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Jeff Quinn April, 20 2011

Posttraumatic Stress Symptoms in a Prospective Gene by Environment Study of a University Campus Shooting

by

Jeff Quinn

Kerry Ressler
Adviser

Department of Psychiatry

Kerry Ressler

Adviser

Astrid Prinz

Committee Member

Gregg Orloff

Committee Member

April, 20 2011

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Jeff Quinn

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Abstract

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On February 18th, 2008 a lone gunman opened fire in a lecture hall at Northern Illinois University (NIU) killing five and wounding 17 students before taking his own life. As part of an ongoing study at that university a large number of female students had completed several trauma related questionnaires before the shooting (n=1045). Follow-up surveys were launched 17 days after the shooting, and at several time points afterwards, providing a unique before and after perspective on these individuals' psychological response to the event. Of the individuals that completed the follow up surveys (n=691) we obtained salivary samples and successfully extracted DNA for 235. We examined this cohort two loci previous linked to PTSD, the serotonin transporter SLC6A4, and the PAC-1 receptor ADCYAP1R1. At SLC6A4, all individuals were genotyped at rs25531, for the VNTR STin2, and for the length polymorphism 5-HTTLPR. At ADCYAP1R1 we genotyped all individuals at rs2267735. Using the Distressing Events Questionnaire as our PTSD measure, we demonstrated that the change in PTSD symptom severity from pre- to post-shooting was significantly associated with rs2267735, rs25531 and the 5-HTTLPR/rs25531 multi-marker genotype ($p < 0.05$). These associations remained significant after controlling for level of shooting exposure, which was assessed during the follow up survey. These results demonstrate gene by environment interactions can be predictive for differential PTSD symptom severity. When examined in a relatively homogenous sample with shared trauma and known prior levels of child and adult trauma, these loci may serve as useful predictors of risk for PTSD-related symptoms in the weeks and months following the trauma.

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Table of Contents

Introduction:

In recent years posttraumatic stress disorder (PTSD) has gained increasing recognition as a major public health issue. PTSD is characterized in the *DSM-IV* by the appearance of three symptom clusters following an acutely traumatic event: re-experiencing, avoidance/numbing, and hyperarousal (American Psychiatric Association [*DSM-IV-TR*], 2000). Re-experiencing includes flashback memories, distressing dreams, or negative psychological or physiological response to reminders of the event. Avoidance includes avoidance of stimuli associated with the trauma, avoidance of places, things, or people that might remind the individual of the trauma, an inability to recall memories related to the trauma, an inability to experience certain feelings, and a feeling of detachment from other people. Hyperarousal includes difficulty sleeping, irritability and outbursts of anger, difficulty concentrating, and an exaggerated startle response (*DSM-IV-TR*, 2000). The persistence of these symptoms beyond a month after the traumatic event is required for the full diagnosis of PTSD. The appearance of these symptoms within a month of the traumatic event is technically classified as acute stress disorder (*DSM-IV-TR*, 2000). In the present study we will be looking at some PTSD symptom data measured within a month following the trauma, and so in those cases we will technically be studying acute stress disorder.

While PTSD has always been associated with combat veterans it is generally accepted that any trauma, including sexual or physical assault, childhood abuse, motor vehicle accident or other type of disaster can also lead to the disorder in at-risk individuals. The

lifetime prevalence of PTSD has been estimated to be 6.8% among adult Americans (Kessler 2000). The rate of PTSD is significantly higher among women compared to men; the lifetime prevalence of PTSD among women is 10.4% while for men it is only 5% (Kessler, Sonnega et al. 1995). While an episode of acute trauma is necessary to bring about PTSD, it is not sufficient. Only a minority of individuals exposed to traumatic or life-threatening events will go on to develop the disorder. Research has shown risk factors for PTSD development to include severity of perceived life threat during trauma, prior trauma, family history of psychopathology, peritraumatic emotional responses, and peritraumatic dissociation (Marmar, Weiss et al. 1994; Ozer, Best et al. 2003).

In addition to these risk factors genetics have been shown to be an important in predicting what individuals will go on to develop PTSD symptoms following a traumatic episode. The heritability of PTSD was first described among veterans of the Vietnam War through twin studies. The original study, which examined 2,224 monozygotic and 1,818 dizygotic male-male veteran pairs, calculated that genetic factors explain approximately 30% of the variance in PTSD symptoms (True, Rice et al. 1993). A more modern twin study examining 222 monozygotic and 184 dizygotic twin pairs taken from the general urban population of Vancouver was able replicate this finding of moderate heritability (Stein, Jang et al. 2002). However, while the heritability of PTSD has been generally accepted for some time, molecular genetic association studies of PTSD have been limited. In this respect, published molecular genetics research into PTSD lags behind similar research on depression, bipolar disorder, and schizophrenia. Several possible loci have been suggested, but few findings have been definitive.

In the present study we examined the relationship between genotypic variation and phenotypic variation in PTSD. We examined genotypic variation based on Single Nucleotide Polymorphisms (SNPs), Variable Number of Tandem Repeats (VNTRs), and Restriction Fragment Length Polymorphisms (RFLPs). All of these different polymorphisms can theoretically have an effect on the cellular processes that contribute to phenotype.

A SNP is a single base within the DNA strand that is commonly found to be variable in the general population. This variation can occur within the portions of DNA that are translated into protein (known as coding regions or exons) and can in turn change the amino acid sequence of a protein or introduce a premature stop codon; these are known as non-synonymous SNPs. It is equally possible for SNPs within the exon to have no effect whatsoever on amino acid sequence due to the inherent redundancy of the genetic code; in this case the SNP is referred to as synonymous. SNPs are more frequently found in the non-coding regions, which include parts of genes that are not translated into proteins (introns) and the intergenic regions. SNPs in these "non-coding" regions can still have a profound effect on protein expression if they are located in certain elements of the DNA sequence, such as those that are recognized and bound to by regulatory proteins.

VNTRs are areas of the genome comprised of one relatively short genetic sequence repeated multiple times. Between individuals the number of repeats can be variable.

VNTRs can theoretically be found in both coding and non-coding regions of the genome but are more commonly found in non-coding regions.

RFLP is a term derived from a laboratory method that involves digesting DNA with restriction enzymes, which occur naturally in bacteria and will cut DNA at very specific sequences. After the DNA is cut the fragments are separated by size via gel electrophoresis. Different sized fragments between individuals indicate a difference in DNA sequence. Restriction followed by gel electrophoresis can theoretically be used to identify both VNTRs and SNPs. If a SNP occurs within the specific sequence recognized by the restriction enzyme it will alter restriction at that site and the length of the fragment will vary allowing one to infer which allele is present. For VNTRs, if the number of repeats between two restriction sites varies the length of that fragment will also vary allowing one can infer the number of repeats present. In the present study we use restriction followed by gel electrophoresis to determine the 5-HTTLPR allele present and to determine the VNTR present at the STin2 locus.

As an alternative to the restriction method, SNPs can be quickly genotyped using fluorescence assays. In this method the sequence surrounding the SNP is amplified and a probe that is complementary to a small length of sequence including the SNP and surrounding bases is added. Depending on what base is present at the SNP the probe will anneal to the sequence and a fluorescent element attached to the probe will be activated. This fluorescence can be read by an optical device allowing one to infer the base present

at that SNP. In the present study we used fluorescence assays to genotype rs25531 within 5-HTTLPR and rs2267735 within ADCYAP1R1.

While identifying polymorphisms within the human genome is relatively straightforward, establishing a link between genotype and phenotype is a complex problem that has been extensively speculated on. Many phenotypes are affected by more than one gene; these are known as polygenic phenotypes and they can be difficult to characterize if all the relevant genes are not accounted for. However, even if all the relevant genes are accounted for measured genetic variation often only accounts for a small percentage of the overall variation of a phenotype within a population. Environmental and unobserved genetic variation can confound an attempt to demonstrate a relationship between genotype and phenotype. Environmental variations between individuals can be a confounding factor because they often mediate the effect of genotype on phenotype. The interactions between genetic variables and environmental variables are known as gene by environment or G x E interactions.

Even if all the relevant genetic and environmental variance can be observed the link between genotype and phenotype can be confounded if genes are interacting in an unknown way. The phenomenon of one gene modifying the effect of another is known as epistasis. These individual interactions are known as gene by gene or G x G interactions, and they can often be difficult to account for statistically as they are representative of complex and poorly understood underlying biological pathways (Cordell 2002). Compounding these problems is the phenomenon of genetic linkage wherein particular

alleles are more commonly found together than they would be by chance. This makes a proof of causality in genetics especially difficult because one must determine if the observed genetic variation is actually causing the phenotypic variation or if it is simply linked to another locus containing truly causal unobserved genetic variation.

Geneticists most commonly rely upon statistical association to establish the relationship between a genotype and phenotype of interest, and that is the approach we have used in the present study. Specifically we used a general linear model to test the significance of our hypothesis. In the model the phenotype as either a categorical (ex. Presence or absence of a disease) or continuous (ex. Height) measure is considered the dependant variable or regressand while genotype (considered categorically) is considered the independent variable or regressor. The general linear model allows for statistical hypothesis testing and produces a measure of significance in the form of a p-value. A p-value of 0.05 or less is generally taken to signal a significant association. This value is the probability of obtaining a result at least as extreme as the one tested. Because the test is done against the null hypothesis of no association between genotype and phenotype a low p-value means it is unlikely that phenotype and genotype are unrelated. If a single locus is found to be statistically associated with a phenotype that locus is said to have a significant main effect on the phenotype.

Using a general linear model an environmental variable can be added to the analysis as an additional regressor. If the genetic variable is still significantly associated after the addition of the environmental regressor one can argue for the presence of a G x E

interaction between the genotype and the environmental variable to predict the phenotype. In the present study we used this method to demonstrate the G x E interaction between 5-HTTLPR and shooting exposure. We also employed a statistical test known as a one-way analysis of variance (ANOVA) when testing for genetic main effects. This test provides essentially the same statistics as the general linear model for our purposes. Additionally we performed permutation analysis for some of our analyses. Permutation analysis involves performing the statistical test many times on different rearrangements of the dependant and independent variables. The proportion of times where the p-value is less than or equal to the original p-value is interpreted at the permutation p-value. This process can be used to rule out the argument that a p-value is influenced by implicit assumptions in the test of significance of an underlying normal distribution in the data.

For psychiatric disorders such as PTSD consideration of G x E interactions is critical to establishing a genetic association. G x E interactions are seen as a promising tool for investigating the two-fold etiology of PTSD because the environmental pathogen (acute trauma) is known, is required for the diagnosis, and has the potential to be measured (Norrholm and Ressler 2009). There is good precedent in the literature for successful G x E studies of psychiatric phenotypes. One study by Caspi et al. looked at the interaction between of stressful life events and 5-HTTLPR genotype and found this G x E to be significantly associated with depression in a study of 1037 individuals (Caspi, Sugden et al. 2003). Xie et al. looked at the interaction between 5-HTTLPR and childhood trauma found and this G x E interaction to be significantly associated with PTSD in a study of 582 European Americans and 670 African Americans (Xie, Kranzler et al. 2009).

Part of the reason for the lack of literature concerning PTSD is the inherent difficulty of studying the disorder. Because PTSD is a psychiatric illness that is dependent on an environmental pathogen, genetic association studies are significantly more complex as compared to other diseases. This complexity has resulted in methodological inconsistencies in the current literature and caused many positive results to be called into question. Because there are so many different types of trauma that can result in PTSD symptoms it is difficult to normalize trauma exposure within a study population. This can be a confounding factor because different types of trauma exposure have been shown to have different effects on PTSD outcomes. For example childhood trauma has been shown to carry a greater risk of developing the disorder compared to adult trauma (Ozer, Best et al. 2003), and trauma involving interpersonal violence has also been shown to carry a greater risk compared to other trauma types (Breslau, Davis et al. 1991). Also heritability studies have demonstrated that greater genetic influence in cases of PTSD resulting from interpersonal trauma compared to other types (Stein, Jang et al. 2002).

In the present study we examine the locus SLC6A4, which codes for the serotonin transporter protein (5HTT). This protein functions to transport serotonin out of the synaptic cleft back into the presynaptic neuron and thus is a regulator of serotonergic neural activity (Ahmed, Bukhari et al. 2009). There is good precedent in the literature for studying this protein. Out of the roughly 30 molecular genetics studies of PTSD conducted so far, 18 have focused on dopaminergic and serotinergic systems (Cornelis, Nugent et al. 2010). Half of these studies have investigated the locus SLC6A4. Out of

these 9 studies that investigated SLC6A4, 8 found significant associations between PTSD symptoms and polymorphisms within the locus (Lee, Lee et al. 2005; Kilpatrick, Koenen et al. 2007; Lee and Santacroce 2007; Grabe, Spitzer et al. 2009; Koenen, Aiello et al. 2009; Mellman, Alim et al. 2009; Thakur, Joober et al. 2009; Xie, Kranzler et al. 2009). SLC6A4 is a good biological candidate gene for PTSD because of the important role serotonin plays in regulating the hypothalamic-pituitary-adrenal axis (HPA axis). The HPA axis controls reactions to stress and is generally thought to regulate mood and emotion. In previous studies SLC6A4 has been linked to a variety of conditions including migraine headaches (Schurks, Rist et al. 2010), alcohol dependence (McHugh, Hofmann et al. 2010), schizophrenia (Vijayan, Iwayama et al. 2009), bipolar disorder (Manchia, Zai et al. 2010), and depression (Caspi, Sugden et al. 2003; Risch, Herrell et al. 2009; Manchia, Zai et al. 2010).

In particular we are examining two polymorphisms within the promoter region of the SLC6A4 gene. While promoter regions do not code for any protein, variation within them can affect the expression proteins downstream from them. The polymorphisms we examined are contained within the 5-HTTLPR (serotonin-transporter linked polymorphic region) and are the most commonly studied polymorphisms within SLC6A4. The first polymorphism is an insertion/deletion within 5-HTTLPR with two common variations known as long (L) and short (S). The presence of the L variation versus the S variation is known affect expression of the serotonin transporter protein, with the S thought to lead to decreased 5-HTT expression, and a higher risk for psychopathology (Heils, Teufel et al. 1996). The second polymorphism is a SNP rs25531 that is found within the variable

length region of 5-HTTLPR. Studies have shown that this SNP may further modulate 5-HTT expression, with L_G alleles being equivalent to S alleles in expression (Hu, Lipsky et al. 2006; Wendland, Kruse et al. 2006). The length polymorphism along with rs25531 are considered together to form a classification of four different alleles ($L_A/L_G/S_A/S_G$) commonly used in research involving this locus (Parsey, Hastings et al. 2006). In this study, we performed analyses using this multi-marker genotype, as well as considering the length polymorphism and rs25531 separately. The third polymorphism we studied is not found in the promoter region; it is a VNTR within the second intron of SLC6A4 known as STin2. This polymorphism is less frequently described in the literature, but has been shown to modulate expression in vitro along with 5-HTTLPR (Ali, Vasiliou et al. 2010). We observed three common copy numbers within STin2 of 9, 10, and 12 repeats.

In the present study we also examine the locus ADCYAP1R1 (pituitary adenylate cyclase activating polypeptide receptor) which codes for the PACAP (pituitary adenylate cyclase activating polypeptide) receptor PAC1. PACAP is a polypeptide that acts as a neurotransmitter and a neuromodulator. It was originally observed to stimulate cAMP production in rat pituitary cells (Miyata, Arimura et al. 1989). PAC1 receptors are abundant in the brain, the pituitary, and the adrenal glands, and PACAP can be found throughout the body and even in the blood (Vaudry, Falluel-Morel et al. 2009). PACAP-PAC1 signaling has been shown to regulate corticotropin releasing hormone and have a role in stress related behavior (Hammack, Cheung et al. 2009; Vaudry, Falluel-Morel et al. 2009). Ressler et al. 2011 has shown that polymorphisms within both ADCYAP1R1

and ADCYAP1 are significantly associated with PTSD in females (Ressler, Mercer et al. 2011).

The particular polymorphism we examined was the SNP rs2267735 which is located within an intron in the ADCYAPR1 locus. Ressler et al. found rs2267735 to be significantly associated with PTSD in females, with the CC genotype the most strongly associated with higher total PTSD symptoms. ADCYAP1R1 expression levels were also found to be associated with rs2267735 with CC individuals having the lowest expression (Ressler, Mercer et al. 2011). The sex-specific nature of this effect may be due to the fact that ADCYAP1R1 expression has been shown to be regulated by oestrogen and rs2267735 is located within a predicted estrogen response element (ERE) (Aenlle, Kumar et al. 2009).

Materials/Methods:

Study Population:

This unique cohort consists of students at North Illinois University who were exposed to the tragic events that unfolded on campus on February 18th, 2008. On that day Steven Phillip Kazmierczak, a former NIU student, walked into a crowded lecture hall where class was in session and opened fire on the audience, killing 5 and injuring 17 before taking his own life. A subset of the students on campus at the time were involved in a previous study by Stephenson et al. (Stephenson, Valentiner et al. 2009). As a result of being involved in this study these students had been interviewed and had filled out

several psychological questionnaires prior to the shooting. These students were then interviewed and completed the questionnaires again several times following the shooting. Additionally, a questionnaire was administered to determine each individual's proximity to the shooting. In total all individuals were interviewed on at least three occasions, prior to the shooting (time point 1), 2-13 weeks post-shooting (mean = 3.2 weeks, time point 2), and 8-12 months following time point 2 (mean 8.4 months, time point 3).

Phenotype Measures:

Previous findings suggest that prior life trauma can strongly impact the development of psychological outcomes such as depression, anxiety or PTSD (Caspi, Sugden et al. 2003). As a result of the previous study pre-trauma measures were taken for each participant at time point 1. These measures included an assessment of child abuse including physical, psychological and sexual abuse. The number of non-child abuse related traumas were also recorded. To measure all previous trauma exposure, we also examined the Traumatic Life Events Questionnaire or TLEQ and the Distressing Events Questionnaire (Kubany, Haynes et al. 2000; Kubany, Leisen et al. 2000). Since childhood sexual abuse and physical abuse are separate line items in the questionnaire, we were able to separate them from other types of trauma in order to examine them separately. We excluded individuals from our final analysis if their DEQ score at time point 1 was greater than or equal to 18. Participants also completed a 12-item measure of exposure, modified from the Littleton, Grills-Taquechel, and Axsom Virginia Tech shooting exposure measure (Littleton, Grills-Taquechel et al. 2009). An exposure variable was created by summing across yes/no items assessing participants' personally experienced exposure to aspects of

the shooting (e.g., on campus, heard gunfire, saw individuals who had been wounded or killed, knew anyone wounded, in building placed on lockdown).

We used the Distressing Events Questionnaire (DEQ) as our measure of PTSD symptoms at all post-shooting time points. Using a 5-point response scale, the DEQ assesses symptoms of PTSD experienced in the past 30 days as specified in the *DSM-IV* (*DSM-IV-TR*, 2000). For Time 2, participants were instructed to answer the DEQ with respect to the campus shooting event. The DEQ has demonstrated very good psychometric properties and provides subscores specific for each symptom cluster: Hyperarousal, Reexperiencing, Avoidance and Numbing (Kubany, Leisen et al. 2000).

An increase of greater than 18 points in DEQ score from time point 1 to time point 2 was considered clinically significant (Kubany, Leisen et al. 2000). Based on the *DSM-IV* diagnosis criteria for PTSD, an individual must have symptoms for 30 days or more (*DSM-IV-TR*, 2000). However, a majority of our sample population had their first post-shooting interview prior to this 1-month cut-off (78%). This limited the power we had to detect factors that could significantly correlate with clinical PTSD. Hence, for some analyses, results presented for those individuals who were interviewed 2-4 weeks post-shooting at time point 2 will refer to acute stress disorder, while results for those individuals who were interviewed 1-month or later post-shooting will refer to PTSD.

<u>DNA Extraction and Genotyping:</u>

Of the original 1045 involved in the study at the time of the shooting, 812 agreed to be re-interviewed and were confirmed as current NIU students at the time of the mass shooting. Seventeen days after the mass shooting, an online follow-up survey was launched and was completed by 85% of those invited (n = 691). Saliva samples were collected from 298 of these individuals using Oragene saliva collection tubes (DNA Genotek, Inc., Ottawa, Ontario, Canada), and DNA was successfully extracted from 235 individuals. This sample was all female, between the ages of 18 and 45 (average age 20.1). Race was self reported as 77.5% White (n=158) and 13.7% Black (n=28). Other races accounted for 8.9% (N=18).

Samples were mailed from NIU to Emory University where DNA was extracted using Agencourt DNAdvance kit (Beckman Coulter, Inc., Brea, California, USA). Samples with DNA concentration below 10ng/ul were excluded. In some cases a second saliva sample was obtained and used for DNA extraction to replace one with a low concentration.

Genotypes at SLC6A4 were successfully obtained for 235 individuals. Individuals were genotyped using duplex PCR with two sets of primers taken from Wendland et al.: one set for the 5-HTTLPR locus (5'TCCTCCGCTTTGGCGCCTCTTCC-3') and (5'-TGGGGGTTGCAGGGGAGATCCTG-3') and one set for the Intron 2 VNTR (5'-GGGCAATGTCTGGCGCTTCCCCTACATA-3') and (5'-TTCTGGCCTCTCAAGAGGACCTACAGC-3') (Wendland, Martin et al. 2006). All primers were obtained from Integrated DNA Technologies, Inc., Coralville, Iowa, USA.

5-HTTLPR PCR resulted in amplicons of 469 bp for the S allele and 512 bp for the L allele, for the Intron 2 VNTR, PCR resulted in amplicons of 250 bp for STin2.9, 267 bp for STin.10, and 300 bp for STin2.12. Genotypes for the 5-HTTLPR and Intron 2 VNTR loci were determined by gel electrophoresis. PCR was then digested using *Msp*I (New England Biolabs, Inc., Ipswich, Massachusetts, USA) resulting in fragments of 402 + 67 bp for S_G and 402 + 10 bp for L_G, samples were run by gel electrophoresis again to determine genotype at rs25531. Out of all samples genotyped 27% (n=75) at random were run in duplicate with 2.6% discordance.

The combined 5-HTTLPR multi-marker genotype is reported to have an effect on 5-HTT mRNA with the order of expression as follows: S/S, L_G/L_G, S/L_G, L_A/L_G, S/L_A, L_A/L_A (Hu, Lipsky et al. 2006). For our analysis we divided these genotypes based on their expression profile and compared S/S, L_G/L_G, and L_G/S against L_A/L_A, L_G/L_A and L/S. Since the S and L_G alleles are reported to have similarly low expression, this model compares low expression homozygotes to high expression hetero- and homozygotes.

Genotypes at ADCYAP1R1 were successfully obtained for 246 out of 251 individuals. Individuals were genotyped using TaqMan® SNP Genotyping Assays (Applied Biosystems). We used made-to-order primers for rs2267735 from Applied Biosystems (Assay ID: C__15872945_10). All individuals were genotyped in duplicate. 7.3% of individuals who went uncalled in one assay were positively called in the alternate assay. There was no discordance in genotype calls between the two assays.

Statistical Analysis:

SPSS 17.0 and R were used to perform all statistical analyses. Individuals who were interviewed 2-4 weeks post-shooting and those who were interviewed greater than 1 month post-shooting were analyzed separately in accordance with the *DSM-IV* definitions of acute stress disorder and post-traumatic stress disorder (*DSM-IV-TR*, 2000). Our dependent variable for all analyses was the difference in DEQ score between time point 2 and time point 1 (delta DEQ). Using univariate ANOVA, we tested for genetic main effects at all three SLC6A4 loci (STin2, 5-HTTLPR, and rs25531) and the 5-HTTLPR/rs25531 multi-marker genotype (L_A/L_A, L_A/L_G, and L/S versus L_G/L_G, L_G/S, and S/S), and also at rs2267735. In our final analysis we used a general linear model and controlled for shooting exposure by including the number of high exposure events experienced as our environmental variable. A high exposure event is defined as any one of the 5 events significantly related to the change in PTSD symptoms from baseline to post-shooting (statistical significance shown in Figure 1). The environmental variable for shooting exposure was created by summing the number of positive responses to all of the 5 exposure types. Individuals who had no positive response to any of the 5 questions were considered to have zero high exposures. Additionally, due to allele frequency differences between races, some analyses were performed for all participants as well as for Whites (the most common self-identified race) separately. We then ran this analysis using the difference in DEQ score between time point 2 and time point 1 for the subcategories of PTSD (hyperarousal, reexperiencing, and avoidance) as our dependant variable.

To confirm that the analytical p-values were not dependent on distributional assumptions, we computed empirical p-values via permutation tests for some analyses. In each of 10,000 permutations of the dataset, we re-performed the data analysis after randomly re-assigning values of the dependent variable. The empirical p-value was then computed as the proportion of permutations for which we observed a genetic association of greater or equal strength to that originally observed.

Results:

Demographics:

The primary study sample (N=691) that was initially interviewed at time point 2 was described in detail by Stephenson et al. (Stephenson, Valentiner et al. 2009). Out of the total study sample, 41% (n=280) had a total score of 18 or more on the DEQ at time point 2, indicating that a large portion of the sample had clinical symptoms of PTSD (Kubany, Leisen et al. 2000). Complete DNA extractions were obtained for 276 participants. We were able to obtain useable DNA for 269 of these participants (based on a qualifying DNA concentration >10ng/μl). In the analysis concerning SLC6A4 only participants with confident genotype calls for all three loci were included. We further limited the study set to 235 participants that had complete phenotype measures. Participants that presented with PTSD symptoms at time point 1, as defined by a DEQ score greater than or equal to 18, were excluded (n=31) so as to only include subjects who did not have clinically significant PTSD prior to the shooting. This left us with a cohort of 204 individuals that were used for SLC6A4 analyses (Table 1). Only 192 individuals out of these 204 were

successfully genotyped at ADCYAP1R1, and thus only 192 individuals were used in the final analyses of rs2267735. All participants were female. Out of the 204 individuals 77.5% (n=158) self-identified as white, and 13.7% (n=28) self-identified as black. The average age was 20 years. Comparisons of the included participants (n = 204), as compared to the other participants (n = 487) on key variables indicated that included participants reported slightly higher PTSD symptoms at time point 2 and were more likely to be White but did not differ in age or the shooting exposure variable.

Pre-Shooting Trauma:

Out of the 204 individuals included in the study, 74.5% (n=152) reported no child abuse before the age of 18. 1.5% of the individuals reported experiencing two or more abuse types that involved fear, helplessness or horror, while 10% experienced sexual abuse involving fear, helplessness or horror before the age of 15, including abuse by a peer. Less than 5% had experienced physical abuse more than once a year. Comparing the number of individuals who had ever experienced child abuse in the form of sexual abuse (before the age of 18), physical abuse (14 or younger) or psychological abuse (14 or younger), there was no statistically significant difference between the group that developed a clinical level of PTSD symptoms following the shooting and the group that did not ($p > .10$). The number of pre-shooting traumatic events as measured by the TLEQ was not significantly associated with more severe PTSD symptoms post-shooting, with the low symptom group experiencing an average of 2.2 traumatic events (standard deviation = 2.04) and the high-symptom group experiencing an average of 2.6 (standard deviation = 2) traumatic events.

Shooting Exposure:

Out of the 11 questions the students were asked regarding the shooting, we found that positive responses to the following 5 were significantly associated with outcome for PTSD at 2-4 weeks: Were you in Cole Hall (where the mass shooting took place) during the shooting? ($p<.05$, #7), Did you hear the sound of gunfire? ($p<.005$, #8), Did you see the gunman fire on someone? ($p<.05$, #9), Did you see the gunman during the shooting? ($p<.0005$, #10), Were you hurt in shooting? ($p<.005$, #11) (Figure 1). For the statistical G x E analysis we used the number of positive responses to these five questions as our exposure variable.

Effect of SLC6A4 on PTSD Symptoms:

Genotypes for all three SLC6A4 loci examined were in Hardy Weinberg equilibrium (for Whites, $p>0.05$, Table 2). 5-HTTLPR allele frequencies match previously reported findings for European or European- Americans (Gelernter, Cubells et al. 1999; Nakamura, Ueno et al. 2000; Koenen, Aiello et al. 2009). The STin2 frequencies closely matched those reported in Europeans (Gelernter, Cubells et al. 1999; Wendland, Kruse et al. 2006; Wendland, Martin et al. 2006). The 5-HTTLPR multi-marker genotype frequencies matched well with the frequencies reported by Wendland and colleagues for a similar sample size of Caucasian Americans (Wendland, Kruse et al. 2006). Our frequencies: SA= 42.4%. SG=0.60%, LA=49.7%, LG=7.3% compared to Wendland et al: SA=43.2%, SG=0.25%, LA=50.0%, LG=6.5%. Genotypes for rs2267735 in ADCYAP1R1 were also in Hardy Weinberg equilibrium ($p = .6649$). Our rs2267735

allele frequencies matched those reported in dbSNP. Our frequencies: C = 50.0%, G = 50.0% compared to C = 53.7%, G = 46.3% (dbSNP, Build ID: 37.2).

We next examined the effect of the 5-HTTLPR genotypes on delta DEQ (n=204). Genotypes were examined for all races together and for self-reported whites only to control for possible population stratification. We did not observe main effects for STin2 or 5-HTTLPR alone on delta DEQ (Table 3). However, both rs25531 and the 5-HTTLPR multi-marker genotype were significantly associated for all races (p=0.032 and p=0.011) (Table 3). rs25531 had a significant main effect on delta DEQ when examined in both an additive allelic (p=0.011) and in a dominant/recessive fashion (p=0.003). Additionally, individuals carrying two risk alleles (L_G or S) had significantly higher PTSD symptoms post-shooting (mean = 23.91) compared to those with one or zero risk alleles (mean =18.42, Figure 2A).

As described above, we had observed a significant correlation between positive responses to certain exposure questions and delta DEQ. We next examined the G x E relationship between the individual 5-HTTLPR genotypes and shooting exposure (variable representing number of positive responses to 5 high-exposure questions) on delta DEQ. We found rs25531 in both dominant and additive models (p=0.006 and p=0.005 respectively) showed a significant G x E association. The 5-HTTLPR multi-marker genotype also showed a significant G x E association (p=0.004, Figure 2). Permutation tests confirmed that this result was not dependent on distributional assumptions (permutation p=0.0069). Surprisingly, despite the low number of individuals that actually

experience the shooting more directly (5%, n=11), this variable still contributes heavily to the genotype effect. It is important to note that there is no correlation between genotype and shooting exposure (r=0.005).

Effect of ADCYAP1R1 on PTSD Symptoms:

We also examined the effect of rs2267735 genotype in ADCYAP1R1 on delta DEQ (n=192, additive model), again looking at all races and self-reported whites only to control for population stratification. We did not analyze individuals separately based on the length of time between the shooting and time point 2. We found rs2267735 to be marginally associated with delta DEQ ($p = 0.0496$ for all races, $p = 0.0144$ for whites only, Table 3). We found GG individuals to have a significantly higher DEQ score (mean = 21.82) at time point 2 compared to CC and GC individuals (mean=17.2) (Figure 2B).

We also examined the G x E relation between rs2267735 and shooting exposure on delta DEQ (additive model). We found a significant interaction for all individuals ($p = 0.00764$, $n = 192$) and whites only ($p = 0.0158$, $n = 146$) (Table 4). Permutation tests confirmed that this result was not dependant on distributional assumptions. The permutation p-value for whites was 0.0153, for all races the permutation p-value was 0.0079.

As mentioned earlier, we were also able to obtain measurements for hyperarousal, reexperiencing and avoidance subscales of PTSD symptoms. These differential symptom clusters as represented on the DEQ were analyzed separately to determine which types of

symptoms were predominant in our genetic risk group. For the 5-HTTLPR multi-marker genotype reexperiencing symptoms were not significantly associated, hyperarousal symptoms were statistically significant ($p < 0.05$), but the greatest effect was seen for avoidance symptoms ($p=0.003$, Table 5). Hyperarousal symptoms were not significantly associated with rs2267735, but avoidance and reexperiencing symptoms were both significantly associated for all races and Whites only ($p < 0.05$) (Table 5).

Discussion:

One of the most critical questions surrounding PTSD and acute stress disorder is why some individuals go on to develop the disorder following a trauma while others are resilient. While twin-studies have established the heritability of PTSD (True, Rice et al. 1993; Stein, Jang et al. 2002) demonstrating how specific genetic factors are involved is complicated compared to similar disorders. It is difficult to normalize the level of trauma exposure across a sample set, self-reported symptoms and personal histories can be unreliable, and baseline levels of PTSD symptoms prior to the index trauma are often impossible to establish. We believe that the unique nature of this cohort, while perhaps not entirely eliminating these problems, may substantially decrease the influence of these confounding factors.

In examining our population of female college students exposed to the tragic NIU campus shooting we were able to establish associations between polymorphisms in the serotonin transporter promoter region and the PAC-1 receptor gene and PTSD outcomes

post-trauma. We were able to establish significant main effects for rs25531, the 5-HTTLPR multi-marker genotype, and rs2267735. After demonstrating that proximity to the shooting was significantly related to PTSD outcomes we were able to account for this proximity variable our final analysis. This strengthened our results for the 5-HTTLPR multi-marker genotype and for rs2267735, allowing us to establish a G x E effect on PTSD outcomes.

Genetics have been clearly shown to play an important role in the differential risk for development of PTSD. The SLC6A4 polymorphisms are of particular interest in relation to not only PTSD but several other psychiatric illnesses. Concerning PTSD and acute stress disorder, the majority of findings implicate the S variant of 5-HTTLPR as a risk factor for developing PTSD. This seems biologically plausible given that the presence of one and two S alleles reduces the expression of 5HTT by 27% and 30%, respectively (Hranilovic, Stefulj et al. 2004), and the S allele associates with greater amygdala activity in response to emotional stimuli (Heinz, Braus et al. 2005). Some studies have offered conflicting evidence against S as a risk allele, but they are in the minority (Grabe, Spitzer et al. 2009; Thakur, Joober et al. 2009). rs25531 (A/G) has also been examined separately for its role in mental health (Mellman, Alim et al. 2009; Bryant, Felmingham et al. 2010). It has been hypothesized that the G allele creates an AP2 binding site which, in turn, can decrease transcription (Hu, Lipsky et al. 2006). Additionally, rs25531 has been shown to have an effect on amygdala activity in response to angry, happy or sad emotions (Dannlowski, Ohrmann et al. 2007). Compared to the other two loci there is less literature concerning STin2, but this polymorphism has been connected with harm avoidance,

suicidality, psychosis, and migraine headaches (Lopez de Lara, Dumais et al. 2006; Proitsi, Lupton et al. 2010; Saiz, Garcia-Portilla et al. 2010; Schurks, Rist et al. 2010). Thus, despite evidence that this polymorphism is likely functional (Karg, Burmeister et al. 2011), to our knowledge there is no strong evidence associating this variant with PTSD.

rs2267735 in ADCYAP1R1 has been shown to be significantly associated with PTSD in an all female cohort by Ressler et al (Ressler, Mercer et al. 2011). This is biologically plausible given that PACAP-PAC1 signaling has been shown to regulate stress related behavior (Hammack, Cheung et al. 2009; Vaudry, Falluel-Morel et al. 2009). Ressler et al. identified C as the risk allele at rs2267735 for increased PTSD symptom levels and CC individuals were shown to have significantly higher PTSD symptoms compared to CG and GG individuals. Our study produced the opposite results, with G emerging as the risk allele and GG individuals having the significantly increased PTSD symptoms. This could be due to several reasons. The study in Ressler et al. used a different scale to measure PTSD symptoms, the Clinician-Administered PTSD Scale (Foa and Tolin 2000), and the study examined a general population unassociated with any common index trauma. More probably however, Ressler et al examined an almost exclusively African-American population while we examined a mostly White population. Population stratification and a resulting difference in allele frequencies could account for our different results. According to dbSNP, the allele frequencies for YRI individuals (Yoruba in Ibadan, Nigeria) are 34.5% for G and 65.5% for C, while the frequencies for CEU individuals (Utah residents with Northern and Western European heritage) are

52.7% for G and 47.3% for C (dbSNP, Build ID: 37.2).

One of the limitations of our study was the fact that we were dealing with an all-female population. However, we believe this is not a major weakness for several reasons. Firstly, the lifetime prevalence of PTSD is known to be significantly higher in women, thus we believe the use of an all female cohort did not diminish our power to detect genetic associations (Kessler 2000). Furthermore, there has been shown to be gender effect with stressful circumstances increasing susceptibility to depression in females who carry the S allele (Sjoberg, Nilsson et al. 2006). Additionally, the previous association between ADCYAP1R1 and PTSD established by Ressler et al. was only significant for females.

Another limitation of our study is our sample size. Although we were unable to obtain saliva samples for the majority of those in the primary study population, we are continuing to make efforts to genotype more members of this study sample for future research. Despite its small size, this study population was uniquely predisposed towards the analysis of PTSD G x E interactions. Most studies of PTSD in the literature that attempt to look at a specific common index trauma have similar or smaller sample sizes (Thakur, Joober et al. 2009; Kolassa, Ertl et al. 2010; Sayin, Kucukyildirim et al. 2010).

Despite these obvious limitations our study had several unique advantages predisposing it to the analysis of PTSD G x E interactions. One major advantage is the fact that we were able to examine individuals who had all been exposed to a similar type of trauma. This is important considering data showing that different trauma types can have differential

effects on PTSD outcomes (Breslau, Davis et al. 1991; Stein, Jang et al. 2002; Ozer, Best et al. 2003). Furthermore, we were able to detail each individual's level of trauma exposure using our shooting exposure survey. The fact that we were able to examine a cohort that had been assessed both prior to and after a trauma for PTSD symptoms carries several advantages. This allowed us to eliminate individuals from the study who already had high levels PTSD symptoms. These types of individuals can act as a confounding factor because they can display a differential response to the index trauma. This also allowed us to look at change in PTSD symptoms as our dependant variable, using the pre-shooting baseline PTSD symptom levels to reduce noise. Additionally the majority of our subjects (78%) were interviewed within 2-4 weeks after the shooting at time point 2. This is an improvement over many studies of PTSD in the literature that rely upon recollections of trauma occurring years before the interview. These types of retrospective measures can be unreliable and can be a major confounding factor in a genetic analysis. We believe establishing a genetic link to PTSD will not be possible without prospective studies such as ours that address some of the numerous confounding factors that surround the disorder.

In summary, the examination of a population for which we had information on lifetime trauma and pre-trauma PTSD symptoms allowed for a unique, prospective G x E study. These data identify SLC6A4 and ADCYAP1R1 as being associated with differential response to a severe trauma. When examined in a relatively homogenous sample with shared trauma and known prior levels of child and adult trauma, the polymorphisms identified in our study may serve as a useful predictor of risk for PTSD-related symptoms

in the weeks and months following the trauma.

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Table 1:

	All Individuals		Individuals with delta DEQ < 18		Individuals with delta DEQ >= 18	
Sample Size	204		138		66	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Age[a]	20.1	2.6	20	2	20.3	3.7
Race	**N**	**%**	**N**	**%**	**N**	**%**
White	158	77.5	105	76.1	53	80.3
Black	28	13.7	21	15.2	7	10.6
Hispanic	4	2	2	1.4	2	3
Asian	6	2.9	5	3.6	1	1.5
Assyrian	1	0.5	0	0	1	1.5
Multiple	5	2.5	4	2.9	1	1.5
Not Reported	2	1	1	0.7	1	1.5
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Pre-Shooting Traumatic Events	2.3	2.04	2.2	2	2.6	2.2
Child Abuse Experienced[b]	**N**[c]	**%**	**N**	**%**	**N**	**%**
Psychological	109	53.4	70	50.7	39	59.1
Physical	21	10.3	15	10.9	6	9.1
Sexual	43	21.1	26	18.8	17	25.8

Table 1. Demographic information for study population (N=204). Study population was an all female, predominantly white, college aged cohort with similar trauma history. [a] measure was taken at time point 2. [b] Excludes traumatic events from child abuse before the age of 15. [c] N represents the number of subjects ever abused; % is the number of abused versus the total.

Table 2:

	All Races		Whites Only	
	N	**%**	**N**	**%**
Stin2				
12/12	75	36.8	51	32.3
12/10	98	48.0	78	49.4
12/9	5	2.5	5	3.2
10/10	26	12.7	24	15.2
5-HTTLPR length polymorphism				
L/L	65	31.9	48	30.4
L/S	106	52.0	84	53.2
S/S	33	16.2	26	16.5
rs25531				
A/A	168	82.4	132	83.5
A/G	31	15.2	23	12.8
G/G	3	1.5	1	0.6
5-HTTLPR multi-marker				
S/S	33	16.2	26	16.5
L_G/L_G	2	1.0	0	0
S/L_G	17	8.3	13	8.2
L_A/L_G	14	6.9	10	6.3
S/L_A	89	43.6	71	44.9
L_A/L_A	49	24.0	38	24.1
rs2267735				
G/G	46	23.9	42	28.8
G/C	100	52.1	77	52.7
C/C	46	23.9	27	18.49

Table 2. Genotype frequencies for study population at three SLC6A4 loci. 5HTTLPR multi-marker genotype refers to the genotype comprised of 5-HTTLPR and rs25531.

Table 3:

	Time point 2 < 4 weeks		Time point 2 > 4 weeks	
	All Races	Whites Only	All Races	Whites Only
Sample Size	161	123	43	35
	p-values			
STin2				
12/12 vs 12/10 vs 12/9 vs 10/10	0.81	.896	.804	.549
5-HTTLPR length polymorphism				
L/L vs L/S vs S/S	.313	.237	.987	.829
L/L and L/S vs S/S	.131	.092	.967	.809
rs25531				
A/A vs A/G vs G/G	0.011*	0.004***	0.651	0.499
A/A vs A/G and G/G	0.003***	0.003***	0.841	0.499
5-HTTLPR multi-marker				
S/S, L_G/L_G, and S/L_G vs L_A/L_G, S/L_A, and L_A/L_A	0.032*	0.006**	0.605	0.395
	All Races, all time point 2	Whites Only, all time point 2		
Sample Size	192	146		
rs2267735				
C/C vs C/G vs G/G	0.0496*	0.0144*		

Table 3. Main effect of genotypes on difference in DEQ score between time points 1 and 2. Sample population was divided into two groups for SLC6A4 analysis; those who were interviewed 2-4 weeks post shooting and those who were interviewed > 1 month post-shooting. Due to potential population stratification all races and whites are analyzed separately (Significance, *p <.05 **p<.01, ***p<.005).

Table 4:

	Whites only, time point 2 < 4 weeks	Whites only, time point 2 > 4 weeks
Sample Size	123	35
	p-values	
STin2		
12/12 vs 12/10 vs 12/9 vs 10/10	0.8	0.464
5-HTTLPR length polymorphism		
L/L vs L/S vs S/S	0.152	0.859
L/L and L/S vs S/S	0.055	0.824
rs25531		
A/A vs A/G vs G/G	0.006**	0.491
A/A vs A/G and G/G	0.005**	0.491
5-HTTLPR multi-marker		
S/S, L_G/L_G, and S/L_G vs L_A/L_G, S/L_A, and L_A/L_A	0.004**	0.413
	All Races, all time point 2	Whites Only, all time point 2
Sample Size	192	146
rs2267735		
C/C vs C/G vs G/G	0.00764**	0.0158*

Table 4. Results for general linear model comparing the effect of genotype and shooting exposure on delta DEQ. Sample population was divided into two groups for SLC6A4 analysis; those who were interviewed 2-4 weeks post shooting and those who were interviewed > 1 month post-shooting (Significance, *p<.05, **p<.01).

Table 5:

	Whites only, time point 2 < 4 weeks	Whites only, time point 2 > 4 weeks
Sample Size	123	35
	p-values	
5-HTTLPR multi-marker		
hyperarousal	0.019*	0.308
reexperiencing	0.071	0.117
avoidance	0.002**	0.140
	All Races, all time point 2	Whites Only, all time point 2
Sample Size	192	146
rs2267735		
hyperarousal	0.298	0.488
reexperiencing	0.00312**	0.0106*
avoidance	0.00891*	0.00891*

Table 5. Results for general linear model comparing the effect of the 5-HTTLPR multi-marker genotype and shooting exposure on delta DEQ subscale symptom score. Sample population was divided into two groups for SLC6A4 analysis; those who were interviewed 2-4 weeks post shooting and those who were interviewed > 1 month post-shooting (Significance, *p<.05, ***p<.005).

Figure 1:

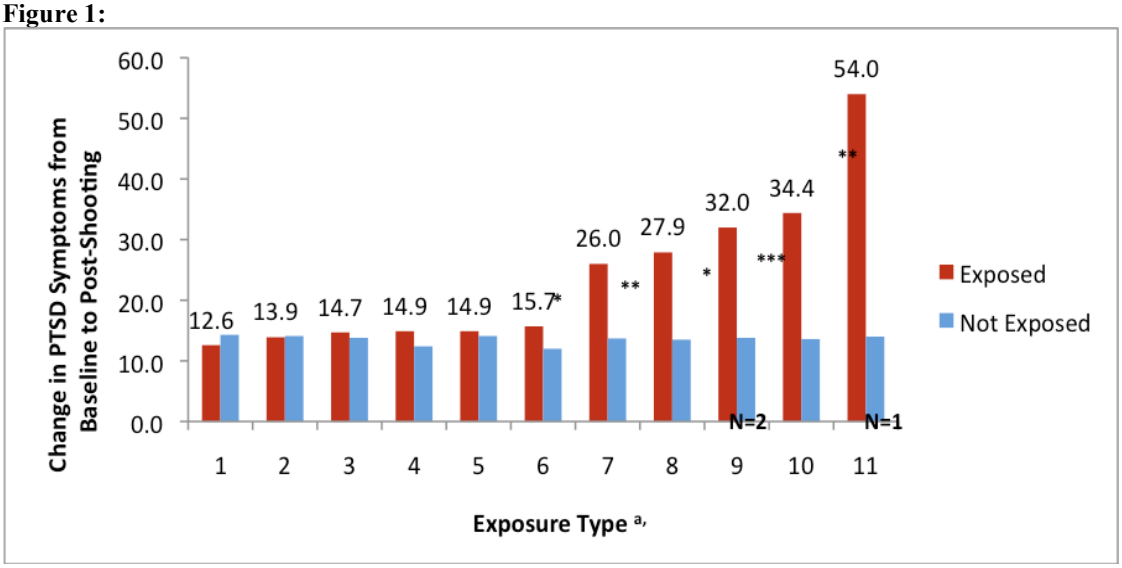
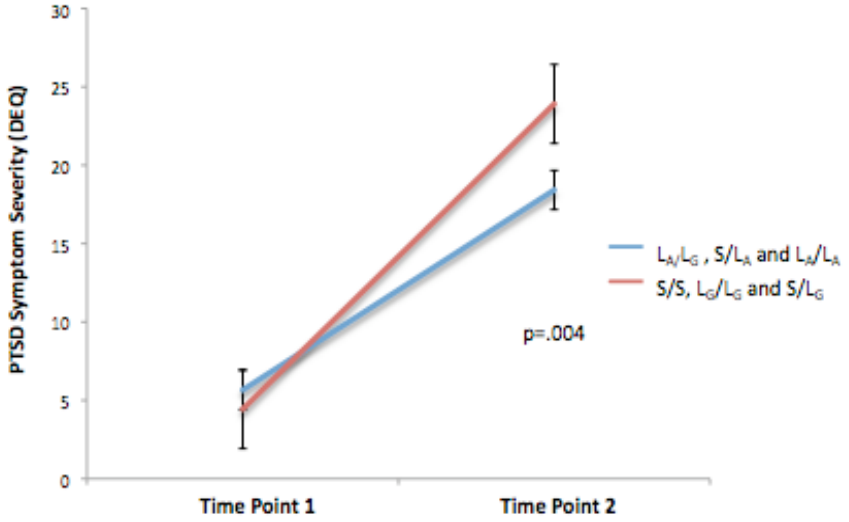


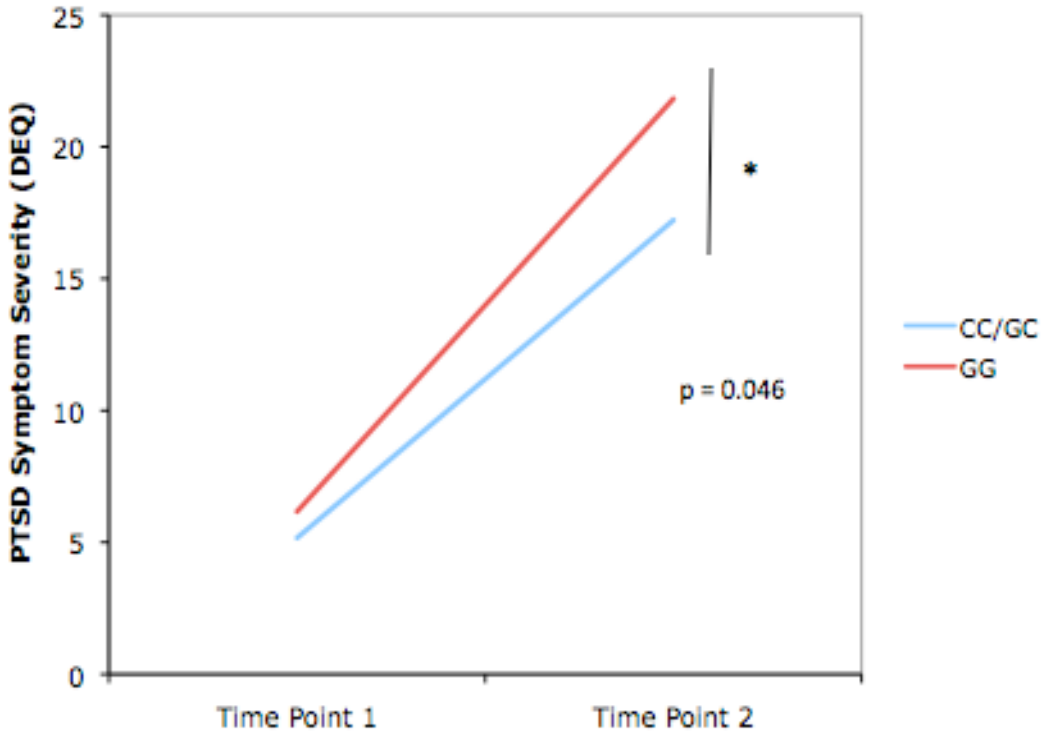
Figure 1. **Effects of Mass Shooting on PTSD Symptoms in a Prospectively Assessed Sample.** Comparison of PTSD Symptom Severity 2-4 weeks after Shooting by Type of Exposure to Shooting. Each number on the x-axis represents a specific yes or no question about each individual's exposure to the shooting: 1. knew someone killed in shooting, 2. knew someone wounded in shooting, 3. were in a building placed on lockdown, 4. on campus during shooting, 5. saw individuals wounded or killed, 6. saw police or other personnel surrounding the building, 7. in Cole Hall during shooting, 8. heard sound of gunfire, 9. saw gunman fire on someone, 10. saw gunman during shooting, 11. were hurt in shooting. [a]All individuals with time point 2 < 4 weeks post shooting.

Figure 2:



5HTTLPR Multi-marker Genotype	Mean	Std. Deviation	N
Time Point 1/Pre-Shooting PTSD score (DEQ)			
L_A/L_G , S/L_A and L_A/L_A	5.66	5.13	89
S/S, L_G/L_G and S/L_G	4.44	4.48	34
Time Point 2/Post-Shooting PTSD score (DEQ)			
L_A/L_G , S/L_A and L_A/L_A	18.42	11.78	89
S/S, L_G/L_G and S/L_G	23.91	14.68	34

Figure 2A. **The 5HTTLPR multi-marker Genotype predicts PTSD Symptom Severity 2-4 weeks Post Trauma**. The Graph shows the mean differences ($\pm$ S.E.) in PTSD symptom severity before and after the shooting relative to genotype. Graph includes whites only with time point 2 < 4 weeks after the shooting. Actual, mean values for the DEQ scores at each time point are given in the table.



rs2267735	Mean	SD	N
Time Point 1/Pre-Shooting PTSD score (DEQ)			
GG	6.17	5.01	46
CC/GC	5.17	4.84	146
Time Point 2/Post-Shooting PTSD score (DEQ)			
GG	21.82	13.62	46
CC/GC	17.21	12.79	146

Figure 2B. **rs2267735 Genotype predicts PTSD Symptom Severity at Time Point 2.** The graph shows the mean differences in PTSD symptom severity before and after the shooting relative to genotype. Graph includes all races and all time points (n = 192). Actual, mean values for the DEQ scores at each time point are given in the table.