FKBP5 Polymorphisms Influence Pre-Learning Stress-Induced Alterations of Learning and Memory

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Abstract

FK506 binding protein 51(FKBP5) is a co-chaperone of heat shock protein 90 and significantly influences glucocorticoid receptor sensitivity. Single nucleotide polymorphisms (SNPs) in the *FKBP5* gene are associated with altered hypothalamus-pituitary-adrenal (HPA) axis function, changes in the structure and function of several cognitive brain areas, and increased susceptibility to post-traumatic stress disorder, major depression, bipolar disorder, and suicidal events. The mechanisms underlying these associations are largely unknown, but it has been speculated that the influence of these SNPs on emotional memory systems may play a role. In the present study, 112 participants were exposed to the socially evaluated cold pressor test (stress) or control (no stress) conditions immediately prior to learning a list of 42 words. Participant memory was assessed immediately after learning (free recall) and 24 h later (free recall and recognition). Participants provided a saliva sample that enabled the genotyping of three *FKBP5* polymorphisms: rs1360780, rs3800373, and rs9296158. Results showed that stress impaired immediate recall in risk allele carriers. More importantly, stress enhanced long-term recall and recognition memory in non-carriers of the risk alleles, effects that were completely absent in risk allele carriers. Follow-up analyses revealed that memory performance was correlated with salivary cortisol levels in non-carriers, but not in carriers. These findings suggest that *FKBP5* risk allele carriers may possess a sensitized stress response system, perhaps specifically for stress-induced changes in corticosteroid levels, which might aid our understanding of how SNPs in the *FKBP5* gene confer increased risk for stress-related psychological disorders and their related phenotypes.

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Graphical Abstract

Exposure to brief stress immediately before learning enhanced long-term memory in non-carriers, but not carriers, of minor alleles for three different *FKBP5* polymorphisms. These findings suggest that carriers of such "risk" alleles for *FKBP5* may possess a sensitized stress response system, which might aid in our understanding of how SNPs in the *FKBP5* gene confer increased risk for stress-related psychological disorders.
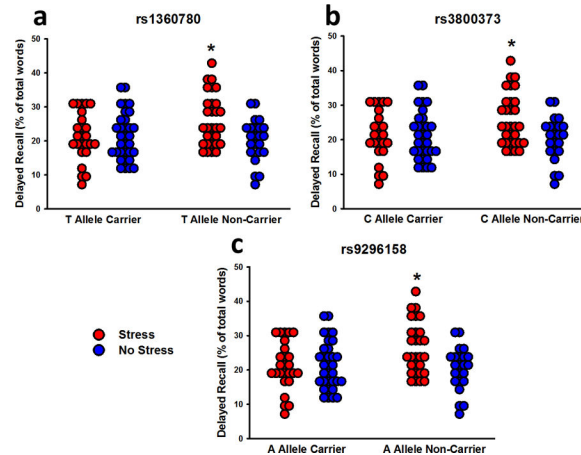
Keywords

stress; cortisol; learning; memory; FKBP5

INTRODUCTION

The effects of stress on learning and memory are profound, yet complex. Research over the past several decades has shown that stress can enhance, impair, or have no effect on learning and memory, depending on several factors (e.g., sex, stage of learning/memory affected by stress, emotional nature of the learned information, etc.) (Diamond *et al.*, 2007; Joels *et al.*, 2011; Schwabe *et al.*, 2012). It has been well-documented that post-learning stress enhances long-term memory consolidation and pre-retrieval stress impairs recall, both effects being attributable to an interaction between corticosteroid and noradrenergic mechanisms in the amygdala and hippocampus (Roozendaal *et al.*, 2009). Pre-learning stress effects on long-term memory are not as well understood and are more inconsistent in the literature. One factor that has emerged in the past decade as a major determinant of pre-learning stress effects on long-term memory is the timing of stress relative to learning (Diamond *et al.*, 2007; Joels *et al.*, 2011; Schwabe *et al.*, 2012). When a brief stressor is administered immediately before learning, long-term memory is generally enhanced (e.g., Diamond *et al.*, 2007; Zoladz *et al.*, 2011; Quaedflieg *et al.*, 2013; Zoladz *et al.*, 2014b; Vogel & Schwabe, 2016). However, when the same stressor is temporally separated from learning (e.g., 30 min before learning), long-term memory is generally impaired (e.g., Zoladz *et al.*, 2011; Quaedflieg *et al.*, 2013; Zoladz *et al.*, 2013). Investigators have contended that these time-dependent effects of pre-learning stress are attributable to a biphasic influence of stress-induced amygdala activation on hippocampal synaptic plasticity, as well as the temporal

profiles of stress-induced noradrenergic and corticosteroid activity (Akirav & Richter-Levin, 2002; Diamond *et al.*, 2007; Joels *et al.*, 2011; Schwabe *et al.*, 2012). Specifically, brief stress experienced immediately before learning enhances long-term memory via the rapid increase in norepinephrine and non-genomic effects of slowly rising corticosteroids exerting excitatory influences on hippocampal synaptic plasticity. In contrast, stress that is temporally separated from learning results in long-term memory impairment due to, at least in part, rising corticosteroid levels exerting gene-dependent, inhibitory influences on hippocampal function.

Our laboratory has been using the pre-learning stress model as a method for better understanding susceptibility factors for stress-induced alterations of learning and memory (Zoladz *et al.*, 2011; Zoladz *et al.*, 2013; Zoladz *et al.*, 2014a; Zoladz *et al.*, 2014b). Because stress-induced alterations of learning and memory are associated with multiple psychological disorders, developing a better understanding of susceptibility factors for stress-induced enhancements or impairments of learning and memory may lend important insight into the mechanisms underlying such illnesses. One susceptibility factor that has garnered a significant amount of attention is the *FKBP5* gene, which codes for FK506 binding protein 51 (FKBP5). FKBP5 is a co-chaperone of heat shock protein 90, which binds to the glucocorticoid receptor (GR). This complex reduces GR nuclear translocation and sensitivity to corticosteroids, thus resulting in reduced negative feedback inhibition of the hypothalamus-pituitary-adrenal (HPA) axis (Binder, 2009). Research over the past two decades has shown that single nucleotide polymorphisms (SNPs) in the *FKBP5* gene are associated with greater risk for PTSD (Binder *et al.*, 2008; Xie *et al.*, 2010; Boscarino *et al.*, 2011; Mehta *et al.*, 2011; Sarapas *et al.*, 2011; Watkins *et al.*, 2016), major depression (Zobel *et al.*, 2010; Appel *et al.*, 2011), bipolar disorder (Willour *et al.*, 2009), and suicidal events (Brent *et al.*, 2010; Roy *et al.*, 2010; Menke *et al.*, 2013), particularly upon interaction with environmental risk factors, such as early life stress (Binder *et al.*, 2008; Roy *et al.*, 2010; Xie *et al.*, 2010; Appel *et al.*, 2011; Watkins *et al.*, 2016). The mechanism by which these SNPs confer increased susceptibility to such illnesses is still largely unknown. However, researchers have shown that these SNPs are associated with altered HPA axis function (e.g., changes in GR sensitivity, dexamethasone-induced suppression of cortisol, recovery of stress-induced increases in cortisol) and significant changes in the volume and connectivity of several cognitive brain areas (e.g., hippocampus, amygdala, frontal cortex), which are characteristic features of stress-related psychological disorders (Binder *et al.*, 2008; Ising *et al.*, 2008; Zobel *et al.*, 2010; Mehta *et al.*, 2011; Sarapas *et al.*, 2011; Fani *et al.*, 2013; Menke *et al.*, 2013; Fujii *et al.*, 2014a; Fujii *et al.*, 2014c; Fani *et al.*, 2016; Hirakawa *et al.*, 2016).

Given the well-established role of the HPA axis, hippocampus, amygdala, and frontal cortex in stress-memory interactions and the association between stress-memory interactions and stress-related psychological disorders, we predicted that SNPs in the *FKBP5* gene might influence how stress affects learning and memory. Investigators have speculated that *FKBP5* polymorphisms promote sensitization of the stress response, thus influencing emotional memory formation (Binder *et al.*, 2008; Binder, 2009; Fani *et al.*, 2013; Cheung & Bryant, 2015; Holz *et al.*, 2015), and some have shown that carriers of *FKBP5* polymorphisms demonstrate an attentional bias to threat (Fani *et al.*, 2013), greater amygdala responses to

emotional stimuli (White *et al.*, 2012; Holz *et al.*, 2015), higher levels of intrusive memories in a laboratory setting (Cheung & Bryant, 2015), and impaired cognition in aged individuals (Fujii *et al.*, 2014b). Thus, we exposed participants to brief stress immediately before learning and hypothesized that three SNPs in the *FKBP5* gene, chosen from previous research (Binder *et al.*, 2008), might prevent the commonly observed stress-induced enhancement of long-term memory via an exaggerated physiological stress response.

MATERIALS AND METHODS

Participants

One hundred and twelve healthy undergraduate students (44 males, 68 females; age: $M =$ 19.79, $SD = 1.59$), predominantly Caucasian (85%), from Ohio Northern University volunteered to participate in the experiment. Individuals were excluded from participating if they met any of the following conditions: diagnosis of Raynaud's or peripheral vascular disease; presence of skin diseases, such as psoriasis, eczema or scleroderma; history of syncope or vasovagal response to stress; history of any heart condition or cardiovascular issues (e.g., high blood pressure); history of severe head injury; current treatment with psychotropic medications, narcotics, beta-blockers, steroids or any other medication that was deemed to significantly affect central nervous or endocrine system function; mental or substance use disorder; regular use of recreational drugs; regular nightshift work. Participants were asked to refrain from drinking alcohol or exercising extensively for 24 h prior to the experimental sessions and to refrain from eating or drinking anything but water for 2 h prior to the experimental sessions. Participants were awarded class credit and $20 cash upon completion of the study. All of the methods for the experiment were undertaken with the understanding and written consent of each participant, carried out in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board at Ohio Northern University; all experimental procedures took place between 1000 and 1700 hours. The overall sample size was based on previous work reporting physiological / behavioral effects associated with the minor alleles for the *FKBP5* polymorphisms (e.g., Ising *et al.*, 2008; Menke *et al.*, 2013; Cheung & Bryant, 2015) and an a priori power analysis (G*Power 3.1.9.2; University of Kiel, Germany) indicating that in order to attain adequate power (i.e., $1 - \beta = 0.80$) to detect moderate effect sizes (i.e., Cohen's $f = 0.25-0.30$) for the stress x genotype interactions, assuming minor allele frequencies ranging from 0.42–0.49 (Binder *et al.*, 2008), we would need a total sample of approximately 90–130 participants.

Socially Evaluated Cold Pressor Test (SECPT)

Following completion of a short demographics survey and the collection of baseline measures (see below), participants were asked to submerge their non-dominant hand in a bath of water for 3 min. Participants who had been randomly assigned to the stress condition ($n = 56$; 20 males, 36 females) placed their hand in a bath of ice cold (0–2°C) water, while participants who had been randomly assigned to the control condition ($n = 56$; 24 males, 32 females) placed their hand in a bath of warm (35–37°C) water. The water was maintained at the appropriate temperature by a circulating water bath (Cole-Parmer; Vernon Hills, IL). If a participant found the water bath too painful, he or she was allowed to remove his or her hand from the water and continue with the experiment. Based on previous work (Schwabe *et al.*,

2008b), a social evaluative component was added to the cold pressor manipulation. Participants in the stress condition were misleadingly informed that they were being videotaped during the procedure for subsequent evaluation of their facial expressions, and throughout the water bath, they were asked to keep their eyes on a camera that was located on the wall of the laboratory.

Subjective and Objective Stress Response Measures

The Positive and Negative Affect Schedule (PANAS) and State Anxiety Inventory (SAI)—Immediately before and approximately 10 min after the water bath manipulation, participants completed the PANAS (Watson *et al.*, 1988) and the SAI (state portion of the State-Trait Anxiety Inventory) (Spielberger *et al.*, 1983). This allowed for a pre-post analysis of stress-induced changes in affect and anxiety, respectively.

Subjective Pain and Stress Ratings—Participants rated the painfulness and stressfulness of the water bath at 1-min intervals on 11-point scales ranging from 0–10, with 0 indicating a complete lack of pain or stress and 10 indicating unbearable pain or stress.

Cardiovascular Analysis—Heart rate (HR) was measured continuously from approximately 1 min before the water bath until its completion via a BioNomadix pulse transducer (Biopac Systems, Inc.; Goleta, CA) placed on the ring finger of participants' dominant hand. The pulse transducer was connected to the PPG module of the MP150 BIOPAC hardware. Average baseline HR (average HR before water bath) and water bath HR (average of HR during water bath) were calculated for statistical analyses.

Cortisol Analysis—On Day 1, saliva samples were collected from participants immediately before and 25 min after the water bath to analyze salivary cortisol levels. On Day 2, saliva samples were collected from participants immediately before and 25 min after the free recall assessment to analyze salivary cortisol levels. Saliva samples were collected in a Salivette saliva collection device (Sarstedt, Inc., Newton, NC). The samples were stored at −20°C until being thawed and extracted by low-speed centrifugation. Salivary cortisol levels were determined by enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's protocol.

Learning and Memory Task

Immediately following exposure to the water bath, participants were presented with a list of 42 words, which were selected from the *Affective Norms for English Words* (Bradley & Lang, 1999). Based on standardized valence and arousal ratings, we chose 14 neutral, 14 positive, and 14 negative words (7 arousing and 7 non-arousing per category), which, across emotional valence and arousal categories, were balanced for word length and word frequency. As per previous methodology (Zoladz *et al.*, 2011; Zoladz *et al.*, 2013; Zoladz *et al.*, 2014a; Zoladz *et al.*, 2014b), participants were instructed to read each word aloud and rate its emotional valence on a scale from −4 (very negative) to +4 (very positive) and its arousal level on a scale of 0 (not arousing) to 8 (very highly arousing), with the aid of self-assessment manikins, on a sheet of paper containing the list of words.

Immediately following word list encoding, participants were given 5 min to write down as many words as they could remember from the list of words they just studied (immediate recall). The next day, participants returned to the laboratory to have their memory for the list of words assessed. Participants were again given 5 min to write down as many words as they could remember from the list of words that they studied on the previous day (delayed recall). Fifteen minutes later, participants were given a recognition test. They were presented with a list of words containing 42 "old" words (i.e., words presented on the previous day) and 42 "new" words (i.e., words not presented on the previous day) and were instructed to label each word as "old" or "new." The "new" words were matched to the "old" words on emotional valence, arousal level, word length and word frequency. To assess participants' ability to discriminate between "old" and "new" words, we calculated a sensitivity index (d' = $z[p(\text{hit}) - p(\text{false alarm})]$) for each category of word to be used for statistical analysis.

Genotyping

On Day 2, during the 15-min delay between free recall and recognition testing, a saliva sample was collected from participants via the OGR-500 Oragene (DNA Genotek, Inc.; Ottawa, ON, Canada). The sample was stored at room temperature, until shipped to DNA Genotek, Inc. for genotyping of polymorphisms rs1360780, rs3800373, and rs9296158 in the *FKBP5* gene. DNA was extracted from 700 μL of saliva, and quantity and quality control procedures were performed before undergoing TaqMan® assay with PCR amplification for genotype. Primers and probes were obtained through Life Technologies, Inc. (Foster City, CA). The call rate for rs1360780 and rs9296158 was 100%, and the call rate for rs3800373 was 99%.

Statistical Analyses

Based on previous work establishing an association between particular *FKBP5* polymorphism alleles, psychological disorders and alterations in the physiological stress response (Binder *et al.*, 2004; Binder *et al.*, 2008; Ising *et al.*, 2008; Binder, 2009; Willour *et al.*, 2009; Brent *et al.*, 2010; Roy *et al.*, 2010; Xie *et al.*, 2010; Zobel *et al.*, 2010; Appel *et al.*, 2011; Boscarino *et al.*, 2011; Mehta *et al.*, 2011; Sarapas *et al.*, 2011; Mahon *et al.*, 2013; Menke *et al.*, 2013; Fujii *et al.*, 2014a; Watkins *et al.*, 2016), we divided participants into "risk allele" carriers [heterozygous, homozygous carriers of the T (rs1360780), C (rs3800373), and A (rs9296158) alleles] and non-carriers [wild type (homozygous for the C (rs1360780), A (rs3800373), and G (rs9296158) alleles] for the purpose of data analysis. Demographic data for each polymorphism can be found in Table 1. The data for each polymorphism were analyzed separately with mixed-model ANOVAs. The between-subjects factors utilized in these analyses were stress (stress, no stress), sex, and polymorphism genotype (risk allele carrier, risk allele non-carrier), and the within-subjects factors were word valence and arousal (for recall and recognition data) or time point [for physiological (heart rate, cortisol) and self-report (PANAS, SAI) data]. Outlier data points that were 3 standard deviations beyond the exclusive group mean were removed from statistical analyses; only 3 data points (1 immediate recall data point from a stressed female risk allele carrier; 1 delayed recall data point from a stressed male risk allele non-carrier; 1 recognition memory data point from a non-stressed female risk allele non-carrier) were classified as outliers. If the assumption of sphericity was violated, Greenhouse-Geisser corrections were

employed, with reduced degrees of freedom reported in the analyses. Genotype-independent effects were reported based on the analysis including rs1360780, but were consistent across all three polymorphisms. Alpha was set at .05 for all analyses, and Bonferroni-corrected post hoc tests were employed when the omnibus F indicated the presence of a significant effect. All statistical analyses were performed in SPSS (version 22.0; SPSS, Inc).

RESULTS

Genotype Characteristics

Chi-square goodness-of-fit analyses revealed that there was no significant deviation from the Hardy-Weinberg equilibrium for any of the polymorphisms: rs1360780 ($\chi^2_1 = 0.21$ ($n = 112$), $P = 0.65$), rs3800373 ($\chi^2_1 = 0.82$ ($n = 111$), $P = 0.27$), and rs9296158 ($\chi^2_1 = 0.35$ ($n = 112$), $P = 0.55$). However, previous work has reported that these SNPs are in linkage disequilibrium (Binder *et al.*, 2004; Binder *et al.*, 2008; Ising *et al.*, 2008; Zobel *et al.*, 2010); thus, the current findings likely reflect one functional effect in the gene.

Subjective and Objective Stress Response Measures

PANAS and SAI—Overall, positive affect decreased and levels of anxiety increased after the water bath manipulation (affect – effect of time point: $F_{1,104} = 12.10$, $P = 0.001$; anxiety – $F_{1,104} = 10.54$, $P = 0.002$). Stressed participants reported lower positive affect than controls (effect of stress: $F_{1,104} = 4.66$, $P = 0.03$; Table 2). Carriers of the *FKBP5* risk alleles for rs1360780 ($F_{1,104} = 5.52$, $P = 0.02$), rs3800373 ($F_{1,103} = 5.64$, $P = 0.02$), and rs9296158 ($F_{1,104} = 6.46$, $P = 0.01$) exhibited significantly lower positive affect than non-carriers. Females (affect: 14.74 ± 0.46; anxiety: 39.07 ± 1.10) reported greater levels of negative affect and anxiety than males (affect: 13.18 ± 0.59; anxiety: 34.34 ± 1.42) (affect – effect of sex: $F_{1,104} = 4.28$, $P = 0.04$; anxiety – effect of sex: $F_{1,104} = 6.89$, $P = 0.01$).

Subjective Pain and Stress Ratings—Overall, pain ratings increased throughout the water bath manipulation (effect of time point: $F_{1.44,149.61} = 4.05$, $P = 0.03$). Stressed participants (PAIN: 6.41 ± 0.21; STRESS: 5.67 ± 0.24) rated the water bath as more painful (effect of stress: $F_{1,104} = 444.63$, $P < 0.001$) and more stressful (effect of stress: $F_{1,104} = 263.71$, $P < 0.001$) than controls (PAIN: 0.23 ± 0.20; STRESS: 0.33 ± 0.23) (p's < 0.001). Stressed females (PAIN: 7.15 ± 0.25; STRESS: 6.71 ± 0.28) also rated the water bath as more painful (effect of sex: $F_{1,104} = 6.68$, $P = 0.01$; Stress x Sex interaction: $F_{1,104} = 6.01$, $P = 0.02$) and more stressful (effect of sex: $F_{1,104} = 11.49$, $P = 0.001$; Stress x Sex interaction: $F_{1,104} = 8.53$, $P = 0.004$) than stressed males (PAIN: 5.67 ± 0.35; STRESS: 4.64 ± 0.39).

Heart Rate—Stressed participants, particularly males, exhibited significantly greater HR following the water bath, relative to controls (effect of time point: $F_{1,104} = 89.19$, $P < 0.001$; Stress x Time Point interaction, $F_{1,104} = 27.50$, $P < 0.001$; Sex x Time Point interaction: $F_{1,104} = 4.58$, $P = 0.04$; Stress x Sex x Time Point interaction: $F_{1,104} = 4.83$, $P = 0.03$; Figure 1a and 1b). The analysis of HR also showed that carriers of the risk alleles for rs1360780 (Genotype x Time Point interaction: $F_{1,104} = 5.19$, $P = 0.03$; Stress x Sex x Genotype x Time Point interaction: $F_{1,104} = 4.74$, $P = 0.03$), rs3800373 (Genotype x Time Point interaction: $F_{1,103} = 4.19$, $P = 0.04$; Stress x Sex x Genotype x Time Point interaction: $F_{1,103} = 6.78$, $P =$

0.01), and rs9296158 (Genotype x Time Point interaction: $F_{1,104} = 4.23$, $P = 0.04$; Stress x Sex x Genotype x Time Point interaction: $F_{1,104} = 5.79$, $P = 0.02$) exhibited greater HR following the water bath manipulation than non-carriers, which appeared to be driven mainly by a greater stress-induced increase in HR in male risk allele carriers, relative to all other groups. To verify the induction of a stress response in all stressed participants, we analyzed HR change scores (average of HR during water bath – average of pre-water bath HR) with a 3-way (stress, sex, genotype) ANOVA. This analysis revealed a greater increase in HR in stressed participants than controls (effect of stress: $F_{1,104} = 27.50$, $p < 0.001$; Figure 1c). Stressed males did exhibit a greater stress-induced increase in HR than females (effect of sex: $F_{1,104} = 4.58$, $P = 0.04$; Stress x Sex interaction: $F_{1,104} = 4.83$, $P = 0.03$), which was driven by the greatest change in HR being observed in stressed male risk allele carriers (rs1360780 – effect of genotype: $F_{1,104} = 5.19$, $P = 0.03$; Stress x Sex x Genotype interaction: $F_{1,104} = 4.74$, $P = 0.03$; rs3800373 – effect of genotype: $F_{1,103} = 4.19$, $P = 0.04$; Stress x Sex x Genotype interaction: $F_{1,103} = 6.78$, $P = 0.01$; rs9296158 – effect of genotype: $F_{1,104} = 4.23$, $P = 0.04$; Stress x Sex x Genotype interaction: $F_{1,104} = 5.79$, $P = 0.02$).

Cortisol—On Day 1, stressed participants exhibited significantly greater salivary cortisol levels than controls following the water bath (effect of stress: $F_{1,103} = 25.28$; effect of time point: $F_{1,103} = 40.42$; Stress x Time Point interaction: $F_{1,103} = 43.22$; P's < 0.001; Figure 2). Across both time points, risk allele carriers in the stress condition exhibited greater salivary cortisol levels than risk allele carriers in the non-stressed condition (rs1360780 – Stress x Genotype interaction: $F_{1,103} = 11.69$; rs3800373 – Stress x Genotype interaction: $F_{1,102} = 10.66$; rs9296158 – Stress x Genotype interaction: $F_{1,103} = 11.12$; P's $= 0.001$). There was no significant influence of stress or genotype on Day 2 salivary cortisol levels (Table 2).

Valence and Arousal Ratings of Learned Words

Valence Ratings—As expected, participants rated negative words more negatively than neutral words, which were rated more negatively than positive words (effect of valence: $F_{1.38,143.62} = 1618.61$, $P < 0.001$). Participants also rated arousing words more negatively than non-arousing words, especially females (effect of arousal: $F_{1,104} = 63.50$; Sex x Arousal interaction: $F_{1,104} = 19.05$; P's < 0.001).

Arousal Ratings—As expected, participants rated arousing words as more arousing than non-arousing words (effect of arousal: $F_{1,104} = 185.74$, $P < 0.001$), and this effect was more pronounced in non-stressed participants (Stress x Arousal interaction: $F_{1,104} = 5.98$, $P = 0.02$). Participants also rated positive words as more arousing than negative words, which were rated as more arousing than neutral words (effect of valence: $F_{1.40,145.59} = 97.19$, $P < 0.001$). Overall, females rated words as more arousing than males, especially positive words (effect of sex: $F_{1,104} = 5.73$, $P = 0.02$; Valence x Arousal interaction: $F_{1.85,192.06} = 12.16$, $P < 0.001$; Sex x Valence x Arousal interaction: $F_{1.85,192.06} = 3.35$, $P = 0.04$), and carriers of the risk alleles for rs1360780 ($F_{1,104} = 10.78$, $P = 0.001$), rs3800373 ($F_{1,104} = 6.40$, $P = 0.01$), and rs9296158 ($F_{1,104} = 9.47$, $P = 0.003$) rated words as less arousing than non-carriers.

Memory Testing

Immediate Free Recall—Participants recalled more positive and negative words than neutral words (effect of valence: $F_{2,206} = 30.88$, $P < 0.001$). They also recalled more arousing words than non-arousing words, particularly when the words were positive (effect of arousal: $F_{1,103} = 62.17$; Valence x Arousal interaction: $F_{2,206} = 50.80$; Ps < 0.001). There was a significant Sex x Arousal interaction ($F_{1,103} = 6.72$, $P = 0.01$) that was dependent on *FKBP5* genotype. Specifically, male risk allele carriers recalled fewer non-arousing words than male non-carriers, while female risk allele carriers recalled more non-arousing words than female non-carriers (rs1360780 – Sex x Genotype interaction: $F_{1,103} = 10.25$, $P = 0.002$; Sex x Genotype x Arousal interaction: $F_{1,103} = 4.89$, $P = 0.03$; rs3800373 – Sex x Genotype interaction: $F_{1,102} = 7.46$, $P = 0.007$; Sex x Genotype x Arousal interaction: $F_{1,102} = 3.52$, $P = 0.06$; rs9296158 – Sex x Genotype interaction: $F_{1,103} = 8.94$, $P = 0.003$; Sex x Genotype x Arousal interaction: $F_{1,103} = 5.35$, $P = 0.02$). The effect of stress on immediate recall was dependent on genotype for rs1360780 (Stress x Genotype interaction: $F_{1,103} = 5.45$, $P = 0.02$; Figure 3a), rs3800373 (Stress x Genotype interaction: $F_{1,102} = 7.61$, $P = 0.007$; Figure 3b), and rs9296158 (Stress x Genotype interaction: $F_{1,103} = 6.45$, $P = 0.01$; Figure 3c). In all three cases, pre-learning stress selectively impaired immediate recall in risk allele carriers.

Delayed Free Recall—Twenty-four hours following learning, participants recalled more positive and negative words than neutral words (effect of valence: $F_{2,206} = 12.64$, $P < 0.001$). They also recalled more arousing words than non-arousing words, particularly when the words were positive (effect of arousal: $F_{1,103} = 84.34$; Valence x Arousal interaction: $F_{2,206} = 56.11$; Ps < 0.001). Females recalled more words than males (effect of sex – $F_{1,103} = 4.49$, $P = 0.04$), particularly female risk carriers for rs1360780 (Sex x Genotype interaction: $F_{1,103} = 5.39$, $P = 0.02$), rs3800373 (Sex x Genotype interaction: $F_{1,102} = 5.56$, $P = 0.02$), and rs9296158 (Sex x Genotype interaction: $F_{1,103} = 4.93$, $P = 0.03$). Similar to immediate recall, the effects of stress on delayed recall depended on genotype for rs1360780 (Stress x Genotype interaction: $F_{1,103} = 7.09$, $P = 0.01$; Figure 4a), rs3800373 (Stress x Genotype interaction: $F_{1,102} = 6.21$, $P = 0.01$; Figure 4b), and rs9296158 (Stress x Genotype interaction: $F_{1,103} = 7.71$, $P = 0.007$; Figure 4c). Pre-learning stress led to a long-term enhancement of recall in non-carriers of the *FKBP5* risk alleles only. That is to say, pre-learning stress exerted no effect on long-term memory in *FKBP5* risk allele carriers.

Recognition—Participants recognized more positive and neutral words than negative words (effect of valence: $F_{2,206} = 35.76$, $P < 0.001$). They also recognized more arousing words than non-arousing words, particularly when the words were negative or neutral (effect of arousal: $F_{1,103} = 36.05$, $P < 0.001$; Valence x Arousal interaction: $F_{2,206} = 3.71$; $P = 0.03$). Females recognized more positive words than males (Sex x Valence interaction: $F_{2,206} = 3.85$, $P = 0.02$), and male risk allele carriers were the only participants to not show greater recognition memory for arousing words relative to non-arousing words (rs1360780: Sex x Genotype x Arousal interaction: $F_{1,103} = 4.78$, $P = 0.03$; rs3800373: Sex x Genotype x Arousal interaction: $F_{1,102} = 4.46$, $P = 0.04$; rs9296158: Sex x Genotype x Arousal interaction: $F_{1,103} = 4.19$, $P = 0.04$). Stress enhanced long-term recognition of non-arousing words, relative to controls, but had no significant impact on the recognition of arousing

words, possibly due to a ceiling effect (Stress x Arousal interaction: $F_{1,103} = 6.48$, $P = 0.01$; Figure 5a). Once again, the effect of stress was dependent on the genotype for rs1360780 (Stress x Genotype interaction: $F_{1,103} = 5.16$, $P = 0.03$; Figure 5b), rs3800373 (Stress x Genotype interaction: $F_{1,102} = 5.50$, $P = 0.02$; Figure 5c), and rs9296158 (Stress x Genotype interaction: $F_{1,103} = 6.04$, $P = 0.02$; Figure 5d). Similar to the effects observed for delayed recall, pre-learning stress enhanced long-term recognition memory in non-carriers of the *FKBP5* risk alleles; this stress-induced enhancement was not observed in *FKBP5* risk allele carriers.

Physiological Correlates of Memory Effects—In order to probe potential physiological correlates of the observed Stress x Genotype interactions, we performed bivariate correlations between our physiological measures (HR and salivary cortisol levels) and memory performance. These analyses revealed significant or marginally significant positive correlations between changes in salivary cortisol levels and recall performance in non-carriers of the risk alleles for rs1360780 (Immediate Recall: $r_{54} = 0.27$, $P = 0.05$; Delayed Recall: $r_{53} = 0.24$, $P = 0.09$), rs3800373 (Immediate Recall: $r_{55} = 0.30$, $P = 0.02$; Delayed Recall: $r_{54} = 0.20$, $P = 0.15$), and rs9296158 (Immediate Recall: $r_{53} = 0.27$, $P = 0.05$; Delayed Recall: $r_{52} = 0.24$, $P = 0.09$) (Figures 6a and 6c), while no significant relationships were observed between such measures in risk allele carriers for rs1360780 (Immediate Recall: $r_{55} = -0.02$, $P = 0.88$; Delayed Recall: $r_{56} = -0.06$, $P = 0.68$), rs3800373 (Immediate Recall: $r_{53} = -0.06$, $P = 0.66$; Delayed Recall: $r_{54} = -0.05$, $P = 0.75$), and rs9296158 (Immediate Recall: $r_{56} = -0.01$, $P = 0.92$; Delayed Recall: $r_{57} = -0.06$, $P = 0.69$) (Figures 6b and 6d). Based on these findings, we divided stressed participants into cortisol responders (stressed participants exhibiting an increase in salivary cortisol of at least 2 nmol/l) and non-responders (stressed participants exhibiting a change in salivary cortisol less than 2 nmol/l), similar to previous work from our laboratory (Zoladz *et al.*, 2011; Zoladz *et al.*, 2013; Zoladz *et al.*, 2014a) and that of others (Schwabe *et al.*, 2008a), and then performed mixed-model ANOVAs on memory performance with responder (responder, non-responder, no stress) as a between-subjects factor in place of stress. These analyses revealed significant Responder x Genotype interactions for immediate recall and delayed recall for rs1360780 (Immediate Recall: $F_{2,98} = 3.97$, $P = 0.02$; Delayed Recall: $F_{2,98} = 5.55$, $P = 0.01$; Figures 6e and 6f), rs3800373 (Immediate Recall: $F_{2,97} = 5.56$, $P = 0.01$; Delayed Recall: $F_{2,97} = 4.74$, $P = 0.01$), and rs9296158 (Immediate Recall: $F_{2,98} = 4.27$, $P = 0.02$; Delayed Recall: $F_{2,98} = 5.58$, $P = 0.01$), revealing that pre-learning stress enhanced short- and long-term recall in non-carriers of the *FKBP5* risk alleles who exhibited greater cortisol responses to the stress (i.e., responders). Pre-learning stress had no impact on short- or long-term memory in non-carriers who exhibited a blunted cortisol response to the stress (i.e., non-responders) or in stressed risk allele carriers overall.

DISCUSSION

Previous work has shown that SNPs in the *FKBP5* gene are associated with altered HPA axis function, changes in the structure and function of several cognitive brain areas, and increased risk for stress-related psychological disorders (Binder *et al.*, 2004; Binder *et al.*, 2008; Ising *et al.*, 2008; Binder, 2009; Willour *et al.*, 2009; Brent *et al.*, 2010; Roy *et al.*,

2010; Xie *et al.*, 2010; Zobel *et al.*, 2010; Appel *et al.*, 2011; Boscarino *et al.*, 2011; Mehta *et al.*, 2011; Sarapas *et al.*, 2011; Mahon *et al.*, 2013; Menke *et al.*, 2013; Fujii *et al.*, 2014a; Fujii *et al.*, 2014c; Fani *et al.*, 2016; Hirakawa *et al.*, 2016; Watkins *et al.*, 2016). Because investigators have speculated that these SNPs may alter emotional memory systems (Binder *et al.*, 2008; Binder, 2009; Fani *et al.*, 2013; Cheung & Bryant, 2015; Holz *et al.*, 2015), thus conferring greater risk for stress-related psychopathology, we examined the influence of three commonly studied SNPs in the *FKBP5* gene on stress-induced alterations of learning and memory. Our results revealed that pre-learning stress impaired short-term memory in carriers of the *FKBP5* risk alleles. More importantly, pre-learning stress enhanced long-term recall and recognition memory in non-carriers of the *FKBP5* risk alleles, while having no long-term effect on risk allele carriers. Follow-up analyses revealed that memory performance was correlated with salivary cortisol levels in non-carriers, but not in carriers. These findings support the notion that *FKBP5* polymorphisms result in a sensitized stress response system, perhaps specifically for stress-induced changes in corticosteroid levels, which could lend insight into how such SNPs are linked to psychological illness.

Risk Allele Carriers Exhibit Hypersensitivity to Stress

Brief stress administered immediately before learning impaired short-term memory in *FKBP5* risk allele carriers and selectively enhanced long-term memory in non-carriers of the risk alleles. The stress-induced enhancement of long-term memory in non-carriers is consistent with our previous work and, as we (Diamond *et al.*, 2007; Zoladz *et al.*, 2011; Zoladz *et al.*, 2013; Zoladz *et al.*, 2014a; Zoladz *et al.*, 2014b; Zoladz *et al.*, 2014c) and others (Joels *et al.*, 2011; Schwabe *et al.*, 2012) have speculated, results from a rapid increase in noradrenergic activity interacting with slowly rising corticosteroid levels that exert non-genomic, excitatory influences on the hippocampus, an area significantly influenced by stress-induced amygdala activity and densely packed with corticosteroid receptors. Partial support for this hypothesis comes from the finding that the stress-induced enhancement of recall in non-carriers was associated with salivary cortisol levels. Specifically, non-carriers exhibiting a greater cortisol response to the stress demonstrated better short- and long-term recall. This association suggests that cortisol exerted excitatory effects on cognitive processing in non-carriers and resulted in a "memory formation" mode that promoted consolidation processes (Schwabe *et al.*, 2012).

Instead of exhibiting behavior consistent with a rapid, stress-induced excitatory phase (and enhanced long-term memory), risk allele carriers demonstrated long-term recall and recognition performance that was unaffected by pre-learning stress, and their short-term memory was actually impaired. We would contend that risk allele carriers retain a sensitized stress response system; thus, the temporal dynamics of stress effects on learning are theoretically shifted, resulting in a much more transient, or potentially absent, excitatory phase of cognitive processing following stress exposure (see Diamond *et al.*, 2007 for graphical illustration of the temporal dynamics model). Specifically, risk allele carriers may exhibit an abnormally intense response that biases them toward an inhibitory phase for learning and memory. The fact that the association between cortisol and memory observed in non-carriers was not observed in carriers implies that the stress-induced change in cortisol had a much different, potentially more adverse, impact in risk allele carriers. Others have

also shown that risk allele carriers exhibit a prolonged cortisol response to stress (Ising *et al.*, 2008), which could have taken place in the present study and resulted in a greater likelihood of GR-dependent, gene-mediated inhibitory effects on hippocampal function, thus preventing the long-term enhancing effects of stress. Differential responses to corticosteroids between risk allele carriers and non-carriers could result from altered *FKBP5* activity. Previous work has associated *FKBP5* polymorphisms with increased transcription of the *FKBP5* gene and elevated levels of the FKBP5 protein (Binder *et al.*, 2004; Binder, 2009; Klengel *et al.*, 2013), which result in GR resistance, increased levels of circulating cortisol (Reynolds *et al.*, 1999; Ising *et al.*, 2008) and negative influences on GR activity and GR-mediated synaptic plasticity (Bennett & Lagopoulos, 2014; Young *et al.*, 2015). Unfortunately, we did not assess the recovery of salivary cortisol levels, *FKBP5* mRNA levels, or FKBP5 protein levels in participants, so we cannot verify this hypothesis. Still, our findings support the notion that *FKBP5* risk allele carriers exhibit altered effects of stress on cognition, possibly as a result of differential sensitivity to stress-induced changes in corticosteroid levels.

Our finding that acute stress impaired short-term memory in risk allele carriers and enhanced long-term memory in non-carriers only is in contrast to the findings of Cheung and Bryant (2015), who reported no *FKBP5* genotype-dependent differences in memory for images. This inconsistency could relate to methodological differences between the two studies, as we employed a list of words, rather than images, as the learning stimulus, and Cheung and Bryant (2015) assessed delayed (48-hr), but not immediate, recall. One interesting, and potentially relevant, finding reported by Cheung and Bryant (2015) is that *FKBP5* risk allele carriers exhibited elevated levels of salivary alpha-amylase, which is an indicator of noradrenergic / sympathetic nervous system (SNS) activity (Nater & Rohleder, 2009). Cheung and Bryant (2015) speculated that *FKBP5* risk allele carriers may have exhibited greater salivary alpha-amylase levels due to a heightened sensitivity to anticipatory stress. In the present study, risk allele carriers exhibited greater HR than non-carriers following the water bath manipulation, providing further support for the possibility of a sensitized stress response system in these individuals. The association between noradrenergic / SNS activity and stress-induced alterations of learning and memory is well-documented in the literature (Roozendaal *et al.*, 2009); however, we did not observe any significant associations between HR and memory performance in risk allele carriers or non-carriers.

A surprising observation was that *FKBP5* risk allele carriers rated the studied words as less arousing than non-carriers. It is possible that this difference contributed to our observed effects, given the well-established finding of superior memory for emotionally arousing material. Specifically, carriers may have been more susceptible to the effects of stress on memory for the word lists because, given the lower arousal ratings, they were already less likely to recall as many words as non-carriers. However, the data observed in non-stressed carriers does not appear to support this line of reasoning, as they exhibited immediate and delayed memory performance that was superior, albeit not significantly, or statistically equivalent to that of non-stressed non-carriers for every memory measure. Another interesting observation in the present study was the sex-dependent effects of *FKBP5* polymorphisms on physiology and behavior, which, to our knowledge, has received minimal

attention in previous work. We observed stress-induced changes in HR that were particularly selective to male risk allele carriers, and we observed differential recall of arousing and non-arousing words in male versus female risk allele carriers. Collectively, these findings support the possibility that the effects of *FKBP5* polymorphisms are influenced by sex differences and the perception of emotionally arousing material.

Relevance for Understanding Psychological Illness

Several SNPs in the *FKBP5* gene have been linked to increased susceptibility for psychological disorders. The SNPs in the *FKBP5* gene studied here have been reported to result in altered GR sensitivity and HPA axis function, and extensive work has shown that individuals with stress-related psychological disorders, such as PTSD and major depression, exhibit altered baseline levels of cortisol, GR density and GR sensitivity (de Kloet *et al.*, 2006; Pariante & Lightman, 2008; Yehuda, 2009). The presently-studied SNPs may confer greater susceptibility to such psychological illness or to disorder-related phenotypes by altering HPA axis and SNS activity, thereby influencing cognitive function. If carriers of the *FKBP5* risk alleles possess a sensitized stress response system, they may be more prone to stress-induced cognitive impairments or the fragmentation of memories that are stored following stress exposure (Cheung & Bryant, 2015). This could ultimately help explain traumatic memory formation and cognitive impairments observed in multiple stress-related psychological disorders.

Limitations and Conclusions

We have shown that three commonly studied SNPs in the *FKBP5* gene influence how pre-learning stress affects long-term memory. Our sample size was large enough to detect moderate effect sizes, but it may not have been large enough to detect smaller effects. On the other hand, it is important to point out that the observed effects were evident for three different assessments of memory across two days. Some limitations of our study include the use of a word list as our learning material and the lack of racial/ethnic diversity in our sample, both of which reduce the external validity of our findings. It is also important to note that, because of the nature of the present study, we are unable to make any causal conclusions regarding the neurobiological mechanisms underlying the observed effects. Finally, the effects observed for these SNPs likely reflect one functional influence, given the high amount of linkage disequilibrium between them. Notwithstanding these limitations, our results may facilitate future endeavors aiming to understand the link between *FKBP5* and psychopathology.

Acknowledgments

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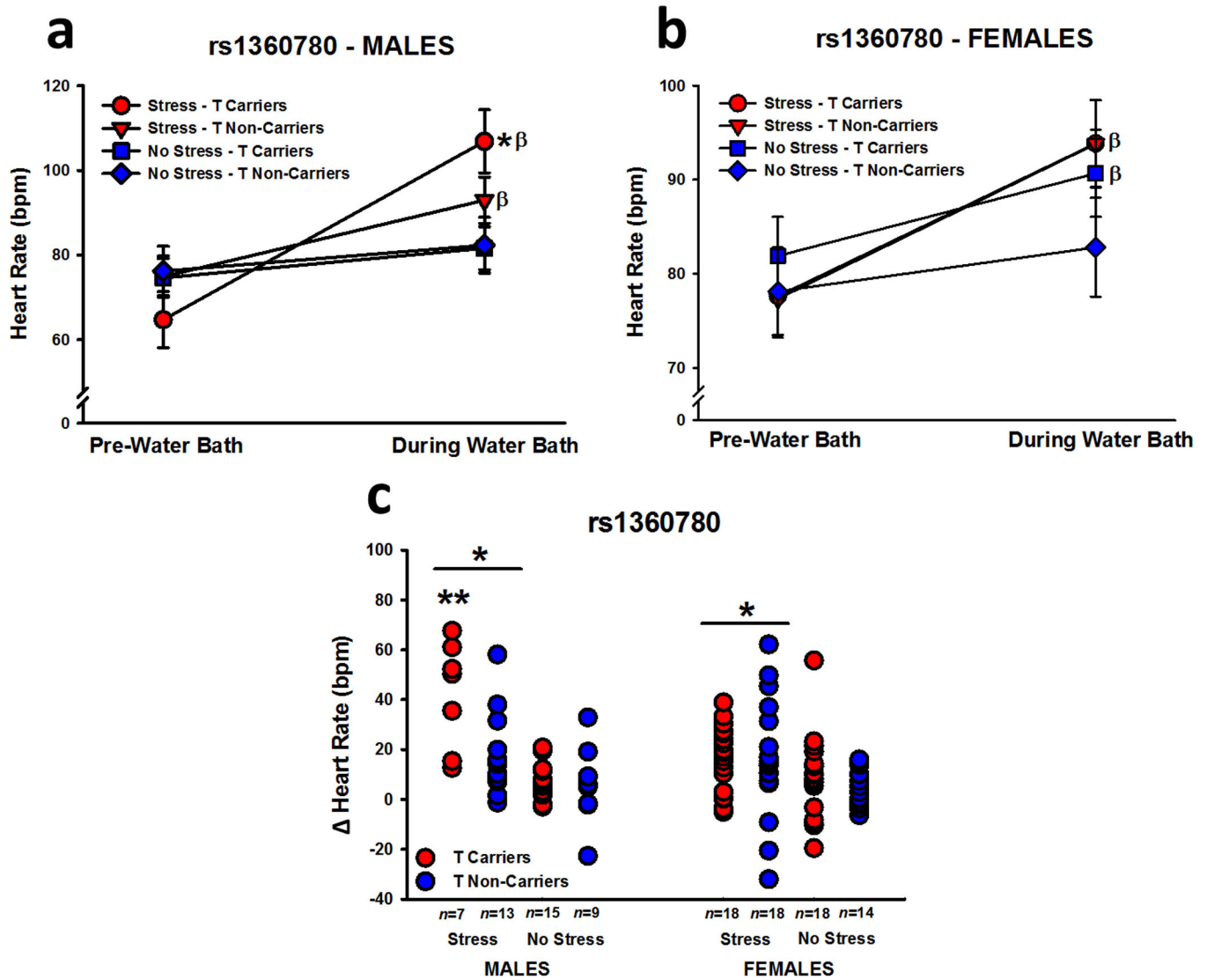
Figure 1.
Heart rate before and during the water bath manipulation. Stressed male (a) and female (b) participants exhibited significant increases in HR during the SECPT, but stressed male risk allele carriers were the only participants to exhibit significantly greater HR after the water bath relative to non-stressed controls. Upon analysis of HR change data (during water bath – pre-water bath), we confirmed that stress led to a greater increase in HR than non-stressed conditions and that this change was greatest in stressed male risk allele carriers (c). Data in insets a and b are expressed as means ± SEM, and the data from rs1360780 are used as a representative example of the effects observed for all *FKBP5* SNPs. * $p < 0.05$ relative to no stress; ** $P < 0.01$ relative to all other groups; β $P < 0.05$ relative to pre-water bath.
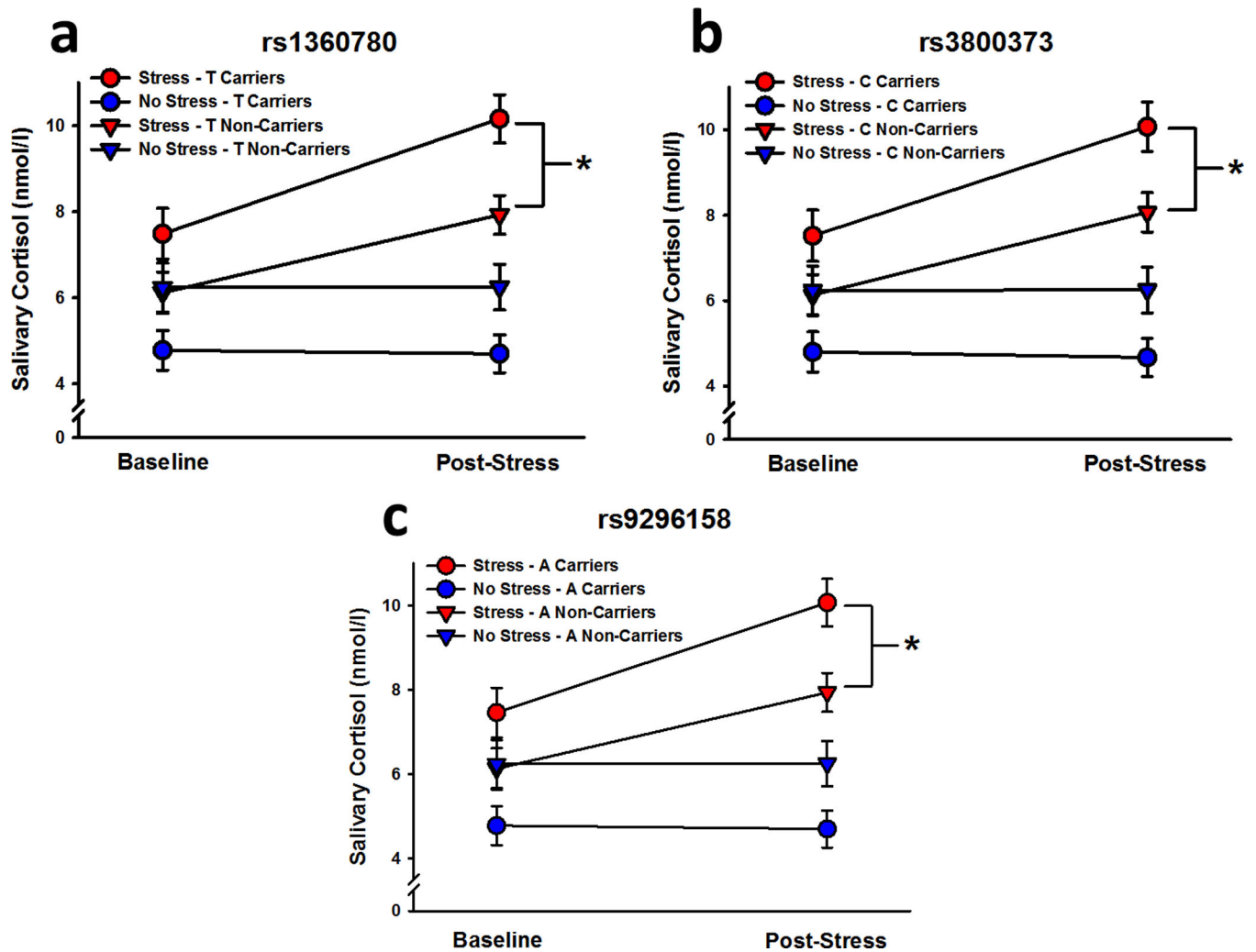
Figure 2.
Salivary cortisol levels before and after water bath exposure. Stressed participants, independent of genotype for each of the three *FKBP5* SNPs (a, b, c), exhibited significant increases in salivary cortisol levels, relative to control (i.e., non-stressed) participants. Stressed risk allele carriers for each polymorphism exhibited greater salivary cortisol levels, overall, than all other groups. Data are expressed as means ± SEM. * $P < 0.001$ relative to no stress.
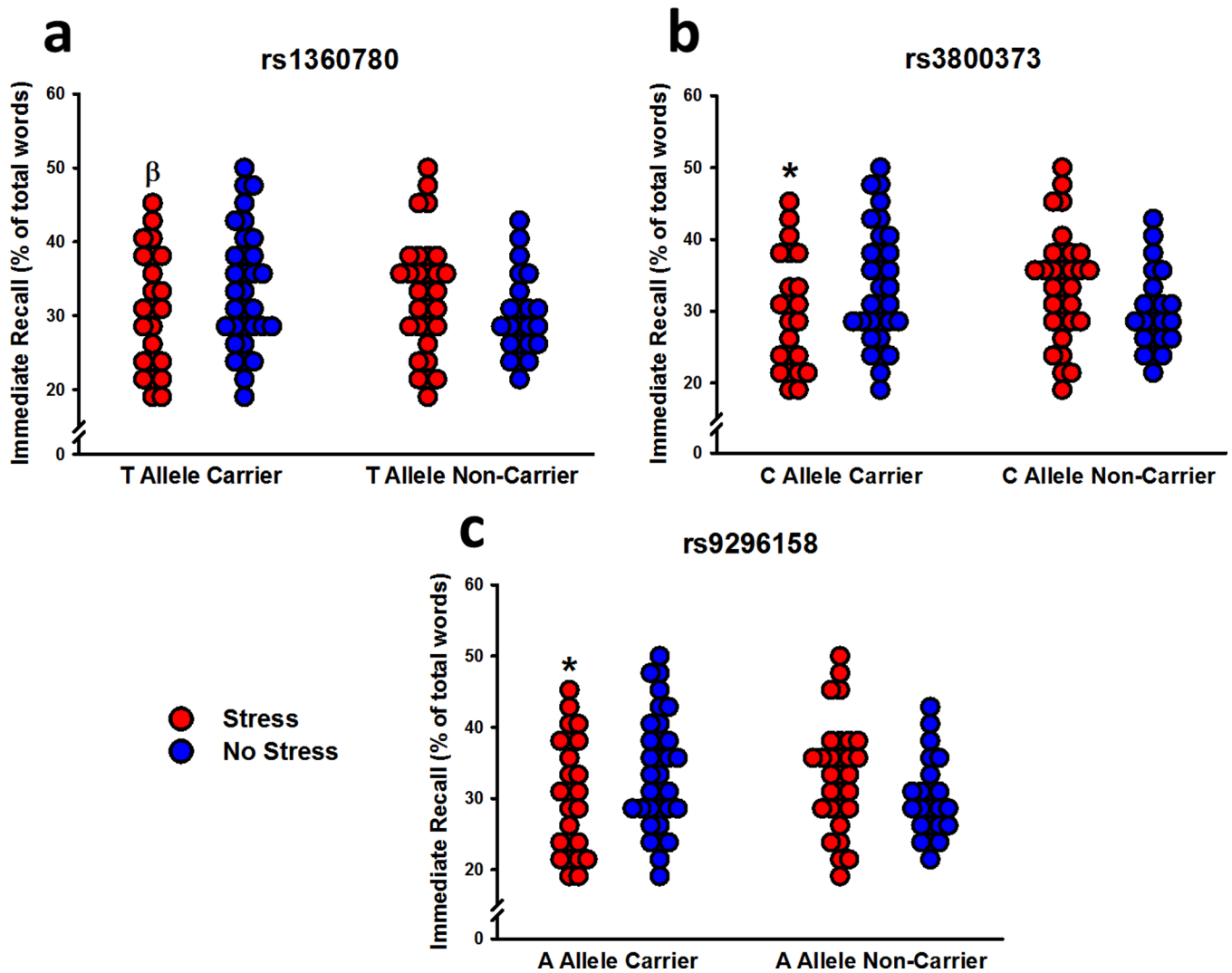
Figure 3.
Immediate free recall performance organized by stress and carriers of the risk allele for the three *FKBP5* SNPs. For each of the three SNPs (a, b, c), exposure to pre-learning stress impaired recall in risk allele carriers. β *P* < 0.05 relative to non-stressed carriers and *P* = 0.068 relative to stressed non-carriers; * *P* < 0.05 relative to non-stressed carriers and stressed non-carriers.
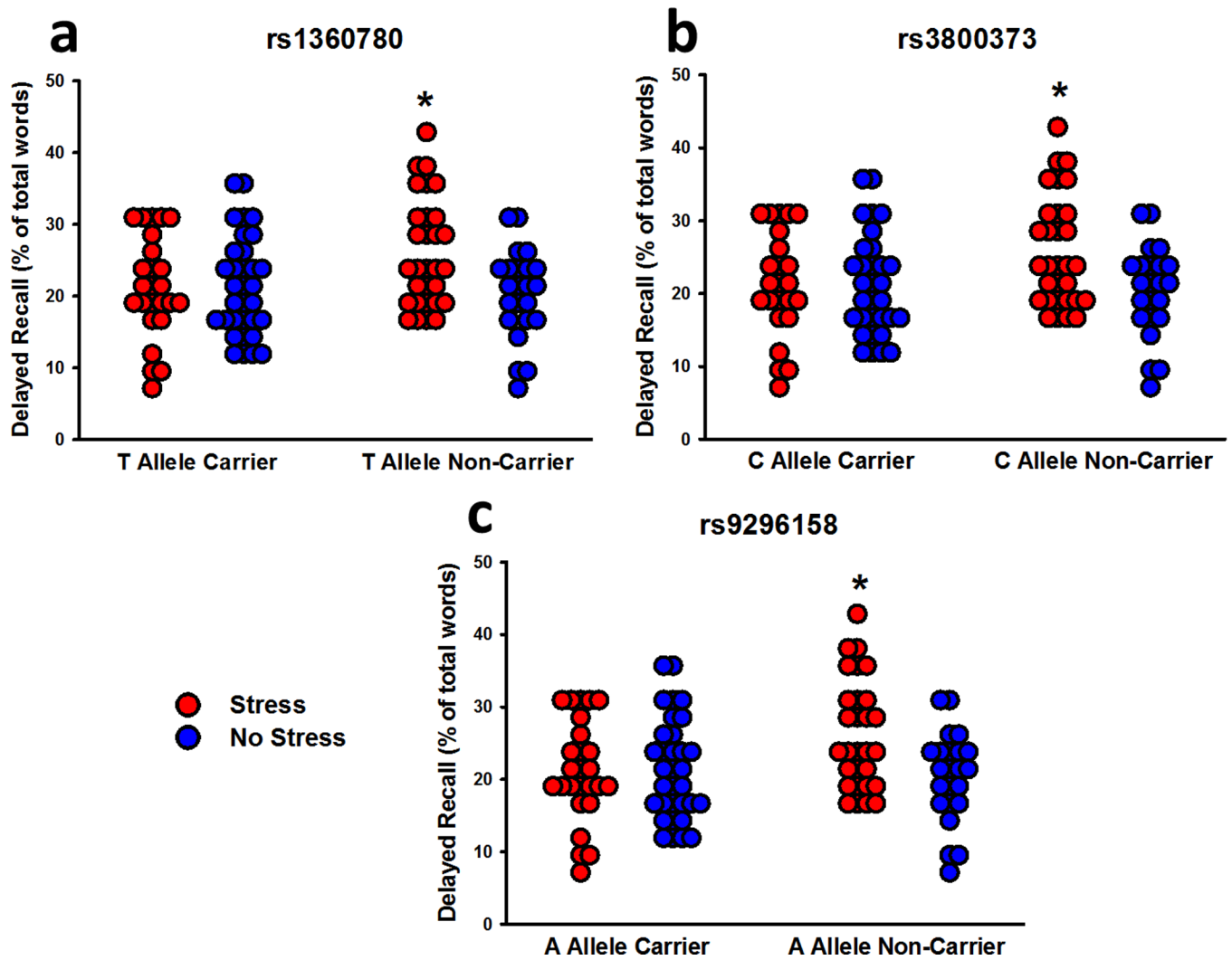
Figure 4.
Delayed free recall performance organized by stress and carriers of the risk allele for the three *FKBP5* SNPs. For each of the three SNPs (a, b, c), stress enhanced long-term recall in non-carriers of the risk allele, but had no effect on carriers. * *P* < 0.05 relative to all other groups.
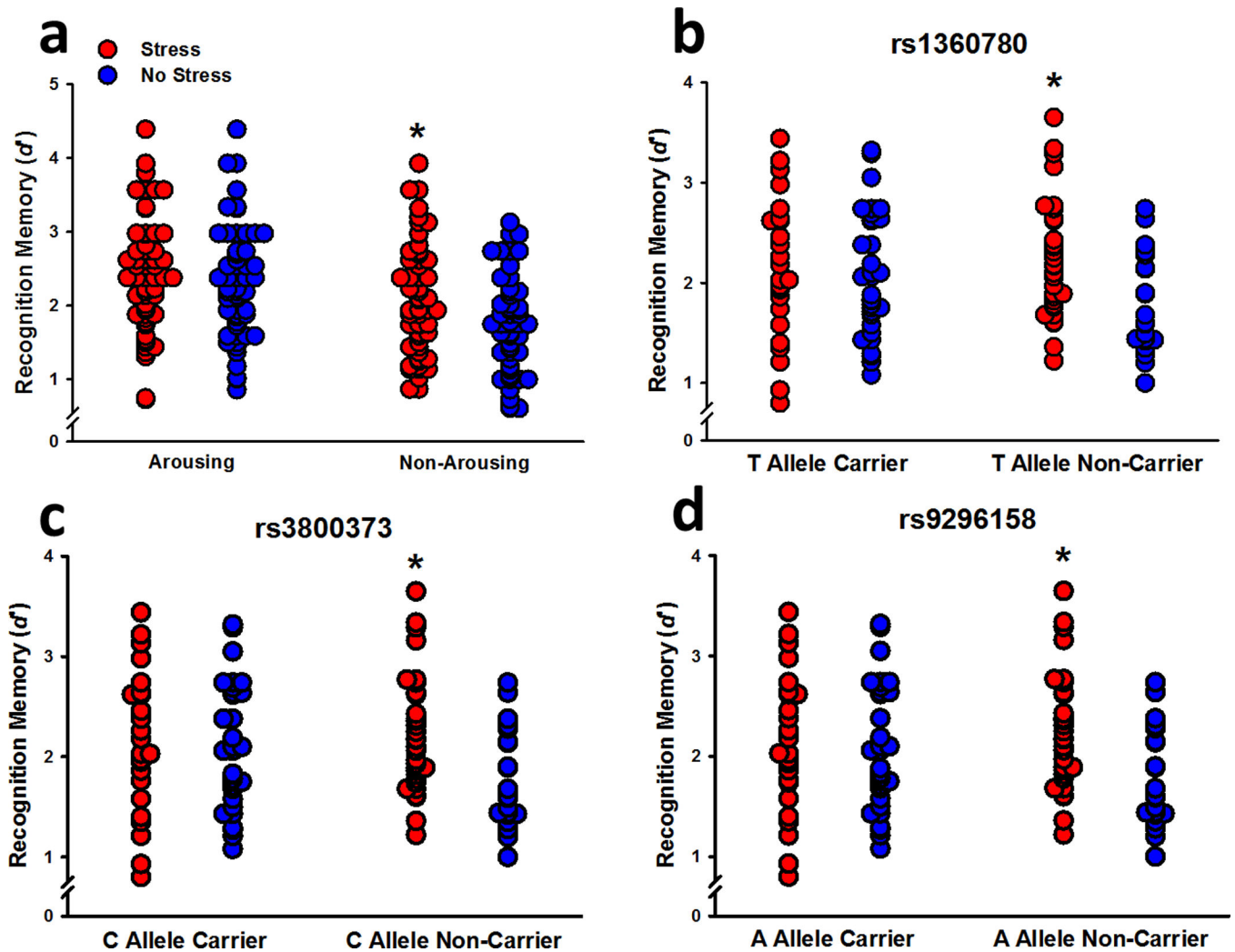
Figure 5.
Delayed recognition memory organized by stress and carriers of the risk allele for the three *FKBP5* SNPs. Stress selectively enhanced long-term recognition memory for non-arousing words (a). For each of the three SNPs (b, c, d), stress enhanced long-term recognition memory in non-carriers of the risk allele, relative to non-stressed non-carriers, but had no effect on carriers. * $P < 0.01$ relative to non-arousing word recognition in non-stressed participants or overall recognition memory in non-stressed non-carriers.
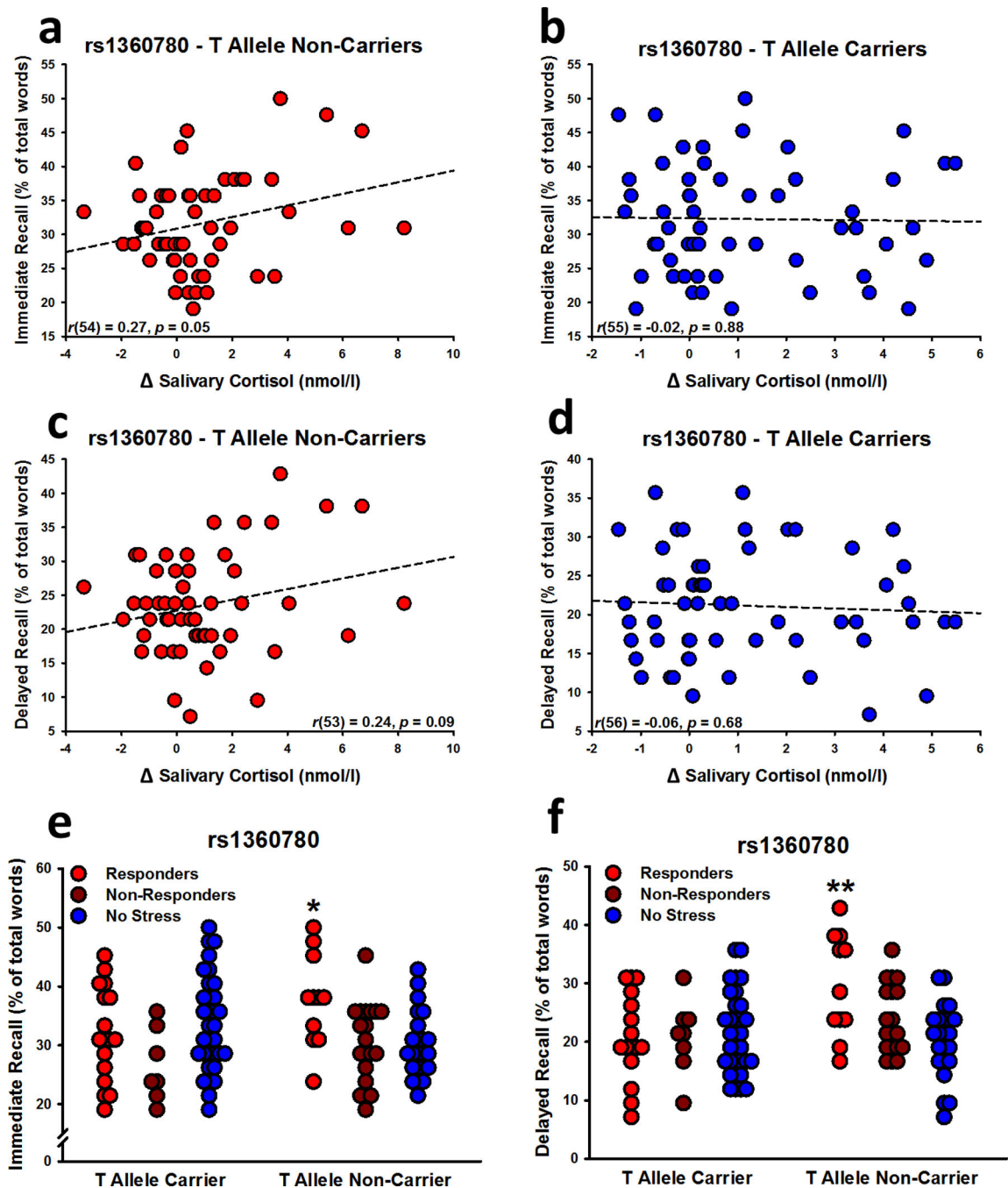
Figure 6.

Relationship between changes in salivary cortisol levels and recall performance, using the rs1360780 polymorphism of *FKBP5* as a representative example. Changes in salivary cortisol levels were positively correlated with immediate (a) and delayed recall (c) in non-carriers of the risk allele for rs1360780. No such relationship was observed in carriers of the risk allele (b, d). Analyses of immediate and delayed recall based on cortisol response to the stress revealed that non-carriers who exhibited a greater cortisol response to the stress had enhanced memory; this effect was no observed in risk allele carriers. * $P < 0.05$ relative to

responders carrying the risk allele; ** *P* < 0.01 relative to non-stressed non-carriers and responders carrying the risk allele.

Table 1

Participant Demographics based on *FKBP5* Polymorphism Genotype

Demographic Measure	rs1360780		rs3800373		rs9296158	
	Carriers (*n* = 58)	Non-Carriers (*n* = 54)	Carriers (*n* = 56)	Non-Carriers (*n* = 55)	Carriers (*n* = 59)	Non-Carriers (*n* = 53)
Age (years ± SEM)	19.88 (0.23)	19.70 (0.20)	19.93 (0.23)	19.69 (0.19)	19.88 (0.22)	19.70 (0.20)
Weight (kg ± SEM)	72.51 (2.27)	74.94 (2.40)	73.98 (2.26)	73.77 (2.42)	72.85 (2.26)	74.60 (2.42)
Height (m ± SEM)	1.72 (0.01)	1.72 (0.01)	1.72 (0.01)	1.72 (0.01)	1.72 (0.01)	1.72 (0.01)
Education (years ± SEM)	15.14 (0.16)	15.24 (0.14)	15.16 (0.16)	15.24 (0.14)	15.15 (0.15)	15.23 (0.15)
Sex (N, %)						
Males	22 (38%)	22 (41%)	22 (39%)	22 (40%)	22 (37%)	22 (42%)
Females	36 (62%)	32 (59%)	34 (61%)	33 (60%)	37 (63%)	31 (59%)
Race (N, %)						
American Indian / Alaska Native	1 (2%)	0 (0%)	1 (2%)	0 (0%)	1 (2%)	0 (0%)
Asian	5 (9%)	3 (6%)	5 (9%)	3 (6%)	5 (9%)	3 (6%)
Black / African American	1 (2%)	7 (13%)	2 (4%)	6 (11%)	2 (3%)	6 (11%)
White	51 (88%)	44 (82%)	48 (86%)	46 (84%)	51 (86%)	44 (83%)

Table 2

Pre-Post Changes (± SEM) in Day 1 Affect and Anxiety and Day 2 Cortisol

Measure/Condition	Pre	Post
Day 1 Positive Affect (PANAS)		
Stress		
Carriers ($n = 25$)	24.37 (1.50) [*],β	22.61 (1.59) [*],β
Non-carriers ($n = 31$)	29.59 (1.22) [*]	26.94 (1.30) [*]
No Stress		
Carriers ($n = 33$)	29.41 (1.17)β	26.64 (1.25)β
Non-carriers ($n = 23$)	29.61 (1.43)	28.67 (1.53)
Day 1 Negative Affect (PANAS)		
Stress		
Carriers ($n = 25$)	13.87 (0.90)	13.54 (0.99)
Non-carriers ($n = 31$)	13.77 (0.74)	14.04 (0.81)
No Stress		
Carriers ($n = 33$)	14.09 (0.71)	13.81 (0.77)
Non-carriers ($n = 23$)	14.22 (0.86)	14.34 (0.95)
Day 1 Anxiety (SAI)		
Stress		
Carriers ($n = 25$)	34.42 (2.24)	40.02 (2.31)
Non-carriers ($n = 31$)	36.43 (1.83)	38.86 (1.89)
No Stress		
Carriers ($n = 33$)	35.57 (1.76)	36.21 (1.81)
Non-carriers ($n = 23$)	34.20 (2.15)	37.92 (2.21)
Day 2 Salivary Cortisol (nmol/l)		
Stress		
Carriers ($n = 25$)	5.81 (0.66)	6.01 (0.57)
Non-carriers ($n = 31$)	5.26 (0.54)	5.44 (0.47)
No Stress		
Carriers ($n = 33$)	4.78 (0.52)	5.57 (0.45)
Non-carriers ($n = 23$)	5.72 (0.64)	5.83 (0.55)

Note: the data for rs1360780 are presented as a representative example of all *FKBP5* SNP effects. PANAS = Positive and Negative Affect Scale; SAI = State Anxiety Inventory;

[*] main effect of stress $p < 0.05$ relative to no stress;

β main effect of genotype $p < 0.05$ relative to non-carriers.