 2007). RSA is often conceptualized as an individual difference factor that reflects one's ability to maintain homeostasis and adaptively respond to environmental demands (Porges, 1995, 2007; Thayer & Lane, 2000). Consistent with this conceptualization, a large body of empirical evidence has demonstrated that low RSA (i.e., low level of vagal influences on the heart's pacemaker) is associated with difficulty regulating emotional states and attention (Berntson, Sarter, & Cacioppo, 1998; Demaree, Robinson, Everhart, & Schmeichel, 2004; Frazier, Strauss, & Steinhauer, 2004; Porges, Doussard-Roosevelt, & Maiti, 1994; Weinstein & Quigley, 2006).

Previous research has also repeatedly shown that anxiety disorders, including PD, are associated with low levels of RSA (see Friedman, 2007 for a review). Similarly, rodent studies have demonstrated that chronic alcohol use leads to enlarged neurons of the nucleus tractus solitarius and the dorsal vagal nucleus, which negatively impacts vagal functioning and lowers RSA (Bañuelos-Pineda et al., 1995). The adverse effects of alcohol on vagal functioning have also been replicated in humans (Ohira et al., 2009; Ryans & Howes, 2002).

It is therefore plausible that the deleterious effects of PD and problematic alcohol use on vagal functioning may be additive and/or interactive, such that individuals with greater concurrent PD symptoms and problematic alcohol use exhibit lower levels of resting RSA relative to individuals with only PD symptoms or only problematic alcohol use.

Moreover, it is possible that *because* of these increased deficits in cardiac vagal tone, individuals with concurrent symptoms display heightened reactivity and/or responding to unpredictable threat. It is hypothesized that greater PD symptoms and problematic alcohol use will be associated with lower levels of resting RSA, which will mediate the relationship between PD symptoms and problematic alcohol use on startle reactivity and/or responding over time during unpredictable threat.

Of note, Gorka and colleagues have also recently found preliminary evidence supporting this tertiary aim. First, in a sample of undergraduates, Gorka et al. (2013d) found that lower resting RSA was associated with increased startle potentiation to unpredictable, but not predictable threat-of-shock. In a separate study, Gorka et al. (2013c) found that lower RSA was associated with less reduction in startle responding over time, during a non-threatening task, in two unselected samples of undergraduates. Therefore, these studies suggest that individual differences in RSA do indeed play an important role in startle responding; however, no study to date has examined whether RSA mediates the relation between psychopathology (i.e., PD and problematic alcohol use) and startle reactivity and responding. If the current hypotheses are supported, this would suggest that vagal enhancement treatments (e.g., low dose of scopolamine; Casadei, Pipilis, Sessa, Conway, & Sleight, 1993) may be beneficial for individuals with concurrent PD and AUD symptoms, as improvements in RSA may lead to decreased responsiveness to unpredictable threat.

1.8 Summary of Aims

In sum, the three primary aims of the current study are to:

1) Examine the unique and interactive effects of PD symptoms and problematic alcohol use on startle potentiation to predictable and unpredictable threat.

2) Examine the unique and interactive effects of PD symptoms and problematic alcohol use on startle responding over time during predictable and unpredictable threat.

3) Examine the unique and interactive effects of PD symptoms and problematic alcohol use on levels of resting RSA, and whether individual differences in RSA mediate Aims 1 and 2.

2. METHODS

2.1 Participants

A total of 176 biological, sibling dyads were recruited for a larger study on familial emotional processes. Individuals were recruited via advertisements posted in the community, local clinics, and in area newspapers/websites. The advertisements were designed to target individuals with a broad range of anxiety and alcohol problems. Inclusion criteria for the larger family study included being between the ages of 18 and 30 years and having at least one biological sibling in the same age range that was also willing to participate. Exclusion criteria included a personal or family history of mania or psychosis, a medical or neurological illness that may impact psychophysiological functioning (e.g., epilepsy), an inability to read or write English, a history of serious head trauma, and left-handedness.

Although 176 sibling pairs were enrolled in the larger study, only one individual from each pair was included in the present study to prevent genetic and psychophysiological homogeneity within participants. The biological sibling included in the current study was chosen at random unless one of the siblings had missing or poor quality data that prevented them from being in the analyses and thereby necessitated the inclusion of the remaining sibling. An additional 42 individuals were excluded from the study due to technical equipment failure during the startle task ($n=21$), missing self-report data ($n=6$), and poor quality startle eyeblink data (i.e., 80% or more of the blinks in any one condition being coded as missing or non-responses [$n=3$] or an inability to visually distinguish eyeblink responses from baseline activity [$n=12$]). The final sample therefore included 134 adults.

All participants provided written informed consent after review of the protocol and all procedures were approved by the University of Illinois-Chicago Institutional Review Board. As

part of the study protocol, participants completed a set of laboratory tasks, a battery of questionnaires, and a semi-structured clinical interview. Laboratory tasks and questionnaires were administered in a counterbalanced order to eliminate potential order effects. Participants received cash as payment for participation.

2.2 Procedure

All interested participants were first screened over the phone to get an initial assessment of the inclusion/exclusion criteria. If it seemed they would quality for the study, there were invited into the laboratory and provided written informed consent. The final eligibility for the study was determined at this initial lab visit. The complete protocol took place during two sessions, separated by approximately 1 week. During Session 1, participants completed a series of diagnostic interviews including a modified SCID designed to capture dimensional assessments of psychopathology. Participants also completed a battery of self-report questionnaires to assess demographics, psychiatric symptoms, and personality constructs. Participants were told at the end of Session 1 to abstain from recreational drug use and alcohol use at least 24 hours prior to the start of Session 2. They were also told to limit their caffeine use the morning of Session 2 to ½ cup. These instructions were provided to limit the impact of drugs and alcohol on psychophysiological data collection.

Individuals that continued to meet eligibility for the study were invited to participate in Session 2. Upon arrival to the lab, all individuals were assessed for acute alcohol intoxication via a breathalyzer and tested negative. After the screening, individuals completed a 3-min resting RSA task in which they were seated, upright, with their eyes closed while electrocardiogram (EKG) data was collected. They also completed a 2.5-min habituation task during which 6 acoustic startle probes were administered. Shock electrodes were subsequently placed on

participants' non-dominant hand and a second habituation task (also with 6 probes) was administered to ensure that attachment of the shock electrodes did not significantly re-potentiate early startle responses. Afterwards, a shock work-up procedure was completed in which participants received increasing levels of shock intensity until they reached a level that they described as "highly annoying but not painful." Ideographic shock levels were used to ensure equality in perceived shock aversiveness (Rollman and Harris, 1987) and to be consistent with prior studies (e.g., Grillon et al., 2004). The maximum shock level a participant can achieve was 5 mA. The mean shock level of the current study was 1.4 mA ($SD = 0.7$).

Participants next completed the NPU-threat task (see below) during which startle eyeblink responses were collected. It is important to note that during Session 2, participants also completed 4 other tasks as part of the larger study. The total time of participation was approximately 3-4 hours for each session.

2.3 Alcohol Use

Current alcohol use was assessed during the structured clinical interview (Session 1). Specifically, participants were asked to indicate their average number of standard alcoholic beverages consumed per week (over the past month) and number of binge episodes within the past 30-days. Binge episodes were defined as having ≥5 standard drinks in one sitting for males and ≥4 drinks for females (Wechsler & Nelson, 2001). Questions were probed using a Time-Line Follow-Back technique (Sobell & Sobell, 1992) such that participants were presented with a calendar of the past 30-days and asked to indicate on what days they consumed alcohol and how many standard drinks they had.

For the current study, current problematic alcohol use was conceptualized as binge drinking, as it has been previously linked to adverse emotional, behavioral, and health-related

outcomes (Jennison, 2004). Notably, because the sample was recruited from the community rather than treatment clinics, the continuous variable of number of binge episodes within the past 30-days was skewed (2.8 [SE = 0.21]) and kurtotic (9.8 [SE = 0.41]). To correct for this distribution issue, number of binges in the past 30-days was re-coded as 0 = no binges and 1 = one or more binges.

2.4 Panic Disorder Symptoms

Current panic disorder symptoms were assessed using the Inventory of Depression and Anxiety Symptoms (IDAS; Watson et al., 2007) – a 64-item self-report measure assessing symptoms of emotional disorders during the previous two weeks. Participants are asked to respond to each item using a 5-point Likert scale ranging from 1 (*not at all)* to 5 (*extremely*) and scores are summed to create 10 subscales related to DSM-IV (APA, 2000) mood and anxiety symptoms including: suicidality, lassitude, insomnia, appetite loss, appetite gain, ill-temper, well-being, social anxiety, traumatic intrusions, and panic. The panic subscale (8 items) was used as the primary panic disorder symptom measure in the current study. Prior research has demonstrated that the IDAS has excellent psychometric properties including internal consistently, test-retest reliability, and convergent and discriminant validity (Watson et al., 2007). Reliability of the panic subscale in the current sample was good ($\alpha = 0.81$).

2.5 NPU-Threat Task

The NPU-threat task is modeled after the one developed by Grillon and colleagues (Schmitz, & Grillon, 2012) and has been previously used in our lab (Gorka et al., 2013d; Shankman et al., 2013). The task includes three within-subject conditions - no shock (N), predictable shock (P), and unpredictable shock (U). Text at the bottom of the computer monitor informs participants of the current threat condition by displaying the following information: "no

shock" (N), "shock at 1" (P), or "shock at anytime" (U). Each condition lasted 145-s, during

which a 4-s visual countdown (CD) was presented six times. The interstimulus intervals (ISIs;

i.e., time between CDs) ranged from 15 to 21-s ($M = 18.0$-s) during which only the text

describing the condition was on the screen. During the N condition, no shocks were delivered.

During the P condition, participants received a shock every time the CD reached 1. During the U

condition, shocks were administered any time during the CD or ISI. Startle probes were

presented during the CD and ISI and the time interval between a shock (and a startle probe) and

the following startle probe was always greater than 10-s to. Each condition was presented two

times in a randomized order (counterbalanced across participants; e.g., PNUPNU; UNPNPU).

All participants received 24 total electric shocks (12 in P and 12 in U) and 60 total startle probes

(20 in N, 20 in P, and 20 in U). After the task, participants reported their 'anxiety' during the

CDs and ISIs for each condition on a scale ranging from 1 (*not at all*) to 7 (*extremely*).

Participants also rated how intense, annoying, and anxiety provoking the shocks were, the degree

to which they would avoid the shocks, and how aversive they found the acoustic startle probes

on a 7-point Likert scale.

2.6 Physiological Data Collection

All stimuli were administered using PSYLAB (Contact Precision Instruments, London,

UK) and psychophysiological data was acquired using BioSemi Active Two system (BioSemi,

Amsterdam, The Netherlands). During the tasks, participants sat in an electrically-shielded,

sound-attenuated booth approximately 3.5-ft from a 19-in computer monitor where visual stimuli

were presented. Acoustic startle probes were 40-ms duration, 103-dB bursts of white noise with

near-instantaneous rise time presented binaurally through headphones. Electric shocks lasted

400-ms and were administered to the wrist of the participants' left hand.

Startle responses were recorded from two 4-mm Ag/AgCl electrodes placed over the orbicularis oculi muscle below the left eye. The ground electrode was located at the frontal pole (FPZ) of an electroencephalography (EEG) cap that participants were wearing as part of the protocol for the larger study. As per published guidelines (Blumenthal et al., 2005), one startle electrode was placed 1-cm below the pupil and the other was placed 1-cm lateral of that electrode. EKG electrodes were placed on the sternum and below the left clavicle. Startle and EKG data were collected using a bandpass filter of DC-500-Hz at a sampling rate of 2000-Hz.

2.7 Physiological Data Processing

Blinks were scored according to guidelines provided by Blumenthal et al. (2005). Data processing included applying a 28 Hz high-pass filter, rectifying, and then smoothing using a 40 Hz low-pass filter. Peak amplitude of the blink reflex was defined within 20-150-ms following the startle probe onset relative to baseline (i.e., average baseline EMG level for the 50-ms preceding the startle probe onset). Each peak was identified by software but examined by hand to ensure acceptability. Blinks were scored as non-responses if EMG activity during the 20-150-ms post-stimulus time frame did not produce a blink peak that is visually differentiated from baseline activity. Blinks were scored as missing if the baseline period was contaminated with noise, movement artifact, or if a spontaneous or voluntary blink began before minimal onset latency and thus interfered with the startle probe-elicited blink response. All presented analyses included blink amplitude (i.e., condition averages do not included non-responses); however, it is important to note that all results were identical using blink magnitude (i.e., condition averages included values of 0 for non-responses).

EKG data was initially processed using QRSTool (Allen et al., 2007) and all artifacts were identified and corrected by hand. After initial correction, inter-beat-interval (IBI) series

data were entered into CardioEdit (Brain-Body Center, Chicago, IL) for further inspection and any additional artifacts were corrected by hand. After data were processed, average RSA was calculated based on methods developed by Porges and Bohrer (1990), using CardioBatch (Brain-Body Center, University of Illinois at Chicago). Specifically, the data were resampled into 30-second epochs and the IBI of each segment was calculated by identifying and averaging the time (in miliseconds) between R-peaks. RSA was then calculated by applying the adult CardioBatch filter to the data, which extracts the variance in the 0.12-0.40Hz frequency band associated with heart-rate variability.

2.8 Data Analysis Plan

For Aim 1, we first confirmed that neither panic symptoms nor alcohol binges were significantly associated with startle amplitude during the no-threat, control conditions of the NPU task (i.e., N_{CD} and N_{ISI}; all $ps > 0.22$). We then created average startle potentiation scores (using the control conditions) for the P- and U-threat conditions to account for baseline individual differences in startle amplitude. Specifically, for P-threat, we subtracted startle amplitude during N_{CD} from P_{CD}. For U-threat, we subtracted average startle amplitude during N_{CD} and N_{ISI} from average startle amplitude during U_{CD} and U_{ISI} because both U conditions had the same meaning during the task. To then test the unique and interactive effects of panic symptoms and alcohol binges on average P- and U-threat startle potentiation, we conducted two hierarchical linear regression analyses – one for P-threat and one for U-threat. All continuous variables were first mean-centered. For both models, covariates including age and gender were entered in Step 1, the main effects of panic symptoms and alcohol binges were entered in Step 2, and the panic symptoms x alcohol binges interaction term was entered in Step 3. Any significant interactions were followed-up using a standard simple slopes approach (Aiken & West, 1991;

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Holmbeck, 2002). The dichotomous variable of alcohol binges was re-coded into two new conditional moderators to reflect "yes binges" or "no binges." Two new interaction terms were also created from these conditional moderators and *post-hoc* additional hierarchical linear regression analyses were run incorporating the aforementioned covariates, panic symptoms, the conditional moderator (i.e., yes or no binges), and the interaction term. Notably, this approach for following up interactions is better than selecting participants in a particular group as the whole sample remains in the analyses, thus increasing power.

For Aim 2, to test the unique and interactive effects of panic symptoms and alcohol binges on the time course of startle amplitude during P- and U-threat, we conducted a series of multilevel mixed growth models. Mixed growth modeling is well-suited for the current aims as it allows time to be modeled continuously, accounts for the variability in durations between startle probes, and handles missing data by weighting slope estimates by the number of observations (Goldstein, 2011). Time was coded as the second the startle probe occurred relative to the startle of the task (task onset = 0-s). There were 10 consecutive startle probes presented in each condition. The dependent variables were individual blinks during P_{CD} (i.e., P-threat) and U_{CD} (i.e., U-threat). Unlike for Aim 1, U_{CD} and U_{ISI} were not collapsed given that averaging blinks across conditions may obscure the slope of responses over time if the two conditions do not have identical time courses. With that in mind, we chose to use U_{CD} (and not U_{ISI}) to limit comparisons and match the two dependent variables on visual stimuli (i.e., a geometric shape was on the screen). Of note, potentiation scores are not used in these analyses as slopes are modeled within person, accounting for potential baseline individual differences in startle amplitude.

Continuous variables were grand-mean centered and dichotomous variables were effects

coded. Age and gender were included as covariates similar to above. Order of NPU task

conditions (e.g., PNUNPU) was dummy-coded and also included as a covariate since order of

presentation could impact the slope of responses over time. Two models were run – one for P-

threat and one for U-threat. Both models used restricted maximum likelihood (REML)

estimation an unstructured covariance matrix. Any significant two-way or three-way interactions

were followed-up using the same simples slopes approach described above. Estimated

coefficients (i.e., 'b') are reported for multilevel mixed growth effects.

In addition to slopes, multilevel mixed growth models estimate and output each

individual's intercept or "starting point." This reflects *initial* P- and U-threat reactivity, which is

a separate measure from average startle amplitude and slope of startle amplitude (Gorka et al.,

2015). Intercepts are a preferable index of reactivity relative to amplitude of the first blink given

that all blinks within the condition, rather than a single blink, are used to estimate the value. We

therefore also tested the unique and interactive effects of panic symptoms and alcohol binges on

initial P- and U-threat reactivity using hierarchical linear regression analyses. The estimated

intercepts from the P_{CD} and U_{CD} covariates-only models (i.e., gender, age, and NPU task version)

were used as separate dependent variables. Alcohol binges and current panic symptoms were

entered in Step 1 and the alcohol binges x panic symptoms interaction term was entered in Step

2.

For Aim 3, 10 individuals were missing resting RSA data due to excessive EKG artifact

or equipment failure. Thus, these 10 individuals were excluded from the analyses for Aim 3. It is

important to note that post-hoc, all analyses from Aims 1 and 2 were re-run with this reduced

sample and the results were identical. Thus, we chose to report the startle findings from the full

sample, and the RSA findings from the reduced sample. For Aim 3a, the unique and interactive effects of panic symptoms and alcohol binges on resting RSA was tested using hierarchical linear regression analyses identical to those described for Aim 1. Like all other models, significant interactions were followed-up using a simple slopes approach. If there were any significant effects of binge drinking or panic symptoms on startle potentiation (Aim 1) or the time course of startle responding (Aim 2), resting RSA was tested as a mediator.

3. RESULTS

3.1 Descriptives and Manipulation Check

57 (42.2%) individuals reported at least one alcohol binge within the past 30-days. The mean number of alcohol binges, within individuals that reported at least one, was 3.1 (SD=2.7, range = 1-14). There was no difference in current panic symptoms between bingers and non-bingers ($ps > 0.47$).

Eight participants were missing self-reported ratings post-threat task due to data collection errors. On average, participants rated the shocks as moderate to extremely intense ($M = 4.56$, $SD = 1.10$), annoying ($M = 5.59$, $SD = 1.29$), and anxiety provoking ($M = 4.73$, $SD = 1.47$), and rated that they would avoid receiving the shocks again to a high degree ($M = 5.01$, $SD = 1.54$). Of note, bingers rated the shocks as more annoying ($F[1, 127] = 8.02$, $p < 0.05$) and intense at a trend-level ($F[1, 127] = 3.66$, $p = 0.06$) than non-bingers. Current panic symptoms were unrelated to shock self-report ratings (all $ps > 0.21$).

3.2 Aim 1: Average Startle Potentiation to U- and P-threat

All results from the hierarchical linear regression analyses are presented in Table 2. For U-threat, there was a main effect of alcohol binges such that individuals who reported one or more alcohol binges within the past 30-days exhibited significantly greater startle potentiation than individuals who did not binge within the past 30-days. There was no main effect of current panic symptoms on U-threat; however, there was a significant alcohol binges x panic symptoms interaction. Follow-up simple slopes analyses indicated that within individuals that did not binge, more panic symptoms was associated with greater startle potentiation ($\beta = 0.22$, $t = 2.00$, $p < 0.05$). Within individuals that did binge, more panic symptoms was associated with less startle potentiation at a trend-level ($\beta = -0.21$, $t = -1.69$, $p = 0.09$; see Figure 1).

For P-threat, there was no main effect of alcohol binges, panic symptoms, or alcohol binges x panic symptoms interaction.

3.3 Aim 2: Time Course of Startle Potentiation to U- and P-threat

All results from the multilevel mixed growth analyses are presented in Table 3. For U-threat, there was a main effect of time such that across participants, startle potentiation decreased over time. There were no main effects of alcohol binges or panic symptoms, or any 2-way interactions with either independent variable. There was a significant time x alcohol binges x panic symptoms interaction.

To follow-up the significant 3-way interaction, using a standard simple slopes approach, we first examined the 2-way, time x panic symptoms interactions for a) those that did not binge and b) those that did binge. Results indicated that for those who had binged, the time x panic symptoms interaction was a trend ($b = -0.01$, $t = -1.61$, $p = 0.10$), whereas for those who did not binge, the time x panic symptoms interaction was non-significant ($b = 0.01$, $t = 1.18$, $p = 0.24$). Although neither two-way interaction was statistically significant, in-order to fully clarify the direction of effects of the three-way interaction, we next examined the effect of time at four levels, using four multilevel mixed models: 1) high panic symptoms and no alcohol binges, 2) high panic symptoms and yes alcohol binges, 3) low panic symptoms and no alcohol binges and 4) low panic symptoms and yes alcohol binges. At 1) high panic symptoms and no alcohol binges there was no main effect of time ($b = -0.09$, $t = -1.56$, $p = 0.12$), indicating a lack of habituation. At 2) high panic symptoms and yes alcohol binges there was a significant main effect of time ($b = -0.18$, $t = -2.74$, $p = 0.01$) such that startle decreased throughout the U-threat condition. At 3) low panic symptoms and no alcohol binges, there was also a significant main effect of time ($b = -0.16$, $t = -2.70$, $p = 0.01$) such that startle decreased throughout the U-threat

condition. At 4) low panic symptoms and yes alcohol binges, there was no main effect of time (b = -0.08, t = -1.36, p = 0.17), indicating a lack of habituation. Results from all four models are displayed in Figure 2.

For P-threat, there was a main effect of time such that across participants, startle potentiation decreased over time (see Table 3). There was no main effect of panic symptoms, main effect of alcohol binges, or any 2-way or 3-way interactions.

Analysis of the U- and P-threat estimated intercepts indicated that for U-threat, there was a positive main effect of alcohol binges on initial startle reactivity (β = 0.17, t = 1.91, $p < 0.05$). There was no main effect of panic symptoms (β = -0.02, t = -0.17, ns); however, there was a significant alcohol binges x panic symptoms interaction (β = -0.77, t = -2.98, $p < 0.05$; Figure 3). For those that did not binge, greater panic symptoms were associated with greater initial startle reactivity at a trend-level (β = 0.20, t = 1.83, p = 0.07). For those who had binged, the greater panic symptoms, the less initial startle reactivity (β = -0.30, t = -2.35, $p < 0.05$).

For P-threat initial startle reactivity, there was no main effect of alcohol binges (β = 0.11, t = 1.23, ns) or panic symptoms (β = -0.05, t = -0.63, ns). There was, however, a significant alcohol binges x panic symptoms interaction (β = -0.53, t = -2.02, $p < 0.05$) such that for those that did not binge, panic symptoms were not associated with initial startle reactivity (β = 0.10, t = 0.86, ns). For those that did binge, greater panic symptoms was associated with less initial startle reactivity (β = -0.25, t = -1.94, $p < 0.05$).

3.4 Aim 3: Resting RSA

Results from the hierarchical linear regression analysis predicting resting RSA is included in Table 2. There were no main effects of alcohol binges or panic symptoms; however, there was a significant alcohol binges x panic symptoms interaction. For those that did not binge,

more panic symptoms was associated with lower resting RSA (β = -0.28, t = 2.33, p < 0.05). For those that did binge, panic symptoms were not associated with resting RSA (β = 0.21, t = 1.58, p = 0.12; see Figure 4).

An additional component of Aim 3 was to examine whether individual differences in resting RSA mediate the findings from Aims 1 and 2. Based on the findings from Aim 1, we tested the moderated mediation model presented in Figure 5 using PROCESS – an SPSS macro for path-analysis based modeling (Hayes, 2013). Panic symptoms was specified as the independent variable, alcohol binges as the moderator of the "a" and "c" paths, resting RSA as the mediator, and average U-threat startle potentiation as the dependent variable. Results indicated that the moderated mediation model was not significant (b = -0.33, 95% CI: -1.77 - 0.69). Although the analyses supported the hypothesized "a" and "c" paths, there was no significant "b" path corresponding to no significant relation between resting RSA and startle potentiation during U-threat.

Of note, although we did not have a hypothesis about RSA mediating the effects of panic symptoms and alcohol binges on average startle potentiation to P-threat, we confirmed that the proposed moderated mediation model was not significant when startle potentiation to P-threat was specified as the dependent variable (b = -0.70, 95% CI: -2.66 – 0.08).

Based on the findings from Aim 2, we would also expect a moderated mediation model analogous to Figure 5 for the time course of startle responding during U-threat. However, because PROCESS cannot model longitudinal data, to test for moderated mediation we used an approach similar to the Baron and Kenny's (1986) method, although using multilevel modeling. Specifically, we tested the significance of a, b, c, and c' paths individually. Results indicated that

there was no significant b path as resting RSA was not associated with the time course of startle

responding during U-threat ($p > 0.24$). Thus, the moderated mediation model was not supported.

4. DISCUSSION

Prior studies suggest that panic symptoms and problematic alcohol use may be associated with a heightened sensitivity to unpredictable threat; however, no study to date has examined the unique and interactive effects of these variables on responding to unpredictable and predictable threat within the same sample, which was the primary aim of the current study. Specifically, the current study examined the impact of panic symptoms and alcohol binges (i.e., problematic alcohol use) on average startle potentiation, the time course of startle responding, and initial startle reactivity to both unpredictable and predictable threat. Resting RSA was also examined as a potential mediator between symptoms/binges and sensitivity to threat. Results indicated that there was a main effect of alcohol binges on average startle potentiation to U-threat, and initial U-threat startle reactivity, such that those that binged exhibited greater average and initial startle responding compared with those that did not binge. There were no significant main effects of current panic symptoms on any dependent variable; however, there were several significant alcohol binges x panic symptoms interactions across measures. Overall, our findings suggest that problematic alcohol use exerts an independent effect on sensitivity to unpredictable threat and moderates (or alters) the relation between current panic symptoms and responding to unpredictable, but not predictable, threat. All findings and their potential implications are discussed below.

4.1 Main Effects of Binge Drinking

As is stated above, we found that binge drinking was associated with greater startle potentiation and initial reactivity to U-threat but not P-threat. This suggests that individuals that binge drink exhibit increased anticipatory anxiety in response to unpredictable, aversive events relative to individuals that do not binge drink. Consistent with this finding, a recent fMRI study

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found that when sober, alcoholic men exhibit abnormal cortical-limbic-striatal response to high intensity, unpredictable threat relative to healthy controls, suggesting deficits in engaging affect regulatory neural circuits (Yang et al., 2013). It is therefore possible that individuals with a greater sensitivity to unpredictable threat are those that are most motivated to engage in alcohol use, as prior studies have shown that it selectively and effectively ameliorates exaggerated anticipatory anxiety (Hefner et al., 2010; Moberg & Curtin, 2009). Thus, in the current sample, sensitivity to unpredictable threat may have preceded the onset of problematic alcohol use and potentially precipitated binge drinking via negative reinforcement processes. Given the cross-sectional design of the study, however, it remains possible that binge drinking caused and/or exacerbated sensitivity to unpredictable threat and that this dysfunction maintains problematic alcohol use rather than having caused its onset. Stated another way, the current study indicates that binge drinkers exhibit a heightened sensitivity to unpredictable threat but it is unclear the extent to which this individual difference emerges pre- and/or post-problematic drinking.

Nevertheless, concurrent binge drinking and sensitivity to unpredictable threat is likely problematic as individuals experience acute relief during intoxication, which could perpetuate continued use and maintain high levels of anxiety in the absence of alcohol (Koob & Le Moal, 2008). Broadly, this implies that binge drinkers experience high levels of anticipatory anxiety when sober and then potentially "normalize" or reduce their anticipatory anxiety when intoxicated. Given the potential clinical significance of these relationships, continued research is critically needed to elucidate the associations between problematic alcohol use and sensitivity to unpredictable threat.

4.2 Interaction of Binge Drinking and Panic Symptoms on Average Startle Potentiation

In addition to a main effect of binge drinking, the current findings indicated a significant alcohol binge x panic symptoms interaction on average startle potentiation to U-threat. For those who did not binge, more panic symptoms were associated with *greater* startle potentiation. For those who had binged, more panic symptoms were associated with *less* startle potentiation (though only at a trend-level). Therefore, among individuals that do not currently binge drink we observed the expected positive association between panic symptoms and startle potentiation to U-threat. This finding is consistent with prior startle studies in those with a diagnosis of PD (Grillon et al., 2008; Shankman et al., 2013), and suggests that both continuous measures of panic symptoms and categorical diagnoses of PD are related to an increased sensitivity to unpredictable threat. As has been suggested by others, individuals with high levels of panic symptoms may experience high levels of anticipatory anxiety in response to unpredictable and irregular panic attacks, and this persistent hypervigilance may sensitize them to all forms of ambiguous or uncertain threat (Grillon et al., 2008). Notably, similar to the AUD literature, it is still presently unclear whether sensitivity to unpredictable threat is a premorbid risk factor for panic symptoms and the development of PD or if it is an acquired propensity in response to repeated unpredictable panic attacks. In light of the accumulating evidence linking panic symptoms with heightened sensitivity to unpredictable threat, it is important for future studies to not only test the extent to which it is a risk vs. acquired factor but also the potential utility of treating or targeting sensitivity to unpredictable threat within anxiety prevention and intervention efforts.

Importantly, however, among individuals that currently binge drink, the opposite association between panic symptoms and startle potentiation to U-threat was observed. This is in

contrast to our initial hypothesis that there would be a positive relation between panic symptoms and startle potentiation to U-threat across all subjects, and that the strength of the association would be stronger for those that binge drink relative to those that do not binge drink. Although unanticipated, this finding highlights that binge drinking impacts the association between panic symptoms and aversive responding, and that there may be important differences between anxious individuals that do and do not binge drink. In regards to this specific finding, it is possible that in the context of problematic drinking, which in-and-of-itself is associated with heightened sensitivity to unpredictable threat, greater panic symptoms alter the way ambiguous cues are interpreted. For instance, binge drinkers that experience high levels of panic symptoms, may not appraise threat-of-shock to be as salient or aversive as those with low levels of panic symptoms. Alternatively, binge drinking may regulate or diminish heightened anticipatory anxiety among individuals with high levels of panic symptoms and thus drinking changed the typical association between panic and response to U-threat.

This hypothesis, however, is somewhat inconsistent with the findings from Gorka et al. (2013b), which found that individuals with PD and a past diagnosis of AD exhibited significantly greater average startle potentiation to U-threat, but not P-threat, compared with individuals with PD-only and healthy controls (who did not differ). If problematic alcohol use regulated anticipatory anxiety amongst individuals with PD, we would not expect greater startle potentiation to U-threat within the comorbid group. Then again, the participants in Gorka et al. (2013b) all had a *past* diagnosis of AD and were not current heavy drinkers so it is possible that within individuals with a history of problematic drinking, the direction of the association between panic and startle potentiation to U-threat depends on whether the problematic drinking is current. Another important point in regards to the findings from Gorka et al. (2013b) is that the

study did not include an AD-only group and it was unclear whether the findings were due to the effects of AD or PD-AD comorbidity. The current study implies that these prior findings may have been due, at least in-part, to the independent effects of AD or a history of problematic alcohol use. However, additional studies are greatly needed to understand the association between panic symptoms and sensitivity to unpredictable threat within the context of problematic drinking, especially given that this finding has important implications for the understanding of both problematic drinking and panic disorder.

4.3 Interaction on Time Course of Startle Responding

The current study indicates that binge drinking and panic symptoms also impact the slope of startle responding to U-threat over time. The multilevel mixed growth analyses revealed a significant alcohol binge x panic symptoms x time interaction for U-threat but not P-threat. At high panic symptoms and no alcohol binges, and low panic symptoms and yes alcohol binges, there was no effect of time suggesting that the amplitude of startle responding remained relatively stable throughout U-threat condition. Meanwhile, at high panic symptoms and yes alcohol binges, and low panic symptoms and no alcohol binges, there was a significant negative effect of time indicating that individuals habituated to the U-threat or at least reduced their startle amplitude over time. Because habituation, defined as the gradual decrease in physiological responding to a stimulus over time (Herry et al., 2010), is considered an adaptive response to a repeatedly presented threat (Groves & Thompson, 1970; Rankin et al., 2009), the current findings indicate that two subgroups of individuals evidenced a maladaptive, or abnormal response to U-threat over time: 1) individuals with high levels of panic symptoms and no alcohol binges and 2) individuals with low panic symptoms and yes alcohol binges. The other two subgroups, however, did exhibit a decline in their startle responding and thus evidenced an

adaptive or "normal" response to U-threat. This includes: 1) individuals with high panic symptoms and yes alcohol binges and 2) individuals with low panic symptoms and no alcohol binges.

In several ways, the current findings are strikingly consistent with the average U-threat startle potentiation findings (discussed above). First, bingers with low panic symptoms displayed a maladaptive response to U-threat; in this instance, a lack of habituation. This provides further evidence that problematic alcohol use is associated with abnormal responding to unpredictable, aversive events, especially among individuals with low levels of current panic symptoms. It is possible that a lack of habituation, or a slower rate of habituation, could reflect a tendency to maintain a high state of threat vigilance over extended periods of time. The fact that this aversive, hypervigilant state is persistent could enhance motivation to engage in alcohol use, especially if these individuals have difficulties modulating anticipatory anxiety using alternative, adaptive emotion regulation strategies.

Interestingly, unlike binge drinkers with low levels of panic symptoms, binge drinkers with high levels of panic symptoms were able to decrease their startle response to U-threat over time. This again suggests that binge drinking alters the typical association between panic symptoms and response to U-threat, as high panic symptoms and no binges was associated with a lack of habituation to U-threat. Moreover, Gorka et al. (in press) found that individuals with a current diagnosis of PD exhibited less of a decline in startle responding to U-threat over time compared with individuals without a current diagnosis of PD. As was noted above, one possible interpretation of the current findings is that binge drinking ameliorates sensitivity to unpredictable threat within individuals with high levels of panic symptoms, despite the fact that among individuals with low panic symptoms binge drinking does the opposite. This pattern of

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effects undoubtedly suggests that the associations between binge drinking, panic symptoms, and sensitivity to unpredictable threat are complex and that symptoms may interact in atypical or unanticipated ways to influence responding to unpredictable, aversive events.

4.4 Interaction on Initial Startle Reactivity

Consistent with both the average startle responding and time course analyses, results of the current study indicated that there was a significant alcohol binge x panic symptoms interaction on initial startle reactivity to *both* U-threat and P-threat. Examination of initial startle reactivity was not an original aim of the current study; however, because multilevel mixed model analyses estimate and output a slope intercept or "starting point" for each individual, we chose to examine the unique and interactive effects of binge drinking and panic symptoms on this measure to try and elucidate the time course results discussed above. Consistent our other findings, we found that for those who binged, greater panic symptoms were significantly associated with less initial startle reactivity to both forms of threat. For those who had not binged, panic symptoms were positively associated with startle reactivity to U-threat, but only at a non-significant trend-level. Notably, Gorka et al. (in press) found that individuals with PD exhibited greater initial startle reactivity – also to both U- and P-threat. We speculated that PD was associated with an increased tendency to appraise all forms of threat as excessively aversive, leading to a heightened initial response to both task conditions. The current study suggests that in the context of binge drinking, this may not be the case as high panic symptoms were associated with less initial startle reactivity to U- and P-threat. Among binge drinkers, high levels of panic symptoms are therefore associated with reduced initial threat reactivity, reduced average startle responding to U-threat, and a typical decline (or habituation) to U-threat.

4.5 The Role of Resting RSA

Because prior studies suggest that anxiety and heavy alcohol use is associated with low resting RSA (Friedman, 2007; Ohira et al., 2009), and low resting RSA is associated with heightened startle potentiation to U-threat (Gorka et al., 2013d), we initially hypothesized that resting RSA would mediate the association between panic symptoms/binge drinking and sensitivity to U-threat. In order to explore these potential associations, we first examined whether panic symptoms and/or alcohol binges were significantly associated with levels of resting RSA and found a significant 2-way interaction. For those who had not binged, greater panic symptoms were associated with lower levels of resting RSA. This is consistent with numerous prior studies suggesting that individuals with current anxiety symptoms, and disorders, exhibit low levels of resting RSA (Friedman, 2007). It also dovetails with the current startle findings indicating that among those that do no binge drink, panic symptoms are associated with heightened aversive reactivity to U-threat, as low RSA is conceptualized as an indicator of difficulties regulating negative emotional states including anticipatory anxiety (Porges, 2007). In other words, when taken together, the current study suggests that individuals with high levels of panic symptoms, that do not binge drink, exhibit elevated aversive responding, that is relatively sustained over time, in response to unpredictable, aversive threats. Moreover, these individuals display evidence of difficulties regulating emotional states *at rest*, reflected in low levels of resting RSA, which could imply that more chronic or stable deficits.

Again, like the startle findings, the typical association between panic symptoms and resting RSA were not observed among individuals that binge drink. In this instance, for those who had binge drank, panic symptoms were not associated with resting RSA (though this statistical association could be considered a trend [$p=0.12$], and was in the positive, rather than

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the expected negative, direction). Thus, binge drinking does not only change the relation between panic symptoms and startle response to threat, it also changes the relation between panic symptoms and a resting-state individual difference factor associated with trait-like abilities to regulate emotions. Although the non-significant relation between panic symptoms and resting RSA among binge drinkers requires extreme caution when interpreting, this positive trend could again reflect that in the context of binge drinking, individuals with high levels of panic symptoms do not exhibit deficits considered "characteristic" of anxiety psychopathology (i.e., sensitivity to unpredictable threat, low resting RSA). As has been stated above, additional research investigating the mechanisms or factors that underlie these somewhat counter-intuitive findings are greatly needed.

Lastly, it is important to note that our hypotheses that RSA would mediate the findings from Aims 1 and 2 were not supported. First, as was briefly mentioned above, RSA was not directly associated with panic symptoms or alcohol binges, though there was a significant panic symptoms x alcohol binges interaction and a moderated mediation model was tested. More importantly, however, in the current study, individual differences in RSA were not related to average startle responding, or the time course of startle responding, to U-threat. This is in contrast to the findings from Gorka et al (2013d), and to some extent, the findings from Gorka et al. (2013c) in which lower levels of resting RSA were associated with less of a decline in startle responding during a non-threat, startle habituation task. Although the reasons for these discrepancies are unclear, it is possible that differences between the NPU-threat task versions (across the studies) could have contributed to the different effects. For example, the NPU-threat tasks administered in both Gorka et al. studies included a 5-min break between task blocks, whereas the current version did not include a break and was one, continuous block. In addition to

task differences, it is possible that unmeasured diagnostic, symptom-level, or personality factors contribute to the association between RSA and startle potentiation to U-threat and these unmeasured factors contributed to differences in findings across the studies. It will therefore be important for future studies to clarify this discrepancy by identifying potential moderators of this association.

4.6 Limitations, Conclusions, and Future Directions

Although the current study had numerous strengths including the use of a well-validated threat task and multiple startle indicators of sensitivity to unpredictable threat, there are also several limitations worth noting. First, the current sample included non-treatment seeking individuals from the community and thus, our binge drinking variable was skewed. To correct for this issue, we dichotomized binge drinking into yes/no, which likely reduced our statistical power and limited our ability to make inferences about how *severity* of problematic drinking may be related to threat responding and RSA. Related to this point, all of the individuals in the current sample were relatively young and thus had limited histories of problematic drinking. Future studies are therefore needed to test whether the present findings are similar in samples of older adults or individuals that have more extensive lifetime alcohol use. Second, although our current panic symptoms variable was normally distributed, the mean level of symptoms was low, and it is thus also unclear whether the pattern of results would generalize to samples with a greater severity of panic symptomology. It is also unknown whether the current findings are specific to panic symptoms, or if similar results would also have been found for broad measures of anxiety or other symptoms of specific anxiety disorders such as social phobia. Third, the current study was cross-section and we are therefore unable to make inferences about the directionality of the associations between binge drinking, panic symptoms, and sensitivity to threat. Future studies

are critically needed to address these gaps and continue to investigate if and how panic symptoms and problematic drinking interact to influence affective responding, cognition, and behavior.

In conclusion, results from the current study suggest that binge drinking is associated with greater initial reactivity and average startle potentiation to U-threat. Binge drinking and current panic symptoms also interact to influence responding to threat – particularly U-threat. Broadly, among individuals that do not binge drink, the characteristic associations between greater panic symptoms, heightened sensitivity to unpredictable threat, and low RSA are observed. However, among individuals that do binge drink, these "typical" associations are reversed such that the pattern of results are in the opposite direction, suggesting that greater panic symptoms in the context of binge drinking is not associated with sensitivity to threat or low RSA. This raises many questions regarding the effects of binge drinking on individuals with high levels of panic symptoms, and/or questions as to who among individuals with high levels of panic symptoms may self-select to binge drink. Given that problematic alcohol use and anxiety symptoms commonly co-occur, and comorbid individuals represent a particularly vulnerable clinical population, continued research surrounding these questions represents a very important avenue of future work.

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Table I.
Demographics and clinical characteristics

Demographics	Mean (*SD*) or %
Age (years)	22.3 (2.9)
Sex (% female)	62.2%
Race/Ethnicity	
Caucasian	41.7%
African American	12.1%
Hispanic	28.0%
Asian	11.4%
Mixed Race	3.0%
Other	3.8%
Clinical Variables	
IDAS panic total score	11.1 (4.0)
Lifetime DSM-5 Panic Disorder diagnosis	8.8%
Alcohol binge within past 30-days	42.2%
Mean drinks consumed per week	4.1 (6.5)
Lifetime DSM-5 Alcohol Use Disorder diagnosis	35.6%
IDAS depression total score	38.2 (11.8)
Daily cigarette smoker	8.1%
Used illicit drug in past 30-days (including cannabis)	25.8%
Psychophysiological Variables	
Startle potentiation during U-Threat (Amplitude)	46.7 (54.3)
Startle potentiation during P-Threat (Amplitude)	24.2 (111.2)
Startle reactivity to U-Threat (Amplitude)	168.9 (96.8)
Startle reactivity to P-Threat (Amplitude)	117.4 (63.1)
Resting RSA	6.9 (1.2)

Note. U-Threat = mean startle potentiation during U_{ISI} and U_{CD}; P-Threat = mean startle potentiation during P_{CD}; RSA = respiratory sinus arrhythmia; IDAS = Inventory of Depression and Anxiety Symptoms.

Table II. *Hierarchical linear regression analyses predicting startle potentiation to U-Threat, startle potentiation to P-Threat and resting RSA*

Variable	U-Threat					P-Threat					Resting RSA				
	β	t	p	R^2	ΔR^2	β	t	p	R^2	ΔR^2	β	t	p	R^2	ΔR^2
Step 1				<0.00					0.03					0.02	
Gender	0.02	0.19	0.85			0.13	1.49	0.14			0.11	1.16	0.25		
Age	-0.02	-0.25	0.81			0.10	1.09	0.28			-0.12	-1.37	0.18		
Step 2			0.01	0.07	0.07[*]			0.44	0.04	0.01			0.48	0.04	0.02
Panic Symptoms	0.03	0.38	0.70			-0.04	-0.46	0.65			-0.06	-0.65	0.51		
Alcohol Binge	0.27[*]	3.06	<0.01			0.11	1.22	0.23			0.10	1.05	0.29		
Step 3			0.01	0.12	0.05[*]			0.35	0.05	0.01			0.01	0.09	0.05[*]
Panic Symptoms x Alcohol Binge	-0.29[*]	-2.59	0.01			-0.11	-0.93	0.35			0.32[*]	2.74	0.01		

Note. * $p < 0.05$; U-Threat = mean startle potentiation during U_{ISI} and U_{CD}; P-Threat = mean startle potentiation during P_{CD}; RSA =

respiratory sinus arrhythmia; Panic Symptoms = Inventory of Depression and Anxiety Symptoms (IDAS) panic subscale; Alcohol Binge =

one or more alcohol binges within the past 30-days (yes/no).

Table III.

Multilevel mixed growth models examining the time course of U-Threat and P-Threat

Variable	U-Threat			P-Threat		
	b	*t*	*p*-value	*b*	*t*	*p*-value
Block 1						
Intercept	155.83*	6.06	<0.01	114.99*	3.50	<0.01
Time	-0.09*	-3.84	<0.01	-0.10*	-2.42	0.02
Age	-0.87	-0.24	0.81	1.23	0.29	0.77
Gender	8.66	0.83	0.41	21.14	1.75	0.08
Task Order	0.48	0.13	0.90	2.22	0.45	0.65
Panic Symptoms	-0.40	-0.16	0.88	-1.07	-0.35	0.72
Alcohol Binge	22.55*	2.18	0.03	15.00	1.26	0.21
Block 2						
Intercept	167.20*	5.09	<0.01	116.91*	3.38	<0.01
Age x Time	0.01	0.61	0.55	<0.01	0.16	0.87
Gender x Time	0.01	0.40	0.69	-0.01	-0.31	0.76
Task Order x Time	<0.01	0.52	0.61	<0.01	0.16	0.87
Panic Symptoms x Time	<0.01	-0.05	0.96	<0.01	-0.25	0.80
Alcohol Binge x Time	<0.01	0.07	0.94	<0.01	0.10	0.92
Block 3						
Intercept	168.11*	5.21	<0.01	117.22*	3.39	<0.01
Panic Symptoms x Alcohol Binge x Time	-0.01*	-2.50	0.03	<0.01	0.34	0.74

Table IV. Summary of significant alcohol binge x panic symptom interaction findings.

|	**Greater Panic Symptoms**	**Lower Panic Symptoms**
Yes Alcohol Binge	1) Less average startle potentiation to U-threat (trend); 2) Less initial startle reactivity to U-threat; 3) Less initial startle reactivity to P-threat	1) Less of a decline in U-threat startle responding over time
No Alcohol Binge	1) Greater average startle potentiation to U-threat; 2) Greater initial startle reactivity to U-threat (trend); 3) Less of a decline in U-threat startle responding over time; 4) Lower resting RSA	

Figure 1.
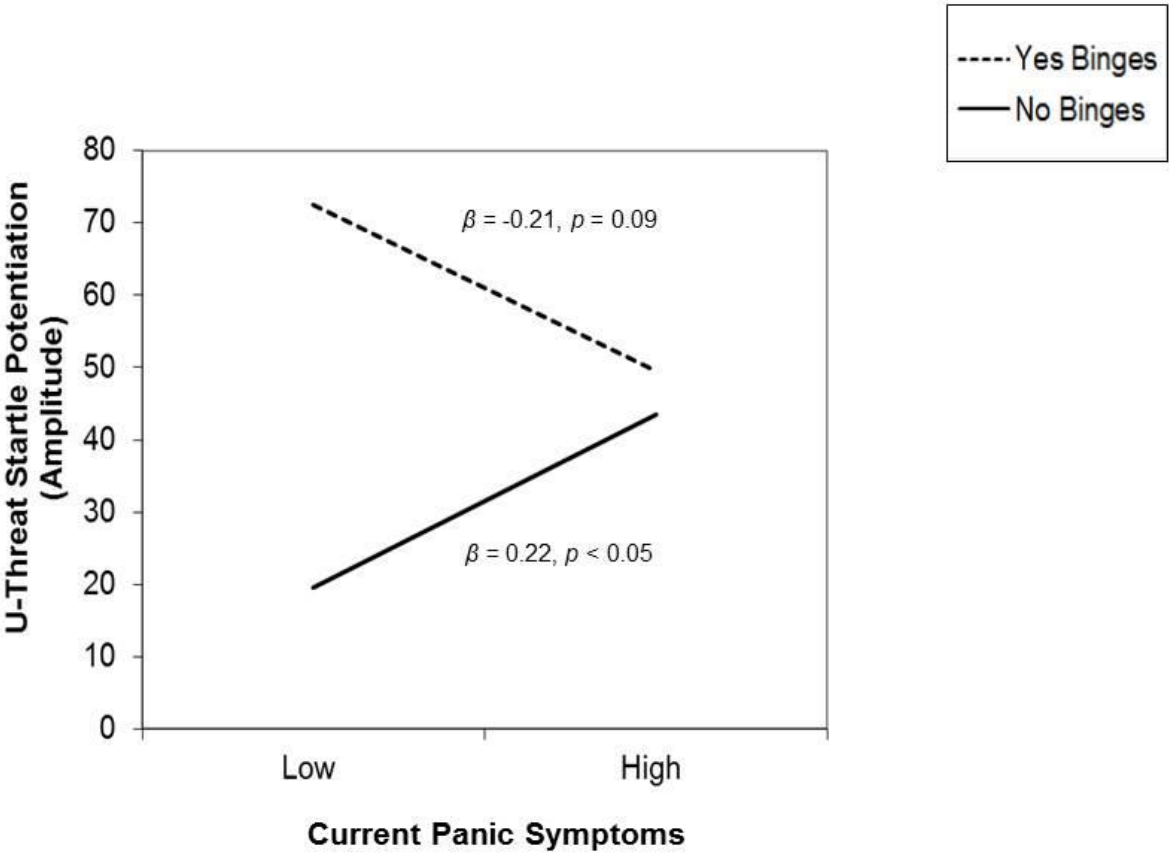
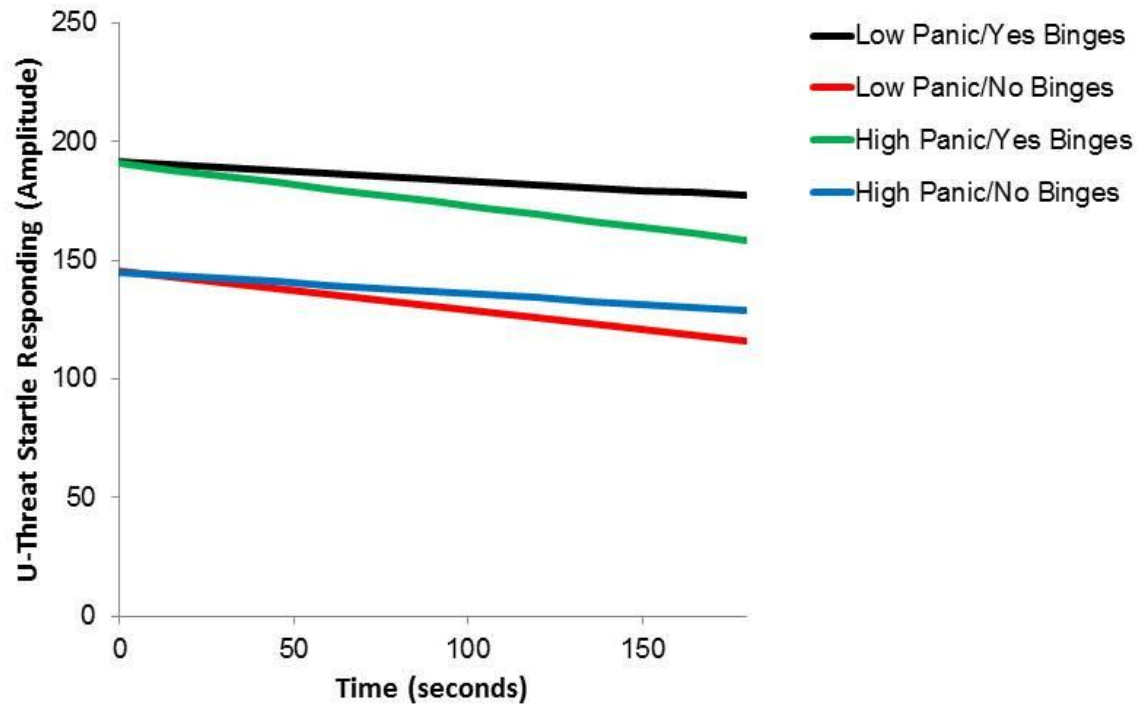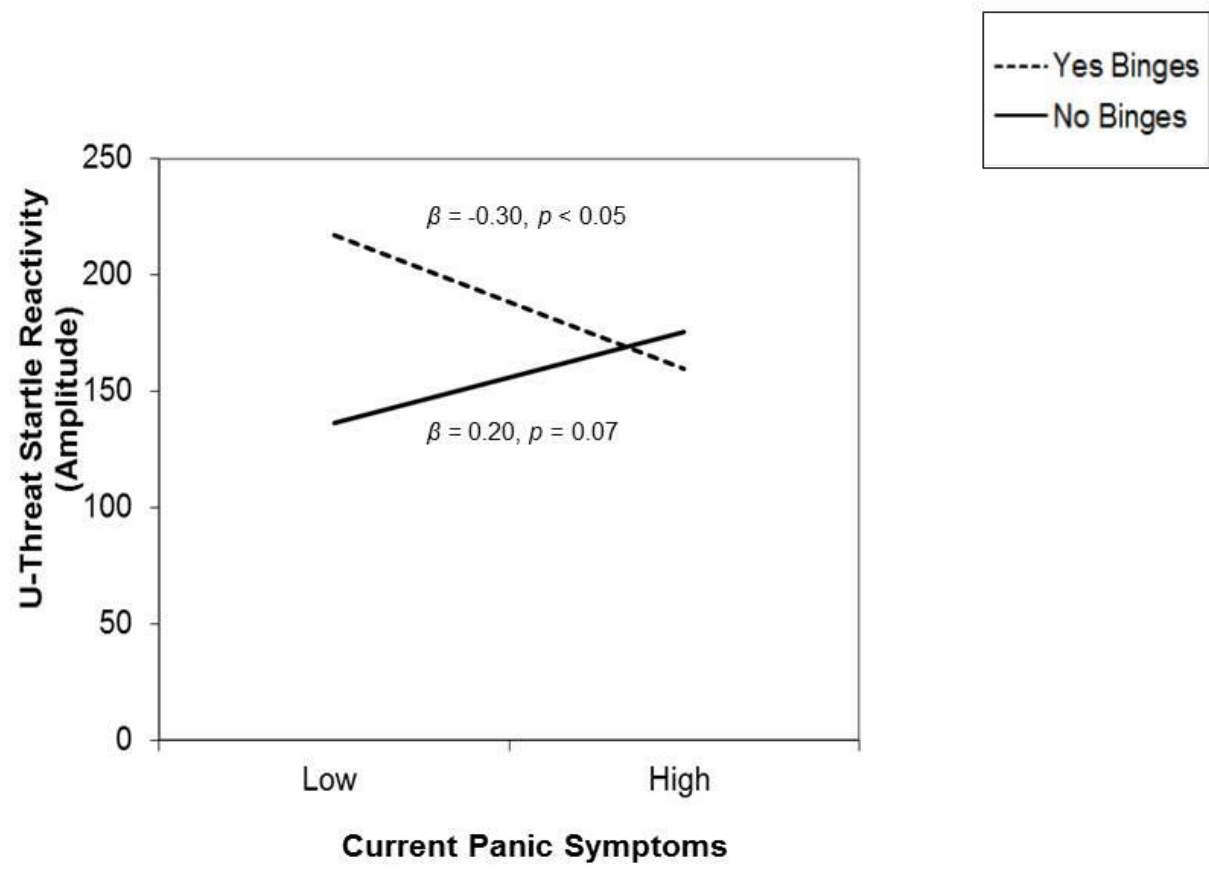**Figure 2.**

Figure 3.

Figure 4.

Figure 5.