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The Role of microRNA in Trait and State Anxiety

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Abstract of the Dissertation

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In humans and other animals, the amygdala has been shown to play a role in implicit emotional learning and memory, emotional modulation of memory, emotional influences on attention and perception, emotion and social behavior. Amygdala function has also been linked to personality traits and neuropsychiatric conditions, including anxiety disorders. The aim of the present study was to investigate the biological underpinnings of trait anxiety through a study of microRNA-mediated gene regulation, which was accomplished by global profiling of post-mortem human brain tissue from the lateral nucleus of the amygdala (LA).

Gene expression in postmortem LA tissue from donors with known antemortem high trait anxiety (n=10) was compared to that in donors with known antemortem low trait anxiety (n=10) through an integration of genome-wide proteomics, mRNA, and microRNA approaches. Gene expression analysis and literature search approaches focused on the synaptic vesicle glycoprotein 2A gene (SV2A) which showed decreased protein expression in trait anxious individuals compared to controls, no difference in

mRNA expression between the two groups, and increased expression of three microRNAs predicted to target SV2A, namely miR-133a, miR-138, and miR-218. Reporter-gene assays and microRNA overexpression experiments in human neuroblastoma cells showed that miR-133a and miR-218, (but not miR-138), functionally target SV2A. The subsequent addition of 26 LA samples (total study n=46) demonstrated that SV2A expression is more complex in the anxious phenotype. Both increased and decreased levels of SV2A showed association with an anxious phenotype, supporting prior *in vitro* and *in vivo* experiments that have demonstrated the necessity of tight regulation of SV2A levels for optimal synaptic functioning.

The impact of psychosocial stress on a variety of negative health outcomes is well documented, with much of the current research efforts directed at possible mechanisms. In an effort to investigate the biological effects of psychosocial stress, we performed gene expression analysis in peripheral blood mononuclear cells. We utilized a validated behavioral social stress paradigm (Trier Social Stress Test) to induce acute psychosocial stress in 36 human participants who completed measures on perceived and chronic stress. Cortisol stress reactivity was measured through continuous saliva collection during the paradigm and subsequent cortisol level analysis. Peripheral blood mononuclear cells were extracted from blood drawn at baseline and at two time points following the stress paradigm. For gene expression analysis, we focused on 12 participants who showed a robust cortisol response to the task, suggesting activation of the hypothalamic-pituitary-adrenal axis. Total RNA was extracted and microRNA microarrays were utilized to assess changes in gene expression between baseline and the two post-stressor time points. Pathway analysis of the predicted target genes of

differentially expressed microRNAs revealed a link between stress and inflammation and highlighted a potential role for miR-9.

The results of this dissertation suggest that microRNAs contribute to both state and trait anxiety.

To my amazing family who have supported, encouraged, and inspired me throughout all of my educational endeavors.

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LIST OF ABBREVIATIONS

(3'UTR) - 3' untranslated region

(5HTTLPR) - serotonin-transporter-linked polymorphic region

(ACTB) - beta actin

(ACTH) - corticotropin

(ANOVA) - analysis of variance

(APE1) - apurinic/apryimidinic endonuclease

(B2M) - beta-2-microglobulin

(BAI) - Beck Anxiety Inventory

(BDI) - Beck Depression Inventory

(BDNF) - brain-derived neurotrophic factor

(CMV) - cytomegalovirus

(COMT) - catechol-O-methyltransferase

(CRH) - corticotropin-releasing hormone

(cRNA) - complementary RNA

(CTQ) - Childhood Trauma Questionnaire

(dlPFC) - dorsomlateral prefrontal cortex

(dmPFC) - dorsomedial prefrontal cortex

(DSM-IV-TR) - Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision

(EMEM) - Eagle's Minimum Essential Medium

(EPSC) - excitatory postsynaptic currents

(EVA) - Extreme Values Analysis

(FCS) - Fetal Calf Serum

(FKPB5) - FK506 binding protein 5

(GABA) - γ-aminobutyric acid

(GAD) - generalized anxiety disorder

(GAPDH) - glyceraldehyde-3-phosphate dehydrogenase

(GUSB) - beta glucuronidase

(GWAS) - genome-wide association study

(HMBS) - hydroxymethylbilane synthase

(HPA) - Hypothalamic-Pituitary-Adrenal Axis

(HPLC) - high-pressure liquid chromatography

(ID) - identification

(IFN-α) - interferon-alpha

(IL) - interleukin

(IPA) - Ingenuity Pathway Analysis

(Kan) - Kanamycin

(LA) - lateral nucleus of the amygdala

(LIMMA) - Linear Models for Microarray Data

(miRISC) - miRNA-induced silencing complex

(miRNA) - microRNA

(MS) - mass spectrometry

(MudPIT) - Multidimensional Protein Identification Technology

(NEAA) - Non Essential Amino Acids

(NEO-PI) – Neuroticism Extraversion and Openness Personality Inventory

(NF-κB) - nuclear factor-κB

(NPY) - neuropeptide Y

(OCD) - obsessive-compulsive disorder

(PBMCs) - peripheral blood mononuclear cells

(PEA15) - phosphoprotein enriched in astrocytes 15

(PFC) - prefrontal cortex

(PKP1) - plakophilin 1

(PMI) - postmortem interval

(PMN) - polymorphonuclear neutrophils

(PTSD) - post-traumatic stress disorder

(pUC) – plasmid, University of California

(RNase) - ribonuclease

(RMA) - Robust Multi-Array Average

(RT-qPCR) – Real-time quantitative polymerase chain reaction

(SDHA) - succinate dehydrogenase complex, subunit A, flavoprotein

(SH-SY5Y) – Human neuroblastoma cell line

(SLE) - stressful life events

(SNP) – single nucleotide polymorphism

(SNRIs) - serotonin–norepinephrine reuptake inhibitors

(SNS) - Sympathetic Nervous System

(SSRIs) - selective serotonin reuptake inhibitors

(STAI) - State-Trait Anxiety Inventory

(SV2A) - synaptic vesicle glycoprotein 2A gene

(SV40) – Simian virus 40

(TBP) - TATA box binding protein

(TdT) - deoxynuclotidyltransferase

(TiCS) - Trier Inventory for the Assessment of Chronic Stress

(TMEM16B) - anoctamin 2

(TNF-α) - tumor necrosis factor-alpha

(UBC) - ubiquitin C

(UDG) - uracil DNA glycosylase

(UNG) - uracil-N-Glycosylase

(vlPFC) - ventrolateral prefrontal cortex

(vmPFC) - ventromedial prefrontal cortex

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The Role of microRNA in Trait and State Anxiety

Chapter 1: Regulatory Role of microRNA in Trait Anxiety

INTRODUCTION

Anxiety can be a defined as a negative cognitive-affective state that includes a sense of unpredictability and fear, along with significant apprehension about the future [1, 2]. The experience of anxiety serves an adaptive role in a myriad of situations, motivating appropriate vigilance or change [2, 3]. It can also be construed as a trait, or a dispositional tendency to frequently experience an anxious state [2]. If experienced frequently, at high intensity, and/or in inappropriate situations, anxiety can be characterized as a disorder [1, 2]. There is a high degree of similarity between normal and pathological anxious reactions which differ largely in timing, duration, and frequency of the experience [2]. The presence of psychopathology can be considered when the anxious experience recurs and is persistent, occurs with inappropriate intensity with regard to the objective danger or threat, leads to feelings of helplessness and inability to cope, and impairs psychological or physical functioning [2, 4].

The term “anxiety disorders” is an umbrella term that describes a set of related disorders, and the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR*)* classifies anxiety disorders into the following categories: anxiety due to a general medical condition, substance-induced anxiety disorder, generalized anxiety disorder (GAD), panic disorder, acute stress disorder, post-traumatic stress disorder (PTSD), adjustment disorder with anxious features, obsessive-compulsive disorder (OCD), social anxiety disorder, and simple phobia [5].

While the various disorders are distinct, they also share a range of common features and often co-occur [2].

Anxiety disorders are the most common psychiatric illness [6], with a lifetime prevalence of 28.8% in the United States [7]. There is a significantly higher risk for disorder development in women than men, with women being 1.6 times more likely to suffer from an anxiety disorder compared to men. Anxiety disorders often result in significant debilitation and chronic medical problems that affect multiple organ systems [8] along with healthcare costs exceeding $40 billion/year [9].

Assessment of Trait Anxiety

Current evidence suggests that personality traits, such as high neuroticism, often precede onset of anxiety disorders [10-13]. Neuroticism can be broadly defined as the tendency to experience negative emotions and to cope poorly with environmental stress. Personality traits are thought to be stable throughout adult life [14], and neuroticism is thought to have a heritability of approximately 40% [15]. Twin studies suggest that there is substantial overlap between genetic factors that influence individual variation in neuroticism and those that increase risk for GAD [10-13]. For example, two adjacent bins on chromosome 1 (60–80cM, 33–54Mb) have been linked to both anxiety and neuroticism [16]. Neuroticism is one of the Big Five personality traits that stem from the Five Factor Model, which also includes extraversion, agreeableness, openness to experience, and conscientiousness [17]. The Neuroticism Extraversion and Openness Personality Inventory (NEO-PI) is an established tool used to assess the Big Five personality traits, including neuroticism. Facets of neuroticism include anxiety,

hostility, depression, self-consciousness, impulsiveness, and vulnerability to stress [17]. The facet of anxiety measured by a subscale of the NEO Personality Inventory is the main phenotype measured by our study.

Genetic Susceptibility

Family history is one of the strongest predictors of likelihood of anxiety disorder onset. Family studies of anxiety showed that first degree relatives of affected probands suffer an increased risk of anxiety disorder development when compared to first degree relatives of unaffected controls, with an approximately 4-6 fold greater risk of the proband's disease (reviewed in [18]). Twin studies aimed at separating genetic from environmental influences found concordance rates for anxiety disorders to be between 12 and 26% for monozygotic twins and between 4 and 15% for dizygotic twins [18]. The heritability estimates of anxiety disorders have been between 20 and 40% [19-21]. Thereby, genetic variation clearly contributes to disease risk.

The search for genes that contribute to development of anxiety disorders has been challenging. Like other complex disorders, anxiety disorders lack a simple genetic architecture, and studies are often faced with the task of detecting small and diverse effects, having to consider epistasis and gene-environment interactions [22]. Linkage studies have pinned anxiety disorders to several chromosomal regions: panic anxiety phenotypes have been potentially linked to 1q[23], 2q[24], 7p[25, 26], 9q[27], 12q[28], 13q[29, 30], 15q[24], and 22q[29], specific phobia has been linked to chromosome 14q [31], social phobia has been linked to 16q [32], agoraphobia has been linked to 3q [23], and OCD has been linked to 3q27–q28[33], 9p24[34, 35], 10p15[36], and 14q[37]

(reviewed in [18]). There is little overlap between the implicated regions and they have often failed to replicate in more than one study. Reasons for this include under-powered study samples, locus heterogeneity, as well as low effect sizes for common alleles in complex disease [38]. More recent association analyses have focused on candidate genes encoding transporters, receptors, and synthetic enzymes that are involved in the neurotransmitter systems targeted by medications- these include the norepinepherine, serotonin, glutamine, and dopamine systems, as well as neuropeptides that have been linked to animal models of anxiety- including neuropeptide Y (NPY), brain-derived neurotrophic factor (BDNF), as well as the corticotrophin releasing hormone system [22].

The serotonin transporter promoter length polymorphism is the most extensively studied variant in genetic studies of mood and anxiety disorders. The variant consists of a dinucleotide repeat present in the promoter region of the serotonin transporter gene. The short allele of the serotonin-transporter-linked polymorphic region (5HTTLPR) is shorter by 43 base pairs when compared to the long form, and its presence leads to decreased transcriptional activity of its promoter. The short allele is fairly common in the general population, with an approximate frequency of 45% in a population of European-American descent. It has been associated with anxiety-related traits such as neuroticism and harm avoidance, and some studies have tied it to anxiety disorders such as OCD, PTSD, and social phobia [39-44]. Nevertheless, replication studies of 5HTTLPR and of other anxiety-related genes have not provided a clear consensus. Other genes associated with anxiety in more than one study include the dopamine receptor D2 (DRD2) gene [45], catechol-O-methyltransferase (COMT) [46], and FK506

binding protein 5 (FKPB5) [47]. The first published genome-wide association study (GWAS) of panic disorder examined 200 cases and 200 controls, identifying anoctamin 2 (TMEM16B) and plakophilin 1 (PKP1) to be associated with the disease phenotype; however, the biological mechanisms are uncertain and the study is still to be replicated [48]. GWAS are prone to false positive discovery due to possible issues with correction for multiple testing, genotyping quality control, confounding due to population stratification, and the 'winner's curse' which is an ascertainment bias wherein the initial effect size is often inflated compared to that seen in the general population (reviewed in [22]). As a result, replication studies are key to establishing an association between a given gene and disease phenotype. In addition, GWAS studies are also prone to false negative results due to inadequate sample sizes to detect smaller effect sizes, along with the difficulty of assessing multiple polymorphisms per gene at a given time [22].

Phenotypic heterogeneity poses a challenge to anxiety research, in that there is often overlap of symptoms and disease diagnostic criteria, and family history of one anxiety disorder often predisposes the proband to other anxiety disorders. Thereby, an endophenotype approach is preferred as genes may have greater effect on fundamental personality traits that might underlie several clinical disorders. Twin studies have shown that the genetic influences on the personality traits of introversion and neuroticism can account for genetic variation in risk for agoraphobia and social phobia [49]. GWAS studies of neuroticism have been conducted [50-52], although loci that would explain more than 1% of the variance in a trait were not seen. Our study investigated the endophenotype of trait anxiety, which is regarded as a stable and biological predisposition [53-56] that reflects an individual's general disposition to experience

anxiety-relevant feelings or thoughts or to show anxiety-related behaviors [57]. Trait anxiety describes the tendency to respond fearfully to a wide variety of unspecific stressors and is closely related to pathological anxiety. It is shown to be influenced by genetic and environmental effects [58].

Anxiety and the Amygdala

The amygdala is a subcortical collection of nuclei found in the medial temporal lobes that is key to establishing the human emotional experience [59-62] and memory consolidation ([61, 63-65], reviewed in [61]). It has been extensively studied in relation to fear and anxiety [66-73].

Studies conducted on nonhuman primates and rodents have led to identification of fear circuitry components [67, 74-78] and have found the amygdala to be critical to the production of fear behaviors ([67] reviewed in [62]. For example, substantial amygdala lesions have been demonstrated to increase the number of times a rat will make contact with a sedated cat (reviewed in [67]). Some animals have been shown to crawl over the cat and to nibble its ear, behaviors that are not seen in non-lesioned rats (reviewed in [67]). Amygdala lesion studies in nonhuman primates have resulted in a significant decrease in fear behavior [75, 79], and under certain conditions, amygdala lesions have led to decreases in social fear along with an increase in fear of objects [80]. The amygdala appears to be most important for stimulus-reinforcement learning in classical conditioning, but it also supports stimulus-reinforcement learning in relation to positive stimuli [62, 81-83], suggesting a broader role in emotional processing [62]. Amygdala damage in humans has led to a decreased ability to recognize fear from

facial expressions along with a lack of fearfulness in social contexts, as well as an inability to acquire conditioned fear responses ([84, 85]).

The prefrontal cortex (PFC) has also been shown to be involved in both automatic and effortful regulation of emotion [62, 86]. Multiple anatomic connections exist between the amygdala [86] and various regions of the PFC, and the PFC has been shown to modulate amygdala responses [86-90]. Recent research efforts have focused on γ-aminobutyric acid-ergic (GABAergic) neurons, emphasizing an inhibitory role for the PFC over amygdala function [91-93]. Neuroimaging studies have shown an inverse relationship between the amygdala and various regions of the PFC, such as the ventromedial prefrontal cortex (vmPFC) [94, 95], ventrolateral prefrontal cortex (vlPFC) [96-98], dorsomedial prefrontal cortex (dmPFC) [99-105], and dorsolateral prefrontal cortex (dlPFC) [100, 106] (as reviewed in [62]). In particular, the vlPFC and vmPFC, for which the PFC-amygdala connections have been mapped, suggest a net inhibitory input [62].

Current functional neuroimaging studies in adults investigating the neural basis of anxiety suggest that adult anxiety disorders can be construed as dysfunction of the brain resulting from a fear production system that is too strong and fear regulation system that is too weak (meta-analysis [68] reviewed in [62]). Thus, amygdala overactivity, when stimulated by fear or anxiety-inducing stimuli and/or stress, along with insufficient inhibition from the medial prefrontal cortex can lead to overexpression of conditioned responses that can contribute to anxiety disorder development [107] .

Indeed, amygdala hyperactivity has been seen during the provocation of symptoms or negative emotional processing in patients with PTSD ([108-110], social

anxiety disorder [111-116], specific phobia [117-120], panic disorder [121], and obsessive-compulsive disorder [121, 122] (as reviewed in [68]), and in spite of some discrepant studies, meta-analysis suggests the presence of amygdala hyperactivity in anxiety disorder patients compared to healthy controls, suggesting an underlying neurobiology that is common to multiple anxiety disorders [68].

Individuals with anxiety-related temperamental traits have also shown increased amygdala reactivity to certain types of emotional processing [123], and the magnitude of amygdala activation was shown to be moderately correlated with measures of anxiety proneness such as anxiety sensitivity and neuroticism. Additional studies have shown an association between amygdala activation to emotional (i.e., fearful) faces and individual differences in anxiety-related personality traits, such as threat sensitivity [124] and social anxiety [125] (reviewed in [123]). Levels of trait anxiety have also been shown to inversely correlate with the structural integrity of an amygdala-prefrontal pathway, such that higher pathway strength is predictive of lower anxiety [126].

A number of specific polymorphisms have been associated with brain measures of emotional reactivity related to anxiety, the most popular of which is 5HTTLPR, wherein the short allele has been associated with increased amygdala reactivity to emotional stimuli [127] and with increased resting activation [128]. Further analyses have demonstrated an association with a reduction in connectivity between the anterior cingulate and amygdala [129]. Thereby, it has been suggested that this variant might exert its effect through an enhancement of fear reactivity by reducing cortical inhibition of amygdala responses to threat [129].

These data suggest that altered amygdala functioning is not specific to a given disorder, and increased amygdala activity is not necessarily indicative of psychopathology. Nevertheless, amygdala hyperactivation may be a key component of a common neurobiological pathway for certain anxiety disorders that stems from the overactivation of a core fear system [68]. In this way, the endophenotye of trait anxiety may underlie mechanisms common to multiple anxiety disorders and so may transcend current diagnostic categories to provide a more fundamental basis for exploration of the molecular underpinnings of anxiety and related psychopathology.

This dissertation is focused on the lateral nucleus of the amygdala, which is the principal input region for visual, auditory, and somatosensory information, and which is also the site for converging Conditioned Stimulus - Unconditioned Stimulus information in associative learning [130-137]. A recent study has specifically linked the lateral nucleus to trait anxiety by demonstrating a significant positive correlation between the surface shape around the lateral and central nuclei of the right amygdala and anxiety score [138]. Future studies will aim to examine gene expression in trait anxious individuals from additional amygdala regions such as the central nucleus as well as regions of the PFC.

Pharmacological Treatment and Anxiety

Anxiety disorders are currently treated through approaches based in pharmaco- and/or psychotherapy. Although there are few studies comparing the efficacy of both treatment modalities, meta-analysis suggests comparable efficacy of both treatment strategies in the case of GAD [139, 140]. Additional studies suggest a benefit of a

combined therapeutic strategy [141, 142]. Currently, GAD is considered to be a potentially chronic disorder with symptoms showing both increased and decreased severity over time [143]. A prospective study in a clinical population discovered that approximately 60% of GAD patients seeking treatment showed recovery over the course of 12 years (defined as a lack of residual symptoms for at least 8 consecutive weeks). However, approximately half of these patients exhibited relapse during the 12-year time period [143] [144]. Also, the decrease in the mean anxiety symptom severity was only modest [143, 145]. Community studies generally reveal a better prognosis than those of clinical populations as a 22 year follow-up study of 105 persons with GAD found that only 20% had persistent disease, which was defined as the presence of daily symptoms over the past 12 months [146].

Limited progress has been made in pharmaceutical interventions for pathological anxiety treatment in recent years, and currently available drug therapies mostly target the serotonergic and GABAergic neurotransmitter systems [147]. First line therapy includes selective serotonin reuptake inhibitors (SSRIs) along with serotonin–norepinephrine reuptake inhibitors (SNRIs), while second line therapy includes tricyclic antidepressants, benzodiazepines, and certain anti-convulsants [143]. These drugs show limited efficacy in a significant proportion of the population, delayed onset of action, along with unwanted side effects that include dependence [147]. Currently, more than 40 percent of patients with GAD fail to show improvement or suffer from residual symptoms in response to multiple trials of first and second line medications and necessitate the administration of drugs in combination [143, 148, 149].

Therefore, there is an unmet need for development of new anxiolytic drugs with novel mechanisms of action [147]. microRNAs (miRNAs) are short (20-23 nucleotides in length), single-stranded, endogenous RNAs that interact with regions of complementarity on mRNA transcripts via Watson-Crick binding within the 3' untranslated region (3'UTR) and regulate post-transcriptional gene expression through translational repression and/or transcript degradation [150]. They have been pursued as novel drug targets due to their involvement in the regulation of almost every biological process in the cell and frequent association with the pathogenesis of human diseases (reviewed in [151]). The first miRNA-targeted drug (Miraversen, which targets miR-122) is currently in phase II clinical trials for the treatment of infection with the hepatitis C virus [151]. miRNAs are pursued as psychiatric drug targets due to their involvement in depression, anxiety, and psychological stress, and pre-clinical models have shown that changes in miRNA levels can affect behavior in a therapeutically relevant way [152].

miRNA genes are located in intronic regions of both protein coding and non-coding genes and in intergenic and exonic regions of the genome (reviewed in [153]). They are transcribed by RNA polymerase II into long primary miRNA transcripts that can range from several hundred nucleotides to kilobases in length containing single miRNAs or miRNA clusters. Primary miRNAs are subsequently cleaved in the nucleus into hairpin precursor miRNAs by a ribonuclease III double-stranded RNA-specific endonuclease called Drosha, and they are in turn exported into the cytoplasm to be further cleaved by the ribonuclease (RNase) III endonuclease Dicer to form mature miRNAs. Mature miRNAs are then loaded into a ribonucleoprotein complex known as

the miRNA-induced silencing complex (miRISC), the key components of which include the miRNA and an Argonaute protein, that functions as an interface for miRNA interaction with its target genes (reviewed in [153]). The miRNA then serves as a guide to areas of complementary found on mRNA transcripts and mediates gene silencing through translational repression and/or mRNA degradation (reviewed in [153]).

Present Study

The aim of this study was to evaluate differences in gene expression in postmortem human lateral amygdala tissue from trait anxious individuals and non-anxious controls in the context of potential regulation by miRNAs. We hypothesized that differences in gene expression according to trait anxiety can be partially explained by miRNA regulation. In our first postmortem sample of 10 trait anxious individuals and 10 non-anxious controls, the synaptic vesicle glycoprotein (SV2A) gene showed decreased protein levels in anxious individuals and similar levels of mRNA transcript in the two groups, along with higher levels of three miRNAs (miR-133a, miR-138, and miR-218) in anxious individuals that could potentially explain decreased SV2A protein levels through a miRNA-mediated translational repression mechanism.

We subsequently aimed to determine whether the three above-mentioned miRNAs functionally target the SV2A 3'UTR with the hypothesis that SV2A protein expression is subject to regulation by these three miRNAs. *In vitro* analyses confirmed that SV2A is indeed targeted by miR-133a and miR-218, but not miR-138.

Our final aim was to investigate whether SV2A, miR-133a and miR-218 remain significantly differentially expressed in an enlarged cohort (N=46) with the hypothesis

that they are indeed differentially expressed. Upon investigation of an enlarged cohort consisting of an additional 26 postmortem samples (total n=46), both higher and lower levels of SV2A were associated with an anxious phenotype, supporting the notion that precise regulation of synaptic SV2A levels is key to optimal synaptic function.

MATERIALS AND METHODS

Cohort 1

Twenty postmortem human lateral amygdala samples were obtained from Caucasian participants in the "Rush Memory and Aging Project" constituting "Cohort 1" in our study. Ten samples were obtained from high trait anxious individuals and 10 from low trait anxious controls. Trait anxiety was assessed ante-mortem by the score on the anxiety facet of the neuroticism subscale of the NEO Personality Questionnaire as described in Table 1.1 [17]. Individuals who scored in the top quartile had anxiety scores greater than 16 and were considered anxious, whereas those who scored in the bottom quartile had anxiety scores less than 9 and were classified as controls. Sample information and information on potential confounding variables such as gender, age at death, the postmortem interval, and use of antidepressants and anticonvulsants is found in Table 1.2. Statistical analysis was conducted with IBM SPSS Statistics version 21.0. Samples were flash frozen in liquid nitrogen and stored in a -70^0C ultra low freezer.

Genome-wide Proteomics

Genome-wide proteomic profiling was done with shotgun proteomics analysis (Multidimensional Protein Identification Technology- MudPIT) at the Stony Brook University Proteomics Center. Frozen human lateral amygdala nucleus samples were prepared with mass spectrometry compatible lysis buffer [154] and quantified for protein yield. To perform shotgun proteomics analysis (MudPIT), 40 µg of protein from each donor brain sample was proteolytically cleaved to generate a mixture of peptides. The resulting peptide mixture was further separated by high-pressure liquid chromatography

(HPLC) using the Waters Delta Prep HPLC System prior to tandem mass spectrometry (MS) analysis [155-157]. An ion-trap mass spectrometer, LTQ^{XL}-Oribitrap (Thermo Fisher Scientific), was used to acquire high mass accuracy MS and tandem MS spectra. The collected MS spectra were matched to a human protein database using the SEQUEST algorithm [158, 159]. Stringent criteria were applied to filter large-scale MS/MS results. Over 2,000 proteins were identified robustly from each human sample by MudPIT analysis. T-test p-value <0.05 and fold change $>|1.5|$ were used to select a set of differentially abundant proteins as a function of trait anxiety for candidate gene selection. Statistical analyses were conducted using IBM SPSS Statistics version 21.0.

Cohort 1: Sample Preparation

Total RNA was extracted from frozen amygdala tissue using the Qiagen miRNeasy Mini Kit with on-column DNAse treatment. RNA quantity and purity were assessed using a Nanodrop Technologies ND-1000 instrument (NanoDrop Technologies).

Cohort 1: mRNA Microarray Profiling

RNA (100 ng) from each individual was analyzed on the Affymetrix U133 Plus 2.0 expression array at the Microarray Core Facility of Stony Brook University. Labelled complementary RNA (cRNA) was prepared using the One-Cycle Target Labeling kit (Affymetrix) according to manufacturer instructions. The cRNA was purified using the GeneChip Sample Cleanup Module (Affymetrix) and the samples were quantitated on a Nanodrop ND1000 spectrophotometer. cRNA from each sample was fragmented at

94°C for 35 minutes in fragmentation buffer (GeneChip Sample Cleanup Module) and hybridized to the array for 16 hours at 45°C. The arrays were then washed in an Affymetrix GeneChip Fluidics Station model 450. Following washing and staining, arrays were scanned on an Affymetrix model 7G scanner. The scans were analyzed using Affymetrix GCOS software. Raw image intensity files were loaded into GenePattern software and normalized using the Robust Multi-Array Average (RMA) method with quantile normalization. Expression values were log2 transformed in order to meet normality requirements for parametric tests. Differential expression was defined as a fold change > |1.5| between anxious and control individuals and t-test p-value <0.05. Statistical analyses were conducted using IBM SPSS Statistics version 21.0.

Cohort 1: miRNA Microarray Profiling

miRNA microarray profiling was performed at the Microarray Core Facility of Stony Brook University using the Affymetrix GeneChip miRNA 1.0 Array according to the manufacturer's protocol. Briefly, 1 µg of total RNA was labelled using the 3DNA Array Detection FlashTag™ Biotin HSR Kit following the manufacturer's recommendations (Genisphere). The Affymetrix GeneChip® Hybridization, Wash, and Stain Kit was used for RNA hybridization to the arrays and for standard Affymetrix array cassette staining and washing according to the manufacturer's instructions. Arrays were scanned and feature extraction was conducted using Affymetrix Command Console software. Robust Multi-Array Average (RMA) normalization was conducted using the Affymetrix package of Bioconductor. Data were analyzed using both parametric and nonparametric tests. Linear scale expression intensities were used to identify

differentially expressed miRNAs with the Wilcoxon rank-sum test. Expression values were then log2 transformed in order to meet normality requirements for parametric statistical tests. Differential expression was defined as a fold change >|1.5| between anxious and control individuals and a t-test p-value <0.05 or a Wilcoxon rank-sum test p-value <0.05. Statistical analyses were conducted using IBM SPSS Statistics version 21.0.

SV2A Candidate Gene Selection

Our aim was to identify novel candidate genes that are associated with the anxious phenotype and show potential for regulation by miRNAs in our postmortem sample. SV2A was selected as a candidate gene according to the following procedure (Figure 1.1):

1) A database search was conducted to identify genes that have greater expression in the human amygdala compared to other tissues with the hypothesis that increased expression in the amygdala compared to other tissues is of functional significance. The Gene Expression Atlas by the European Bioinformatics Institute (EMBL-EBI) revealed 1872 genes that met these criteria. The search was conducted in October 2009 using the following URL: http://www.ebi.ac.uk/gxa/ with the following search criteria: “all genes” “up” in “Homo sapiens” “amygdala.”
2) Genes identified in Step 1 were referenced against the list of differentially expressed proteins identified by the proteomics data (criteria for differential expression: fold change >|1.5| between anxious and control individuals and

student's t-test $p<0.05$ resulting in 292 proteins) to create a subset of genes that have greater expression in the human amygdala when compared to other tissues and show differential expression in the lateral amygdala according to trait anxiety in our postmortem samples. This resulted in 75 genes.

3) In an effort to identify novel candidate genes for anxiety, a Pubmed literature search was conducted to identify a subset of genes identified in Step 2 with published animal models showing an anxious phenotype but lacking human studies. This resulted in two genes, namely SV2A and phosphoprotein enriched in astrocytes 15 (PEA15), that have greater expression in the human amygdala when compared with other tissues, have published animal models of anxious behavior, and lack human studies.
4) Genes identified in Step 3 were then integrated with miRNA and mRNA data from the postmortem tissue using Ingenuity Pathway Analysis (IPA) with a focus on potential for miRNA regulation.

SV2A emerged as a candidate gene as it satisfied the above criteria and showed decreased protein expression in controls compared to anxious individuals (Figure 1.2A), no difference in mRNA expression between the two groups (Figure 1.2B), along with increased expression of three miRNAs computationally predicted to target SV2A, namely miR-133a, miR-138, and miR-218 in anxious individuals (Figure 1.3B), supporting a hypothesis of translational repression (additional detail can be found in the results section). PEA15 was not pursued further as it was not targeted (nor predicted to be targeted) by any of the differentially expressed miRNAs in our study. Other candidate

genes identified by this postmortem sample will be investigated in further studies in the context of additional gene regulatory mechanisms.

Adjustment for Confounding Factors

Factors such as gender, age, the postmortem interval, social isolation, total adversity, anticonvulsant use, and antidepressant use could potentially confound the relationship between SV2A expression and anxiety, as well as the relationship between the expression of selected miRNAs and anxiety. Therefore, SV2A protein expression was adjusted for the above confounding variables using stepwise linear regression with the following simple linear model with log transformation (log transformation was done to stabilize variance): $$\log_2 protein = \alpha + \sum_i \beta_i Variable_i + \epsilon$$ Criteria for variable inclusion consisted of probability of F to enter ≤ 0.05 and probability of F to remove ≥ 0.10.

The same model was used to adjust miR-133a and miR-218 expression for the same confounding variables. miR-138 could not be tested using this model as it is not significantly associated with anxiety when log 2 transformed to meet normality requirements for parametric tests, showing borderline significance with a p=0.051. It was included in the study due to its fold change and significance in the non-parametric Wilcoxon rank-sum test. All analyses were conducted in IBM SPSS version 21.0.

Luciferase Assay

The computational miRNA target prediction tool Targetscan (www.targetscan.org), predicted SV2A targeting by miR-133a, miR-138, and miR-218 (Figure 1.3A). Dual luciferase assays with an SV2A 3'UTR clone (Genecopoeia) were

used to test the hypothesis that the SV2A 3'UTR is directly targeted by miR-133a, miR-138, and miR-218. The full SV2A 3'UTR was cloned donwstream of the firefly luciferase reporter gene in a dual luciferase (firefly/renilla) vector (Figure 1.4A). Human embryonic kidney (HEK 293, Sigma-Aldrich) cells under passage 10 were plated in 96-well plates at a density of 5 x 10^4 cells/well in medium containing Eagle's Minimum Essential Medium (EMEM) + 2 mM glutamine + 1% non essential amino acids (NEAA) + 10% fetal calf serum (FCS) and co-transfected with 100 ng of the SV2A-3'UTR vector and either 100 nM miRNA mimic (miR-133a, miR-218, or miR-138) or 100 nM of miRNA negative control mimic using the DharmaFECT Duo Transfection Reagent (Dharmacon). A control condition (SV2A) with only the SV2A-3'UTR vector without miRNA co-transfection was also included.

miRNA mimics are double-stranded oligonucleotides that are designed to mimic the function of endogenous miRNA and are chemically modified to engage miRISC with the active miRNA strand (Dharmacon). The sequence of the negative control is based on miRNAs native to *C. elegans* and possesses minimal sequence identity with human, mouse, or rat. Transfection was accomplished following the Express Transfection protocol (Dharmacon) with 24 hr incubation. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega) and the FLUOstarOptima microplate reader (BMG Laboratories).

Transfection conditions were optimized using siGLO Green Transfection Indicator (Thermo Scientific). Each experiment was repeated independently three times, and each condition was tested in pentuplicate. Outliers were formally excluded according to the following procedure [160]:

1) In a set of experimentally derived data points, the boundaries of each of the four quartiles were first determined within each tested condition. Thus, the maximum (Qmax), the 75th percentile (Q75), the median (x~), the 25th percentile (Q25), and the minimum values (Qmin) were found for each condition tested.
2) The fourth spread (fs) was calculated by fs = Q75 - Q25.
3) The standard upper and lower outlier boundaries were then calculated by:

$O_U = (x\sim) + 1.5 \times fs$

$O_L = (x\sim) - 1.5 \times fs$

Data points lying above the upper boundary or below the lower boundary were considered outliers and were excluded from further analysis as suggested by Jacobs and Dinman (2004) [160]. In each pentuplicate measurement set, between 0 and 2 observations were excluded based on the above criteria. Statistical tests were conducted using one-way analysis of variance (ANOVA) with the Tukey post-hoc test.

Site-directed Mutagenesis of miR-133a and miR-218 Binding Sites

Site-directed mutagenesis was performed using KOD Xtreme polymerase (Clontech) as shown in Figure 1.5A. A Targetscan predicted binding site for miR-218 on the full length SV2A 3'UTR beginning at position 1051 was mutated from "A**GC**ACA" to "A**CG**ACA" as described previously [161], and a predicted miR-133a binding site beginning at position 38 was mutated from "GGAC**CAA**A" to "GGA**GGG**AA" in a separate construct. Transfection in HEK 293 cells was performed as described above co-transfecting 100 ng of the mutant construct along with 100 nM of the respective miRNA mimic. Each experiment was independently repeated 3 times, and each

condition was repeated in pentuplicate. Statistical analysis of the firefly/renilla ratio followed exclusion of outliers as described above, excluding at most 2 observations per pentuplicate measurement set. Statistical tests were conducted using one-way ANOVA with the Tukey post-hoc test.

Neuroblastoma Cell Culture and Transfection

One hundred nM of miR-133a, miR-138, and miR-218 miRIDIAN miRNA mimics and inhibitors (Dharmacon), 100 nM of negative control mimic (Dharmacon), and 100 nM of negative control inhibitor (Dharmacon) were transfected into human neuroblastoma SH-SY5Y cells (Sigma-Aldrich) as separate experimental conditions using Lipofectamine 2000 (Invitrogen) for 24 hours in order to assess changes in mRNA levels and for 48 hours to assess changes in protein levels. miRNA inhibitors are RNA oligonucleotides with a proprietary secondary structure designed to inhibit the function of endogenous miRNAs (Dharmacon). The negative control inhibitor is an RNA oligonucleotide with a sequence based on miRNAs native to *C. elegans* and possesses minimal sequence identity with human, mouse, or rat (Dharmacon). SH-SY5Y cells below passage 10 were plated in 6-well plates in pentuplicate (for mRNA analysis) and quadruplicate (for protein analysis) at a density of $5x10^5$ cells/well in medium containing Ham's F12:EMEM (1:1) + 2mM glutamine + 1% NEAA + 15% FCS. Cells were plated directly into the transfection mix according to the Lipofectamine 2000 protocol (Life Technologies). Amounts of lipofectamine and miRNA mimics and inhibitors were optimized using the siGLO Green transfection indicator (Dharmacon). Each experiment

was independently repeated three times and each condition was repeated in pentuplicate for mRNA analysis and in quadruplicate for protein analysis.

Real-time quantitative polymerase chain reaction (RT-qPCR)

One hundred nM miR-218, miR-133a, and miR-138 mimics and inhibitors (Dharmacon) were transfected into SH-SY5Y cells using Lipofectamine 2000 along with relevant controls as described above. Cells were incubated for 24 hours in order to assess changes in SV2A mRNA levels. Total RNA was collected using the Qiagen miRNeasy kit using on column DNase treatment according to manufacturer instructions (Qiagen). Reverse transcription was accomplished using the QuantiTect Reverse Transcription Kit (Qiagen) with an input of 250 ng of total RNA for each reaction. RT-qPCR was done using the QuantiTect SYBR Green kit with Uracil-N-Glycosylase (UNG) (Qiagen) in a Roche Lightcycler 480 with the following cycling conditions: Step 1: UNG 2 min at 50ºC, Step 2: PCR initial activation 15 min at 95ºC, Step 3 (cycling): denaturation 15s at 94ºC, annealing 30s at 55ºC, extension 30 s at 72ºC, Step 4: melting curve analysis. RT-qPCR primers were designed with the Universal Probe library (Roche), and RT-qPCR normalization was done by geometric averaging of multiple internal control genes according to Vandesompele et al. [162]. Eight housekeeping genes [beta actin (ACTB), TATA box binding protein (TBP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta glucuronidase (GUSB), beta-2-microglobulin (B2M), hydroxymethylbilane synthase (HMBS), succinate dehydrogenase complex, subunit A, flavoprotein (SDHA), and ubiquitin C (UBC)] were evaluated for stability with the geNorm algorithm (Biogazelle), identifying GUSB and

B2M as fitting controls for mRNA expression analysis in SH-SY5Y cells (Figure 1.S1). SV2A primers are intron-spanning between exons 3 and 4, and the amplified sequence is found in each of the two described SV2A variants. The sequence of the forward primer is: ccagggttacggcactttc, and that of the reverse primer is: gagaagacaatggggatgga. The sequence of the forward primer for the control gene GUSB is cctgtgacctttgtgagcaa, and the reverse primer sequence is aacagatcacatccacatacgg. The sequence of the forward primer for the gene B2M is ttctggcctggaggctatc, and the reverse primer sequence is tcaggaaatttgactttccattc. Gene specific amplification efficiencies were found using the specified procedure in GeNorm software (Biogazelle), and RT-qPCR analysis was conducted using these gene-specific amplification efficiencies. Each experiment was repeated independently three times, each condition was repeated in pentuplicate in each experiment, and each RT-qPCR reaction was performed in triplicate. Outliers were formally excluded as described above with at most one measurement excluded per pentuplicate set. Statistical tests were conducted using one-way ANOVA with the Tukey post-hoc test.

Quantitative Immunoblotting

One hundred nM miR-218, miR-133a, and miR-138 mimics and inhibitors (Dharmacon) were transfected into SH-SY5Y cells using Lipofectamine 2000 (Life Technologies) along with relevant controls as described above. Cells were incubated for 48 hours in order to assess changes in SV2A protein levels. Protein was collected using the cOmplete Lysis-M kit (Roche), and concentrations were established using the Bradford Protein Assay (Bio Rad). Quantitative immunoblotting was performed using

100 µg of protein from each condition, which was repeated in quadruplicate. A polyclonal rabbit SV2A antibody (Santa Cruz SC28955) was used at a dilution of 1:100, along with a fluorescently tagged goat anti-rabbit secondary antibody (Rockland 611-130-122) at a dilution of 1:10,000. A monoclonal mouse β-actin antibody (Sigma A4700) was used at a dilution of 1:200, along with a fluorescently tagged goat anti-mouse secondary antibody that was used at a dilution of 1:10,000 (Rockland 610-131-121). SV2A levels were normalized to β-actin to ensure equal loading, and mouse brain lysate served as a positive control to ensure the ability of the antibody to bind SV2A protein. Signal intensity was assessed with the Odyssey Infrared Imaging System, and band quantification was performed with Odyssey Infrared Imaging System Software according to manufacturer instructions. Outliers were formally excluded as described above with at most one measurement excluded per quadruplicate set. Results from three independent experiments were included in the analysis of miR-133a and miR-218, and one independent experiment was done to confirm lack of targeting by miR-138. Statistical tests were conducted using one-way ANOVA with the Tukey post-hoc test.

Enlarged Cohort

Twenty-six additional postmortem human lateral amygdala samples were obtained from Caucasian participants in the "Rush Memory and Aging Project." Only 9 of these samples satisfied the anxiety score selection criteria (either Top or Bottom Quartile) employed in Cohort 1. Four of these were anxious and 5 were classified as controls. The remaining 17 samples belonged to individuals with a wide spectrum of

anxiety scores spanning the second and third quartiles. As a result, anxiety was treated as a continuous variable in subsequent analyses by using the anxiety score.

Sample Preparation: Enlarged Cohort

Samples were flash frozen in liquid nitrogen and stored in a -70^{0}C ultra low freezer. Proteomics analysis was performed at Stony Brook University in the same manner as for Cohort 1, and the two data sets were integrated. For mRNA and miRNA profiling, total RNA from the additional samples was extracted using the Qiagen All-Prep kit with manufacturer recommended modification to extract small RNA and on-column DNAse treatment (Qiagen). RNA quantity and purity were assessed using a Nanodrop Technologies ND-1000 instrument (NanoDrop Technologies). RNA quality was measured using the Agilent Bioanalyzer 2100A (Agilent Technologies).

mRNA Microarray Profiling: Enlarged Cohort

mRNA microarray profiling was performed at the Cold Spring Harbor Laboratory Microarray Facility using Affymetrix Human Gene ST 1.0 Arrays according to the manufacturer's protocol. Briefly, 500 ng of total RNA was used to synthesize cDNA by reverse transcription using random hexamers tagged with a T7 promotor primer sequence. The double-stranded cDNA was subsequently used as a template and amplified by T7RNA polymerase, producing several copies of antisense complementary RNA (cRNA). In the second cycle of cDNA synthesis, random hexamers were used to prime reverse transcription of the cRNA from the first cycle to produce single-stranded DNA in the sense orientation. The DNA was fragmented with a combination of uracil

DNA glycosylase (UDG) and apurinic/apryimidinic endonuclease (APE1). DNA was labled by terminal deoxynuclotidyltransferase (TdT), and hybridization was performed according to the manufacturer's protocol. The arrays were subsequently washed, stained, and scanned according to standard Affymetrix protocols. Images were processed using Affymetrix Microarray Suite 5.0. Expression Console, and image quality was subsequently assessed. Raw image files were loaded into GenePattern software and normalized using the Robust Multi-Array Average (RMA) method with quantile normalization. Expression values were log2 transformed for further analysis.

miRNA Microarray Profiling: Enlarged Cohort

miRNA microarray profiling was performed at the Cold Spring Harbor Laboratory Microarray Facility using the Affymetrix GeneChip miRNA 2.0 Array according to the manufacturer's protocol. Briefly, 500 ng of total RNA was labelled using the 3DNA Array Detection FlashTag™ Biotin HSR Kit following the manufacturer's recommendations (Genisphere). The Affymetrix GeneChip® Hybridization, Wash, and Stain Kit was used for RNA hybridization to the arrays and for standard Affymetrix array cassette staining and washing according to the manufacturer's instructions. Arrays were scanned on the Affymetrix GeneChip Scanner 3000, and feature extraction was conducted using Affymetrix Command Console software. Microarray background correction, quantile normalization, probe set summarization, and log2 transformation were performed with the miRNA QC Tool Version 1.1.1.0 (Affymetrix).

Cross-Platform Integration of mRNA and miRNA Array Data to Form the Enlarged Cohort

The 26 additional samples that were added to form the enlarged cohort were analyzed using updated Affymetrix mRNA and miRNA array platforms compared to those utilized in Cohort 1. The change of mRNA array platforms was made for the following reasons: the classical 3' expression U133 Plus 2.0 mRNA array used in Cohort 1 does not discriminate between alternatively spliced transcripts that share identical 3' ends, nor does it discriminate transcripts that lack a 3' exon due to alternative splicing, non-polyadenylation, genomic deletions, or other non-canonical genomic events that are not detected in 3' based expression experiments [163]. The Human Gene ST 1.0 array enables exon-level analysis with insight into alternative splicing and differential expression of each exon within a gene, enabling more precise characterization of gene expression [163]. miRNA profiling was conducted with the updated Affymetrix 2.0 miRNA array as opposed to the Affymetrix 1.0 miRNA array since it includes twice the number of probesets along with probes for pre-miRNA and can thereby provide more expression information [164].

In a recent review of cross-platform integration approaches by Rudy et al. [165], the XPN (Cross-Platform Normalization) [166] method showed the highest inter-platform concordance. Therefore, XPN was used to normalize arrays from two platforms. XPN is based on an assumption that the samples of each set fall into one of several statistically homogenous groups, and each group is associated with a set of genes. K-means clustering was applied independently on genes and samples to find blocks. Each expression value was fitted in a linear model, represented by block mean and offset and

variance. The function ReadAffy**()** in package Affy was used to load array data. The Robust Multi-array Average (RMA) algorithm was applied to adjust the background and perform quantile normalization.

SV2A and miRNA Expression Analysis in the Enlarged Cohort

Protein expression data from the enlarged cohort were examined to determine whether SV2A protein levels correlate with anxiety score using the Pearson correlation coefficient. The inverse relationship between SV2A protein expression and anxiety score seen in Cohort 1 did not hold (Pearson correlation coefficient= -0.067 with p=0.657); however, visual inspection of the data suggested the possibility that both increased and decreased SV2A levels correlate with high anxiety score (Figure 1.8). An alternative method of analysis was employed to determine whether individual differences in SV2A protein expression from the sample mean might correlate with an increase in anxiety score. This type of analysis enabled determination of whether both increased and decreased levels of SV2A correlate with anxiety. Mean SV2A protein expression was calculated for the entire enlarged cohort, and the absolute value of the expression difference between each observation and the sample mean was calculated. The Pearson correlation coefficient was then used to correlate the deviation from mean SV2A protein expression with anxiety scores.

The Pearson correlation coefficient was also used to examine potential correlation between SV2A mRNA levels and anxiety score, as well as individual deviation from mean SV2A mRNA expression and anxiety score. The Pearson correlation coefficient was also used to determine whether there is a correlation

between miR-133a, miR-138, and miR-218 expression and anxiety score. Seven individuals with increased anxiety scores and increased SV2A protein expression were tracked in each graph according to participant identification (ID) number in order to enable detection of possible differences from the remaining sample.

Exploratory Graphical Analyses

Several graphical approaches were undertaken in order to determine whether the seven individuals with high anxiety scores and high SV2A protein expression differ from the remaining study sample in a significant way. SV2A protein expression was graphed as a function of SV2A mRNA expression and as a function of miR-133a, miR-138, and miR-218 expression. In addition, miR-133a expression was graphed as a function of miR-138 and miR-218 expression, and miR-138 was graphed as a function of miR-218 expression.

Adjustment for Confounding Factors

Variables such as gender, age, the postmortem interval, social isolation, total adversity, anticonvulsant use, and antidepressant use could potentially confound the relationship between miR-133a and miR-218 expression and anxiety. Therefore, the relationship between both miR-133a and miR-218 expression and anxiety was adjusted for the above confounding variables using stepwise linear regression with the same simple linear model utilized for Cohort 1. The relationships between both SV2A protein and mRNA expression and anxiety did not require adjustment because they did not show a significant correlation with anxiety score in the enlarged cohort. However, the association between the deviation from mean SV2A protein expression and anxiety was

adjusted for the above confounding variables, with the deviation from mean SV2A protein expression serving as the dependent variable.

In order to determine whether the seven individuals with increased anxiety scores and increaed SV2A protein expression differ from the remaining sample in significant ways, each confounding variable was plotted against anxiety score, SV2A protein and mRNA expression, and miRNA expression, and those seven individuals were tracked in each graph according to participant ID number.

Expression of Additional miRNAs Predicted to Target SV2A

Targetscan predicted high probability of targeting of the SV2A 3'UTR by the following miRNAs: miR-128, miR-27ab, miR-19, miR-137, miR-22, miR-139-5p (Figure 1.3A). miRNA expression data from the enlarged cohort was examined for expression values for each of the above-mentioned miRNAs. miRNA expression values were plotted against SV2A protein and mRNA expression in order to determine whether these miRNAs could potentially play a regulatory role in SV2A levels. Each of the seven individuals with increased anxiety score and increased SV2A protein expression was tracked in each graph according to participant ID number in order to determine whether aberrant expression of any of these miRNAs could potentially contribute to the increase in SV2A protein seen in these individuals.

Synaptotagmin Expression

SV2A mediates the effectiveness of calcium in inducing vesicle fusion by regulating the expression and trafficking of synaptotagmin, a major calcium sensor protein [167]. Therefore, synaptotagmin expression was correlated with anxiety score as well as SV2A expression. Deviation in SV2A expression from the sample mean was also calculated and correlated with anxiety score.

RESULTS

SV2A is a Candidate Gene for Anxiety-related miRNA Regulation

The selection process depicted in Figure 1.1 identified SV2A as a candidate gene for trait anxiety. SV2A demonstrates greater expression in the human amygdala compared to other tissues, and mice heterozygous for one functional copy of SV2A have a normal lifespan but develop an anxiety-like phenotype [168]. In Cohort 1, SV2A protein levels were significantly higher in controls when compared to anxious individuals (Figure 1.2A) (fold change comparing control to anxious individuals = 1.94 and t-test p-value <0.05), whereas mRNA levels did not differ significantly between the two groups (Figure 1.2B). Three miRNAs (miR-133a, miR-138 miR-218) that were expressed at higher levels in anxious individuals (Figure 1.3B) are predicted to target SV2A (Figure 1.3A). miR-133a and miR-218 show greater expression in anxious individuals as evidenced by a fold change of 1.53 and 1.81, respectively, comparing anxious individuals to controls, as well as student's t-test and Wilcoxon rank-sum test p-values <0.05. miR-138 shows borderline higher expression in anxious individuals compared to controls with a fold change of 1.52, a Wilcoxon rank-sum test p-value <0.05 and a t-test p-value = 0.051. miR-138 was included in further analysis despite borderline significance in order to avoid a potential type II error due to the small sample size of the study. It is possible that miR-138 would show a significant association with anxiety in a larger sample with greater power and so it was included as a candidate gene in order to minimize the likelihood of classifying it as a false negative.

Taken together, the genome-wide data suggest a model wherein the decrease in SV2A protein levels seen in anxious individuals potentially results from increased expression of these three miRNAs suggesting a translational repression mechanism of miRNA-mediated gene regulation.

Adjustment for Confounding Factors

Anxious individuals did not differ from the control group in potential confounding variables such as gender composition, age at death, the postmortem interval (PMI), and psychological variables such as total adversity, anticonvulsant use, or antidepressant use. They differed significantly in terms of social isolation. Anticonvulsant use was considered to be a possible confounding variable since SV2A is the target of the antiepileptic drug Levetiracetam [169], which has also been used in the treatment of anxiety disorders. Antidepressants were treated as a potential confounding variable due to their ability to alter protein and miRNA expression in the brain. For example, the SSRI Fluoxetine has been shown to alter the expression of the serotonin transporter through an increase in the expression of miR-16 [170].

Results of stepwise linear regression analyzing SV2A protein expression and anxiety provided the following model: log2 SV2A protein expression = 4.26 – 0.064 (anxiety score) with an R^2 value of 0.232. All of the potential confounding variables (postmortem interval, age, total adversity, social isolation, anticonvulsant use, and antidepressant use) were excluded as they did not significantly add to the descriptive value of the model. Stepwise linear regression provided the following model for miR-133a expression: miR-133a expression (log2 microarray intensity) = 2.05 + 0.03(anxiety

score) with an R^2 value of 0.241. The same confounding variables were excluded as they did not significantly contribute to the model. Results of stepwise linear regression for miR-218 expression generated the following model: miR-218 expression (log2 microarray intensity) = 7.969 + 0.036(anxiety score) with an R^2 of 0.210. The same confounding variables were excluded as they did not contribute to the descriptive value of the model. Thereby, the variables listed in Table 2 do not confound the relationship between SV2A protein expression and anxiety, nor the respective relationships between miR-133a and miR-218 and anxiety.

The SV2A 3'UTR is Targeted by miR-133a and miR-218, but not miR-138

Transfection with miR-133a and miR-218 significantly decreased relative luciferase activity from the SV2A dual luciferase construct ($p<0.05$) (Figure 1.4B). There was a statically significant difference in relative luciferase activity between co-transfection with both the SV2A construct and miR-133a and transfection with the SV2A construct only ($p<0.00001$). This was also the case for miR-218 ($p<0.00001$ vs transfection with SV2A only), and there was no difference between transfection with the SV2A construct and co-transfection of the SV2A construct with the negative control mimic ($p=0.134$). In contrast, miR-138 did not decrease relative luciferase activity as determined by one-way ANOVA ($p=0.054$) with a Tukey post-hoc test p-value of 0.51 versus transfection with the sole SV2A construct, suggesting that it does not target the SV2A 3'UTR.

To confirm that the predicted target sequences of miR-133a and miR-218 in the SV2A 3'UTR are functional, the sites were mutagenized using site directed mutagenesis (Figure 1.5A). Notably, neither miR-133a nor miR-218 could inhibit luciferase activity

from the mutagenized SV2A construct (Figures 1.5B), suggesting that the predicted sequences are genuine binding sites for the respective miRNAs.

Transfection with miR-133a and miR-218, but not with miR-138, Leads to a Reduction in Endogenous SV2A mRNA and Protein levels

Since luciferase assays showed targeting of the SV2A 3'UTR by miR-133a and miR-218, the effects of miRNA transfection on SV2A mRNA and protein levels were subsequently investigated in human neuroblastoma SH-SY5Y cells. Transfection with either miR-133a or miR-218 elicited a significant decrease in SV2A mRNA compared to controls. Transfection with miR-138 mimic did not produce a decrease in SV2A mRNA, further suggesting that the miRNA does not target the SV2A 3'UTR (Figure 1.6). There was no significant difference in SV2A mRNA levels following transfection with miR-133a and miR-218 inhibitors compared to controls (Figure 1.6).

Transfection with miR-133a and miR-218 mimics showed a significant decrease in SV2A protein (one-way ANOVA $p<0.05$ and Tukey post-hoc test $p<0.05$ versus untreated control and mock transfection with negative control mimic) (Figure 1.7A) while transfection with miR-138 did not (one-way ANOVA $p>0.05$) (Figure 1.7B).

Anxious Individuals in an Enlarged Cohort Demonstrate both Increased and Decreased SV2A Protein Expression and No Association with SV2A mRNA Expression

SV2A protein expression in the enlarged cohort did not support the inverse relationship between SV2A protein levels and anxiety seen in Cohort 1 (Pearson correlation coefficient between SV2A protein expression and anxiety score = -0.067 with

p = 0.65) (Figure 1.8). Visual inspection of the graph depicting SV2A protein expression as a function of anxiety score suggested the possibility that both increased and decreased SV2A levels correlate with anxiety score. This hypothesis is supported by *in vitro* and *in vivo* studies that suggest the necessity of precise SV2A levels for optimal synaptic functioning, with defects in neurotransmission observed due to both under- and over-expression of SV2A [171]. Individual differences from mean SV2A expression in the enlarged cohort were shown to positively correlate with anxiety score, in that larger deviations from the mean correlated with higher anxiety scores (Pearson correlation coefficient = 0.37 with p=0.01 for the correlation between anxiety score and deviation from mean SV2A expression). Seven individuals showed increased anxiety scores with increased SV2A expression. These individuals (Participants 3, 9,18, 20, 25, 26, and 27) were highlighted according to participant ID number and investigated further, as it was imperative to determine whether they differ in any way from the rest of the sample since the results from Cohort 1 suggested a decrease in SV2A protein levels in high anxious individuals.

In the enlarged cohort, there was no significant correlation between SV2A mRNA expression and anxiety score (Pearson correlation coefficient = -0.088 with a p-value of 0.559) (Figure 1.9), nor was there a significant correlation between individual differences from mean SV2A mRNA expression and anxiety score (Pearson correlation coefficient = -0.122 with a p-value of 0.420). The seven participants with increased SV2A protein expression and increased anxiety scores are identified by participant ID number in Figure 1.9 and did not show an aberrant pattern of SV2A mRNA expression compared with the rest of the sample. This observation suggests that the higher levels

of SV2A protein expression seen in those individuals are not due to higher levels of SV2A mRNA expression and might be a reflection of decreased protein turnover or translational efficiency.

miR-133a and miR-218 Expression, but not miR-138 Expression, Increase with Anxiety Score in the Enlarged Cohort

There was a significant positive correlation between miR-133a and miR-218 expression and anxiety score (Pearson's correlation coefficient= 0.383 and 0.475 respectively, both with $p<0.05$), whereas miR-138 did not show significant correlation with anxiety score (Pearson's R is 0.202 and $p>0.05$) (Figure 1.10). The seven individuals with high anxiety scores and high SV2A protein levels were tracked according to participant ID number in Figure 1.10.

Exploratory Graphical Analyses

Several exploratory graphical approaches were undertaken in order to identify trends in the expression data relating to SV2A and to determine whether the seven individuals with increased anxiety scores and increased SV2A protein expression differ from the remaining sample in a significant way. Several individuals demonstrated an inverse expression pattern for miR-133a and miR-218. For example, Participant #3 showed a higher level of miR-133a and a lower level of miR-218 expression; Participant #24 showed a lower level of miR-133 and a higher level of miR-218 expression, Participant #25 showed a lower level of miR-133a and a higher level of miR-218

expression. Participant #26 showed a lower level of miR-133a and a higher level of miR-218 expression.

A plot of SV2A protein expression as a function of SV2A mRNA expression (Figure 1.11) revealed that there is no significant correlation between the two variables (Pearson's R = -0.017 and p=0.910). The seven individuals with increased anxiety scores and increased SV2A levels did not show abnormally high or low levels of SV2A mRNA when compared to the rest of the sample. SV2A protein expression was also graphed as a function of miR-133a, miR-138, and miR-218 expression, and none of these miRNAs showed significant correlation with SV2A protein levels (Figure 1.12). Finally, miR-133a expression was graphed as a function of miR-138 expression and miR-218 expression, and miR-138 was graphed as a function of miR-218 expression. Only miR-218 and miR-138 expression were found to be positively correlated with each other (Pearson's R=0.593 p=0.000014) (Figure 1.13), implying the possibility of co-regulation by a common upstream factor.

Confounding Variables

Results of stepwise linear regression provided a model wherein anxiety score = 35.7(2.051- (miR-133a expression) with an R^2 value of 0.138. The following variables are excluded since they did not significantly add to the descriptive value of the model: postmortem interval, age, total adversity, social isolation, anticonvulsant use, and antidepressant use. Stepwise linear regression provided a model wherein anxiety score = 21.73 (7.819 – miR-218 expression) with an R^2 value of 0.223. The same confounding variables were excluded as they did not significantly contribute to the

model. SV2A protein and mRNA expression, as well as miR-138 expression were not significantly associated with anxiety in the enlarged cohort, and so they were not adjusted for confounding variables. The association between the deviation of SV2A protein expression from the sample mean and anxiety was adjusted for the above confounding variables generating the following model: anxiety score = 10((log2 (deviation of SV2A protein expression from the sample mean) + 0.82 (total adversity) - 3.395) with an R^2 value of 0.205, suggesting that greater deviation of SV2A protein expression from the sample mean and an increased number of adverse life events are associated with an increase in anxiety score. All other confounding variables did not significantly add to the descriptive value of the model and were excluded.

In order to determine whether the seven individuals with increased anxiety scores and increased SV2A protein expression differ from the remaining sample in significant ways, each confounding variable was plotted against anxiety score, SV2A protein and mRNA expression, and miRNA expression, and the seven individuals with increased SV2A protein expression and increased anxiety score were tracked in each graph.

In the enlarged cohort, anxiety score was not significantly correlated with postmortem interval, age, or total adversity, but it did show significant correlation with social isolation (Pearson's R=0.551 p=0.000086) (Figure 1.14). Anxiety score was not associated with gender, anticonvulsant use, or antidepressant use (t-test p value>0.05). The seven individuals with increased anxiety scores and increased SV2A protein expression did not show aberrant expression patterns for the confounding factors in relation to anxiety score (Figure 1.14).

SV2A protein expression was not significantly correlated with postmortem interval, age, total adversity, or social isolation (Figure 1.15). SV2A protein expression was not significantly associated with gender or antidepressant use, but there was significant association with anticonvulsant use (t-test p-value <0.05). The seven individuals with increased anxiety scores and increased SV2A protein expression did not show aberrant expression patterns for the confounding factors in relation to SV2A protein expression (Figure 1.15).

SV2A mRNA expression was not significantly correlated with postmortem interval, age, total adversity, or social isolation, and it was not significantly associated with gender or antidepressant use. The seven individuals with increased anxiety scores and increased SV2A protein expression did not show aberrant expression patterns for the confounding factors in relation to SV2A mRNA expression (Figure 1.16).

miR-133a expression was not significantly correlated with postmortem interval, age, total adversity, or social isolation and it was not significantly associated with gender or antidepressant use. The seven individuals with increased anxiety scores and increased SV2A protein expression did not show aberrant expression patterns for the confounding factors in relation to miR-133a expression (Figure 1.17).

miR-138 expression was not investigated in relation to potential confounding variables as its expression was not signifiantly associated with anxiety in the enlarged cohort.

miR-218 expression was not significantly correlated with postmortem interval, age, total adversity, nor social isolation and it was not significantly associated with gender or antidepressant use. The seven individuals with increased anxiety scores and

increased SV2A protein expression did not show aberrant expression patterns for the confounding factors in relation to miR-218 expression (Figure 1.18).

Expression of Additional miRNAs Predicted to Target SV2A

Targetscan predicted high probability of targeting of the SV2A 3'UTR by the following miRNAs: miR-128, miR-27ab, miR-19, miR-137, miR-22, and miR-139-5p (Figure 1.3A). miRNA expression data from the enlarged cohort was examined for expression values of each of the above-mentioned miRNAs, and miRNA expression levels were correlated with SV2A protein and mRNA expression. Interestingly, miR-137 showed a positive correlation with SV2A mRNA expression (Pearson's $R=0.344$ and $p=0.019$) and no significant correlation with SV2A protein expression (Figure 1.19H). Positive correlation between miRNA and SV2A mRNA expression was also found for miR-22 (Pearson's $R=0.375$ and $p=0.01$) and for miR-139-5p (Pearson's $R=0.687$ and $p<0.000001$), both of which did not show significant correlation with SV2A protein expression (Figures 1.19J and L, respectively). There was no significant correlation between the expression of the other miRNAs and SV2A protein or mRNA expression. Each of the seven individuals with increased anxiety score and increased SV2A protein expression was tracked in each graph in order to determine whether aberrant expression of any of these miRNAs could potentially contribute to the increase in SV2A protein, and no aberrant expression patterns were observed (Figure 1.19).

Synaptotagmin Expression

There was no correlation between anxiety score and synaptotagmin protein expression (Pearson's R = -0.209, p = 0.164). There was also no significant correlation between the deviation from mean synaptotagmin expression and anxiety score (Pearson's R = -0.264, p = 0.074).

DISCUSSION

The genome-wide approach utilized in this dissertation identified the SV2A gene and its potential regulation by miR-133a and miR-218 in the development of an anxious phenotype. Both increased and decreased expression of SV2A is associated with increased anxiety, suggesting that precise levels of SV2A are necessary for proper synaptic function.

SV2A is one of three genes of the membrane glycoprotein SV2 that encode the isoforms SV2A, SV2B, and SV2C and is expressed exclusively in neurons and endocrine cells [172]. SV2A is the most widely expressed isoform [173] and is the only isoform that is expressed in many GABAergic, inhibitory neurons [173, 174]. Indeed, SV2A shows greater expression in the human amygdala when compared to other tissues, based on genome-wide expression profiling data [175, 176].

SV2A mediates the effectiveness of calcium in inducing vesicle fusion by regulating the vesicle content of synaptotagmin, a major calcium sensor protein [167]. In this way, it functions as a positive modulator of calcium-dependent exocytosis [171]. A lack of SV2A results in decreased secretion of neurotransmitter in excitatory [177] and inhibitory neurons [178] and in cultured chromaffin cells [179] (reviewed in [171]). Most systems show a decreased number of vesicles that are capable of responding to increased levels of presynaptic calcium. Thereby, a decrease in SV2A leads to a decrease in the readily releasable pool of vesicles [177, 179, 180].

SV2 shows structural similarity to the major facilitator transporter family, suggesting that SV2A functions as a transporter. However, its described functions

include regulating both expression and trafficking of synaptotagmin [167] as well as affecting the concentration of presynaptic calcium [180]. Thereby, it is plausible that the mechanism by which SV2A regulates neurotransmission is rooted in SV2A's control of the vesicle's ability to detect changes in levels of presynaptic calcium [171]. Mice that lack SV2A develop severe seizures and die within 3 weeks of birth [178, 181], whereas mice heterozygous for one functional copy of SV2A have a normal lifespan, but develop an anxiety-like phenotype as evidenced by an increase in open-arm avoidance in the elevated plus-maze test along with shorter escape latency from a lit area in the inhibitory avoidance procedure [168].

The majority of anxiety-related behavioral tests in mice employ approach-avoidance behaviors that are designed to mimic the animal's response to conflict in the natural environment [182]. Both approach and avoidance behaviors are evolutionarily conserved from nematodes to mammals and the neural organization of fear is comparative across species (reviewed in [182]). Paradigms that test anxiety with approach-avoidance behavior have been validated with pharmacological interventions used in humans and are therefore considered to be appropriate animal models of human anxiety (reviewed in [182]). These include the elevated plus maze, the light dark box, the open field test, as well as novelty induced hypophagia tests. In this battery of tests, mice choose between exploration or remaining in a safe environment. Notwithstanding differences in cognition between humans and mice, anxiety genes discovered in mice with the aid of the above tests have been shown to translate to humans (reviewed in [182]). To date, there have been no human studies investigating

the potential link between SV2A and anxiety and thus we aimed to examine its role in human anxiety.

Interestingly, increased SV2A expression exhibits a similar phenotype in cultured cells to that seen with decreased expression [171]. The reduced synaptic release probability measured in neurons that overexpress SV2A is nearly identical to that seen in neurons from SV2A/B knockout mice that experience severe seizures [178, 181] [171]. In cultured neurons overexpressing SV2A, the action potential-induced excitatory postsynaptic currents (EPSC) are reduced by an average of 35% compared to control neurons, and responses to a 10 Hz stimulation train fail to decline to the extent seen in the control [171]. The reduction in synaptic depression (implying reduced synaptic release probability) is a hallmark feature of the SV2A knockout phenotype that is also present in neurons that overexpress SV2A [171].

Decreased SV2A expression leads to a decrease in expression and trafficking of synaptotagmin [171, 183, 184] whereas SV2A overexpression leads to an increase in cellular synaptotagmin levels. Thereby, both increased and decreased SV2A and synpatotagmin expression lead to a common phenotype of reduced synaptic release probability. Interestingly, overexpression of SV2A results in a small but significant decrease in the colocalization of SV2A and synaptotagmin. These results imply that optimal levels of both proteins are necessary for proper neurotransmission, a notion that is further supported by the observation that the amount of SV2s and synaptotagmin per vesicle is under tight regulation [185]. Interestingly, there was no significant association between synaptotagmin expression and anxiety in our sample. However, we did not distinguish between protein expression in the cell body and neurite from protein

expression in the synapse, and it is possible that changes in levels of both proteins occurred in the synapse.

Changes in SV2A expression in either direction have similar impact on synaptic function and are consistent with the observation that both increased and decreased levels of SV2A have been associated with seizures [171]. Kindling of seizures in rats leads to increased SV2A expression [186-188], whereas qualitative expression analyses show decreased SV2A expression in temporal lobe epilepsy [189].

SV2A is the binding site of the anticonvulsant drug Levetiracetam ((S)-α-ethyl-2-ox-pyrrolidine acetamide) [169], which shows promise in anxiety disorder treatment [190-192]. Levetiracetam reduces kindling-induced appearance of seizures [193]. In cultured neurons overexpressing SV2A, treatment with Levetiracetam results in a decrease in the amount of both SV2A and synaptotagmin at the synapse and a decreased amount of total synaptotagmin per length of neurite. However, Levetiracetam treatment fails to restore lower levels of total SV2A and did not alter the ratio of SV2A and synaptotagmin at the synapse [171]. Interestingly, Levetiracetam has not been found to restore normal EPSC amplitude nor synaptic depression in hippocampal neurons that lack SV2A, suggesting that Levetiracetam works by reversing the effects of SV2A overexpression rather than its insufficiency [171]. Since SV2A is part of a protein complex, it is possible that Levetiracetam alters SV2A's ability to bind to proteins that influence protein trafficking or localization, such as clatherin adaptor proteins or cytoskeletal proteins [171]. In this way, Levetiracetam might work to decrease inappropriate protein interactions that occur due to SV2A over-abundance [171].

Levetiracetam has demonstrated promise in treatment of anxiety disorders [194]. Preclinical studies have shown potential for Levetiracetam as a mood stabilizer in animal models of anxiety, depression, and mania (reviewed in [195, 196]). A three month add-on trial of Levetiracetam for drug resistant epilepsy demonstrated improvement in both anxiety and depressive symptoms [197]. An open-label multicenter study found that Levetiracetam monotherapy and add-on therapy for epilepsy contributed to an improvement in psychiatric symptoms while reducing the frequency of seizures [198]. A 24-week prospective open-label study of Levetiracetam adjunctive therapy for epileptic patients saw improvements on the Beck Anxiety Inventory score, as well as in measures such as obsessive-compulsiveness, depression, and anxiety, along with an improvement in the overall quality of life, emotional well-being, seizure worry, energy-fatigue, and social function [196].

Concurrently, several studies have found negative psychotropic effects that include irritability, aggressiveness, suicidality, mood disorders, and other psychiatric symptoms in both children and adults on Levetiracetam therapy [199-206]. Psychiatric side-effects can be seen in more than 10% of patients using the drug. These include behavioral symptoms such as “agitation, aggression, anger, anxiety, apathy, depersonalization, depression, emotional lability, hostility, hyperkinesias, irritability, nervousness, neurosis and personality disorder” [207]. Risk factors for developing psychiatric adverse effects include poor seizure control and previous psychiatric history [196].

It is likely that the conflicting effects of Levetiracetam on anxiety-related symptoms support the necessity of a precise SV2A stoichiometry at the synapse for normal

neurotransmission. It is possible that both increased and decreased SV2A levels can lead to symptoms of anxiety, as is the case with epilepsy, and response to treatment with Levetiracetam will depend on whether the particular anxious phenotype results from too much or too little SV2A. *In vitro* studies suggest that Levetiracetam corrects the neurotransmission defect seen with SV2A overexpression, and so it is likely that individuals with epilepsy or anxiety resulting from overabundant SV2A might be more likely to respond positively to the drug, whereas those with disease due to insufficient SV2A levels might experience an exacerbation of psychiatric symptoms.

The deviation from mean SV2A expression seen in our study in anxious individuals was modified by the number of total adverse life experiences. Participants with greater life adversity were more likely to experience increased anxiety, which is congruent with current research that shows a positive association between negative life events and anxiety disorder onset [208]. There was no difference in SV2A mRNA expression between anxious individuals and controls in our sample, and there was an increase in the expression of miR-133a, MiR-138, and miR-218 in Cohort 1. miR-133a and miR-218 continued to be associated with anxiety in the enlarged cohort. These miRNAs are computationally predicted to target SV2A and suggested a mechanism of translational repression. Results from cell culture experiments demonstrated that miR-133a and miR-218, but not miR-138 target the SV2A 3'UTR, leading to decreased SV2A mRNA and protein levels, supporting a mechanism of mRNA degradation.

There is currently substantial debate whether miRNA induced target silencing occurs primarily through mRNA degradation or at the level of translation [209, 210]. It is possible for mRNAs to be repressed exclusively at the level of translation, exclusively at

the level of mRNA stability, or by a combination of both mechanisms [211] and the precise determinants for this are not fully understood [212, 213]. Some studies have suggested that the mechanism of repression might be influenced by 3'UTR context-dependent features and mRNA-binding factors [209, 214]. For example, it is possible that miRNA-mediated deadenylation (direct or indirect) might have a different effect on mRNA stability, depending on the cellular environment. A system that experiences robust decay would see rapid destruction of mRNA transcript following deadenylation [215]. However, a system wherein mRNA decay is not as prevalent following deadenylation might experience mRNA target storage [215]. The effect of a given miRNA on its target mRNA might also be linked to the specific 3'UTR context in that the miRISC complex interacts with mRNA decay factors such as the deadenylase and decapping complex, and it also binds regulators of translation [215]. 3'UTRs can be filled with proteins capable of exerting positive or negative effects on stability and/or translation, as well as transport, localization, and polyadenylation/deadenylation [215]. In this way, a particular miRNA/mRNA relationship can be complex, malleable, and unique to a particular biological system. It is therefore likely that miR-133a and miR-218 contribute to the anxious phenotype by decreasing SV2A protein levels, but the mechanism of miRNA-mediated gene regulation might differ in the brain from that seen in cell culture.

Interestingly, positive correlation was observed between SV2A mRNA levels and several miRNAs computationally predicted to target SV2A (miR-22, miR-137, and miR-139-5p). Several groups have found significant positive correlation between miRNA and target mRNA levels in gene expression data (as reviewed in [216]). This could result

from co-regulation of a miRNA and its target by a common upstream factor [217] or might support potential positive regulation as suggested by Vasudevan et al. [218]. Positive regulation might be less likely in our study, since SV2A protein levels did not significantly correlate with the expression of these miRNAs. It is also likely that the positive correlation with mRNA levels might reflect an inaccuracy in the prediction algorithm for some of the miRNA/mRNA associations.

FUTURE DIRECTIONS

An increase in the number of lateral amygdala samples from anxious individuals would improve study power and facilitate determination of whether trait anxiety is truly associated with both a decrease and an increase in SV2A protein.

RT-qPCR will be performed shortly in order to confirm the differences in miR-133a and miR-218 expression seen in anxious individuals. RNA from these participants is in limited supply and so RNA amplification must be conducted prior to RT-qPCR analysis. Immunohistochemistry of lateral amygdala tissue would enable examination of the spatial distribution of SV2A proteins and should be prioritized in additional samples that will be obtained at a future date. Quantitative immunoblotting will be done from tissue lysates in an effort to confirm differences in protein levels between anxious individuals and controls. These experiments have not yet been conducted due to the scarcity of the postmortem tissue, along with interest in additional gene targets that requires prioritization. An animal model of miR-133a and miR-218 infusion into the LA would be key to understanding whether these two miRNAs in fact lead to a decrease in SV2A protein levels in the amygdala and result in an anxious phenotype, a conclusion that is currently based on correlation. In addition, Levetiracetam should be administered to SV2A heterozygous mouse in order to assess effects on anxiety with the hypothesis that the drug might exacerbate the anxious phenotype in this mouse model.

Additional transfection experiments can be done to further clarify the targeting relationship between miR-133a, miR-218 and SV2A. Transfection of HEK 293 cells with

increasing concentrations of miR-133a and miR-218 would enable determination whether miRNA-mediated repression is dependent on miRNA concentration. This experiment was attempted once with no observed effect of concentration (data not shown). In addition, SH-SY5Y cells can be transfected with increasing concentrations of the two miRNAs to determine whether there are concentration-dependent effects on SV2A mRNA and protein levels. In addition, co-transfection of miR-133a and miR-218 should be done to assess for additive effects. This experiment was performed once in HEK 293 cells, and enhanced repression due to co-transfection was not seen (data not shown). miR-133a and miR-218 co-transfection should be done in SH-SY5Y cells in order to determine whether there is an additive effect of the two miRNAs on SV2A mRNA and protein levels. Finally, luciferase constructs with mutation of miR-218 and miR-133a sites that are not as strongly predicted to target SV2A should be made, along with a construct that mutates both of the functional miR-218 and miR-133a sites. Some of these experiments were attempted, and unfortunately many of the constructs failed to induce luciferase activity, indicating a technical problem that requires additional troubleshooting.

Future experiments will aim to determine the mechanism of miRNA-mediated SV2A gene silencing by miR-133a or miR-218 in HEK 293 cells. Transfection experiments in SH-SY5Y cells suggest a mechanism of mRNA degradation (or perhaps translational repression with subsequent mRNA degradation) and the mechanism of miRNA-mediated gene silencing in HEK 293 cells could be determined experimentally and compared to that seen in SH-SY5Y cells in an effort to better understand miRNA-mediated gene silencing by these miRNAs.

Future experiments will also examine causes of the increased miR-133a and miR-218 expression seen in anxious individuals. It is possible for increased miRNA expression to be caused by single nucleotide polymorphisms (SNPs) that influence miRNA gene expression and/or processing. Two genes encode miR-133a; they are MIR133A1 (which encodes miR-133a-1) and MIR133A2 (which encodes miR-133a-2). The mature sequences of both miR-133a-1 and miR-133a-2 are identical. MIR133A1 is found on chromosome 18 and MIR133A2 is located on chromosome 20 (reviewed in [219]). Previous studies have shown that the 79 Thymine > Cytosine MIR133A2 variant (substitution of Thymine for Cytosine at the 79th position in the miRNA stem-loop sequence) is associated with an increase in miR-133a-5p levels [219]. Seventeen SNPs are found in the MIR133A1 gene and flanking regions 1 kilobase up and downstream of the gene. There are 41 such SNPs in the MIR133A2 gene [220]. miR-218 is encoded by two genes- MIR218-1 (chromosome 4) and MIR218-2 (chromosome 5) [220]. There are 16 SNPs in the MIR218-1 gene and surrounding regions 1 kilobase up and downstream of the gene. There are 32 SNPs in the MIR218-2 gene and surrounding regions [220]. Future work will aim at genotyping select SNPs that are most likely to lead to differences in gene expression in the postmortem sample. In addition, SNPs in the miRNA-binding sites of SV2A will be analyzed for possible differences in miRNA sensitivity.

Finally, co-immunoprecipitation experiments of SV2A and synaptotagmin would enable determination of the association of the two proteins in each individual according to anxiety score. In addition, separation of the synaptic fraction from that of the cell body

and neurite in future samples would enable investigation of SV2A, synaptotagmin, and miRNA levels at the synapse where they are most functionally relevant.

FIGURES

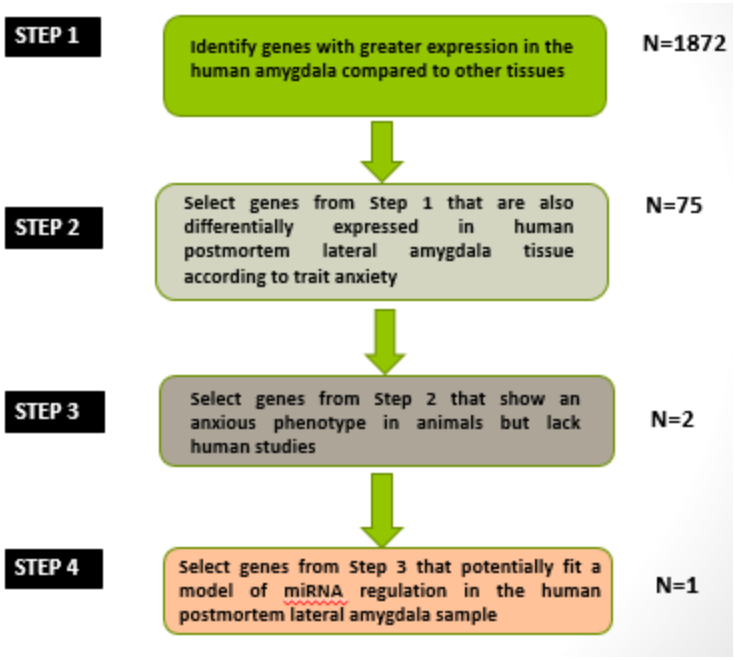
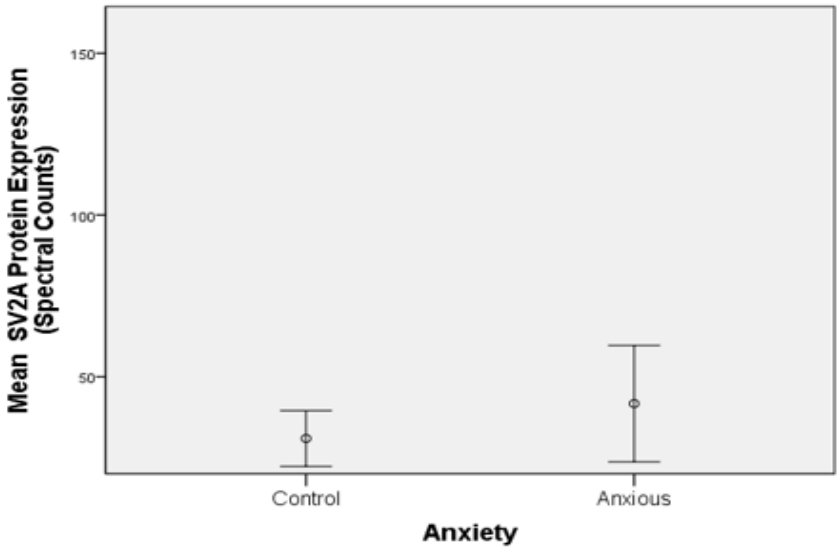


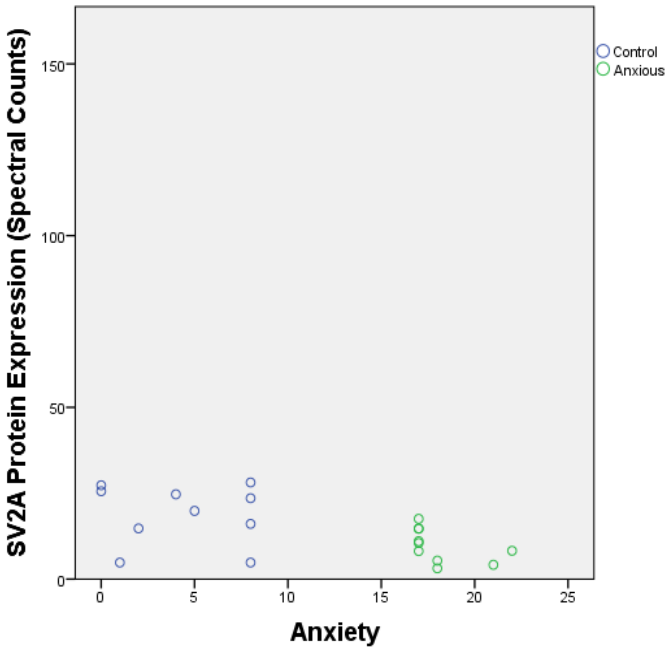
Figure 1.1. Selection of Candidate Gene SV2A

The candidate gene SV2A was selected according to the following criteria: increased expression in the human lateral amygdala compared to other tissues, differential expression according to trait anxiety status in human postmortem sample, published animal models of anxious behavior with a lack of human studies, and potential for miRNA regulation in the postmortem sample.

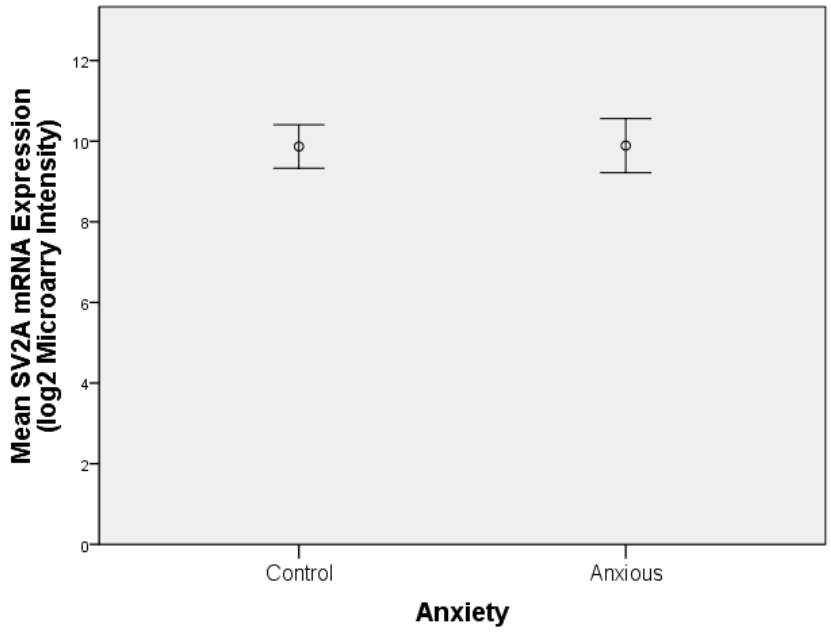
Figure 1.2. SV2A Protein and mRNA levels in Human Postmortem Lateral Amygdala

A. SV2A Protein Levels are Lower in Anxious Individuals Compared with Controls





B. SV2A mRNA Levels Do Not Differ Significantly Between Anxious Individuals and Controls



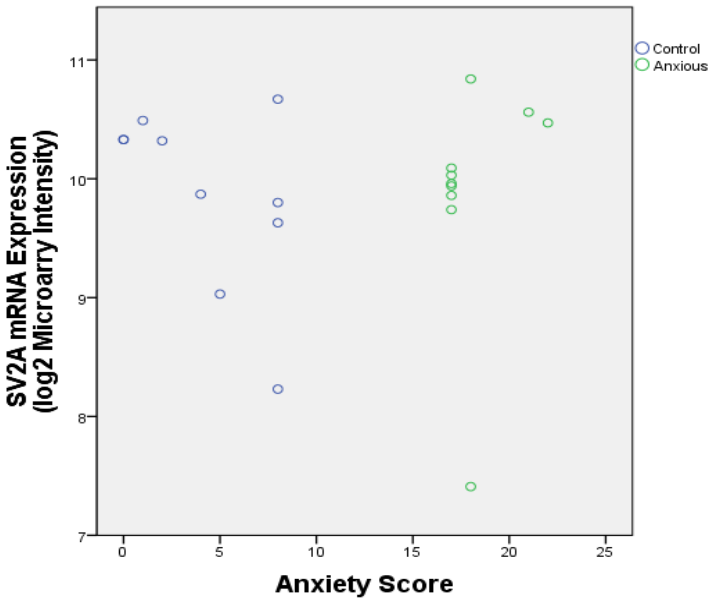
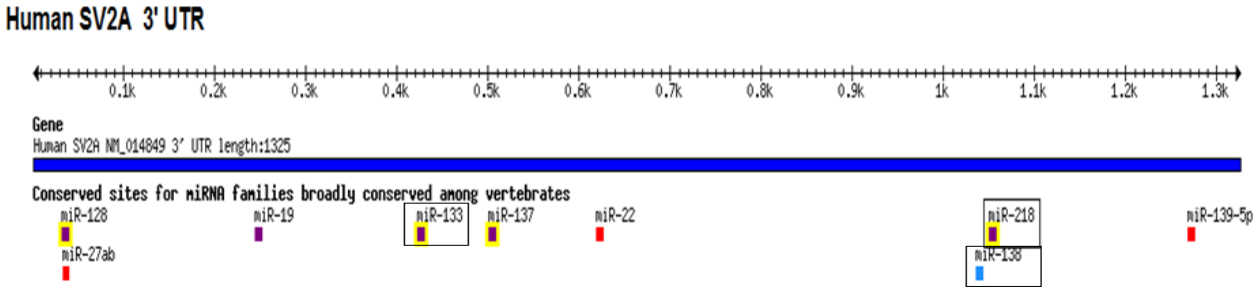


Figure 1.2. SV2A Protein and mRNA levels in Human Postmortem Lateral Amygdala.

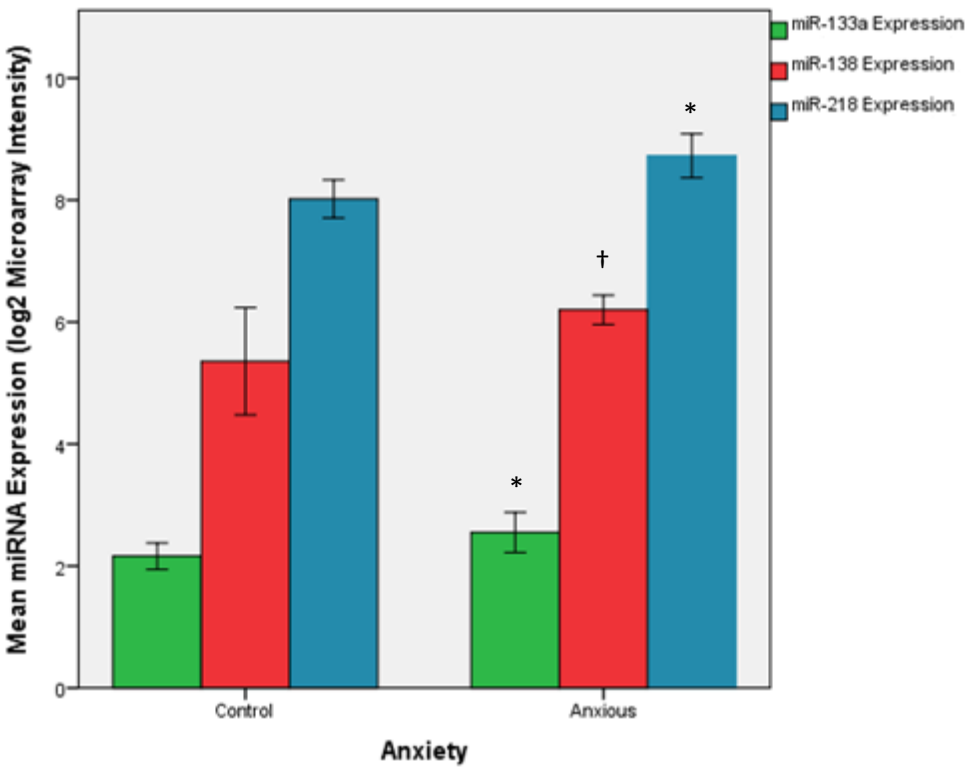
A). Mean SV2A protein levels are lower in anxious individuals when compared to controls. T-test p-value < 0.05. **B).** Mean mRNA levels do not differ significantly between controls and anxious individuals. T-test p-value= 0.87. Error bars are 95% confidence interval of the mean.

Figure 1.3. miR-133a, miR-218, miR-138 are Differentially Expressed According to Trait Anxiety and are Predicted to Target SV2A

A. Targetscan Prediction of SV2A Targeting by miR-218, miR-133a, and miR-138



B. miR-133a, miR-138, and miR-218 Levels are Higher in Anxious Individuals than Controls



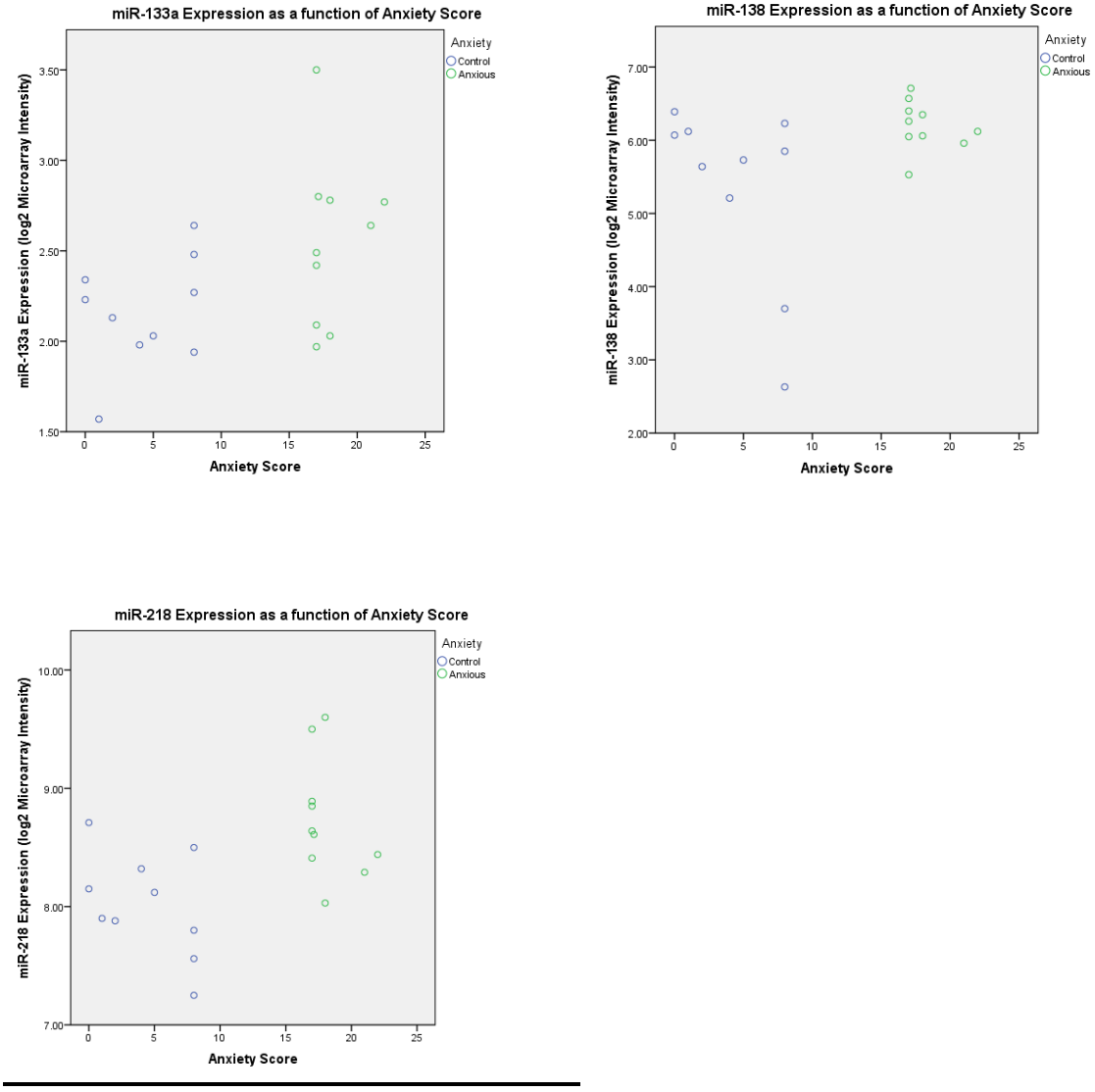
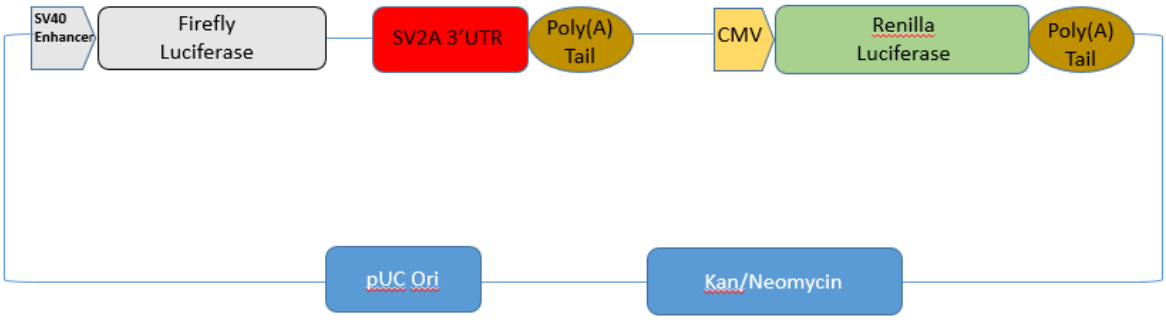


Figure 1.3. miR-133a, miR-218, miR-138 are Differentially Expressed According to Trait Anxiety and are Predicted to Target SV2A.

A). The three miRNAs (miR-133a, miR-138, and miR-218) are highly likely to target the SV2A 3'UTR according to Targetscan prediction algorithms. **B).** Significantly higher levels of the three miRNAs (miR-133a, miR-138, and miR-218) are found in anxious individuals when compared to controls. Error bars are 95% confidence interval of the mean. *t-test p-value <0.05 and Wilcoxon rank-sum p-value <0.05 for miR-133a and miR-218 expression in anxious individuals versus controls. † t-test p-value = 0.051 and Wilcoxon-Rank p-value <0.05 for miR-138 expression in anxious individuals versus controls.

Figure 1.4. miR-218 and miR-133a Target the 3'UTR of SV2A, whereas miR-138 Does Not Target the SV2A 3'UTR

A. Schematic of the Dual Luciferase (Firefly/Renilla) vector



B. miR-133a and miR-218 Target the SV2A 3'UTR, whereas miR-138 does not Target the SV2A 3'UTR

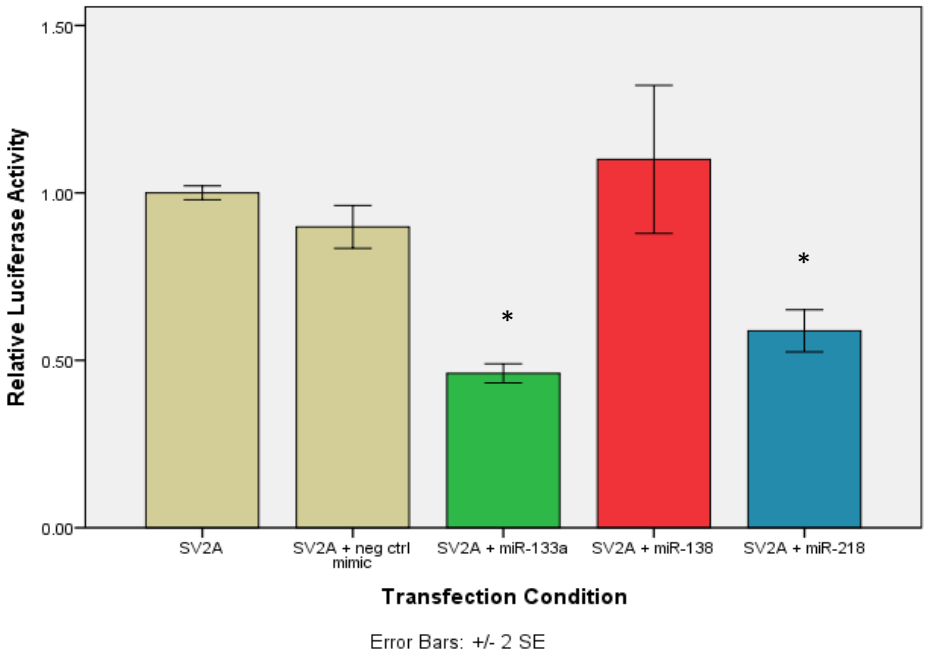


Figure 1.4. miR-218 and miR-133a Target the 3'UTR of SV2A, whereas miR-138 Does Not Target the SV2A 3'UTR.

A). The SV2A 3'UTR clone was obtained from Genecopoeia. Schematic Key: SV40 Enhancer: Simian virus 40 enhancer, functions as a weak promoter. CMV: cytomegalovirus promoter, functions as a strong prmoter. Kan/Neomycin: Kanamycin/Neomycin resistance gene, pUC Ori: origin of replication (pUC = plasmid University of California). **B).** HEK 293 cells were transfected with the SV2A-3'UTR vector and respective miRNA mimics and incubated for 24 hrs. All firefly/renilla ratios are normalized to the SV2A 3'UTR clone. Each experiment was repeated independentenly three times and each condition was performed in pentuplicate with exclusion of at most two outlying measurements per pentuplicate measurement set. Error bars are two standard errors of the mean. * Statistical significance is based on one-way ANOVA with Tukey post-hoc test, p-value < 0.05 vs SV2A control.

Figure 1.5. Mutation of miR-218 and miR-133a Binding Sites in the SV2A 3'UTR Prevents Targeting

A. Mutation of miR-218 and miR-133a Binding Sites

miR-218 binding site was mutated from "A**GC**ACA" to "A**CG**ACA"

Clone: "SV2A 218 mutant"

miR-133a binding site was mutated from "GGAC**CAA**A" to "GGA**GGG**AA"

Clone: "SV2A 133a mutant"

B. miR-133a and miR-218 do not Target the Mutated SV2A 3'UTR

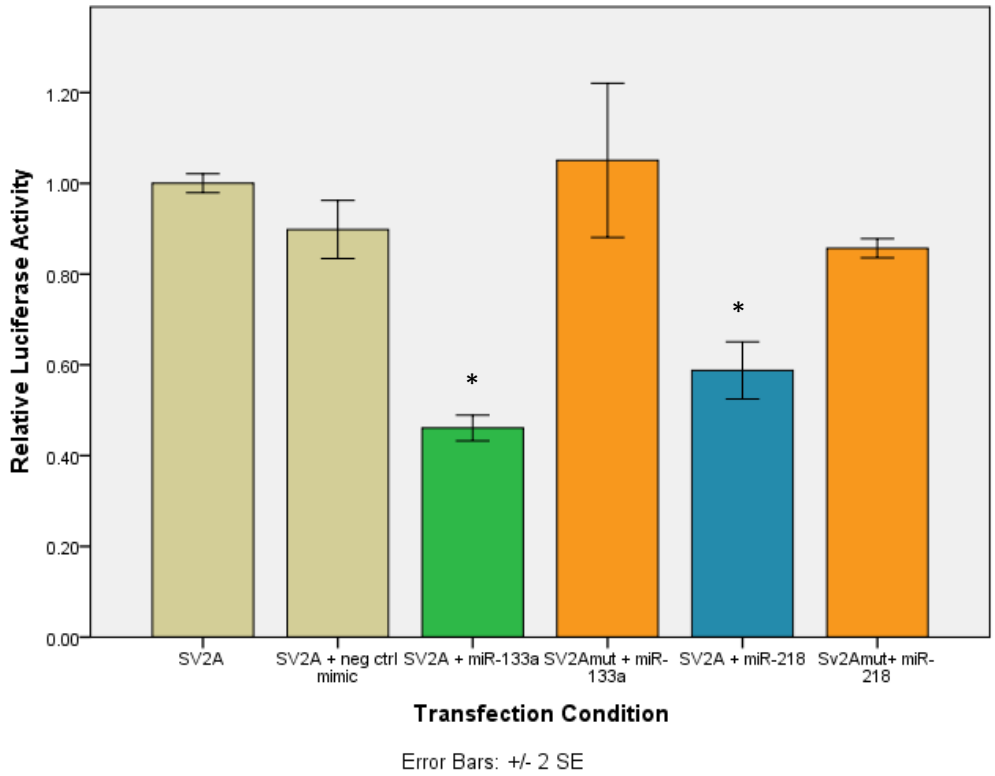


Figure 1.5. Mutation of miR-218 and miR-133a Binding Sites in the SV2A 3'UTR Prevents Targeting.

A). miR-218 and miR-133a binding sites were mutated in the SV2A 3'UTR Dual Luciferase clone. The miR-218 binding site was mutated from "A**GC**ACA" to "A**CG**ACA" as in [161]. The miR-133a binding was mutated from "GGAC**CAA**A" to "GGA**GGG**AA. **B).** Disruption of the predicted miRNA binding sites prevents a decrease in relative luciferase activity, suggesting specific targeting of the SV2A 3'UTR. All firefly/renilla ratios are normalized to the SV2A 3'UTR clone. Each experiment was repeated independentenly three times and each condition was performed in pentuplicate with exclusion of at most two outlying measurements per pentuplicate measurement set. Error bars are two standard errors of the mean. * Statistical significance is based on one-way ANOVA with Tukey post-hoc test, p-value < 0.05 vs SV2A control.

Figure 1.6. Transfection with miR-133a and miR-218, but not miR-138, Decreases Endogenous SV2A mRNA Levels in SH-SY5Y Cells

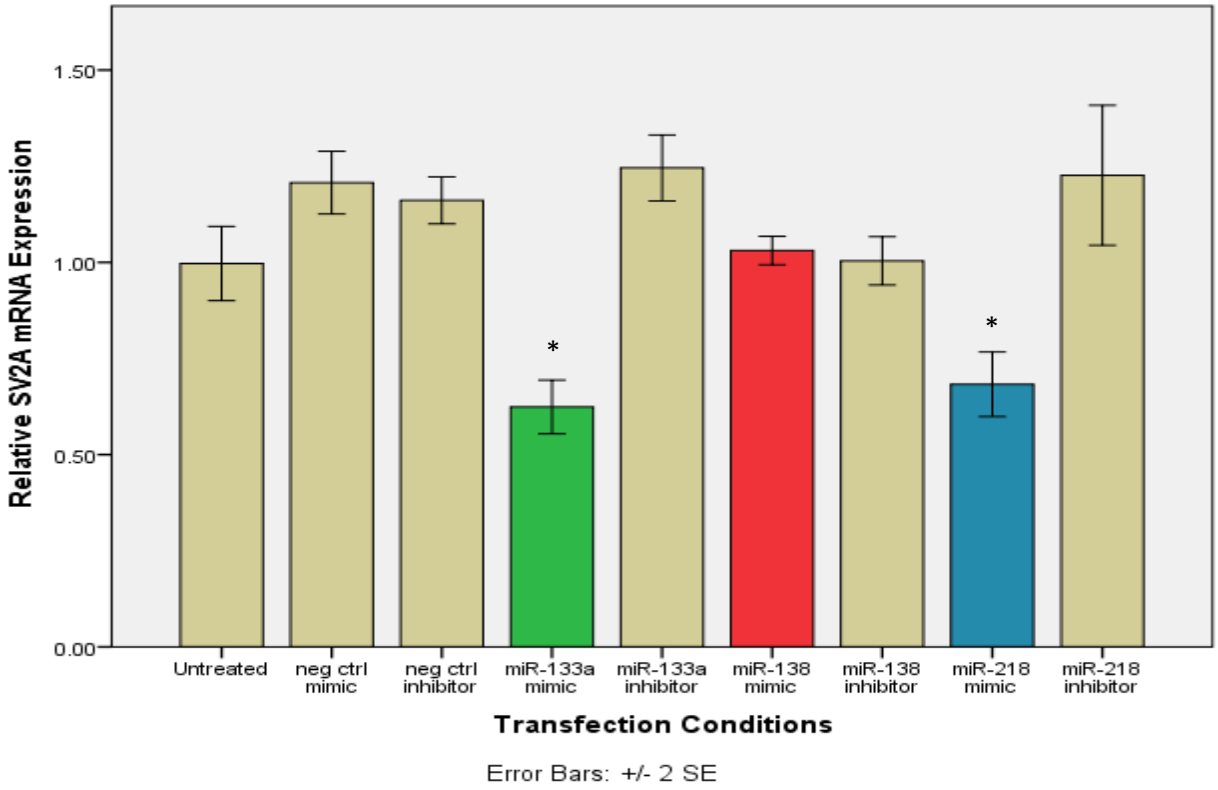
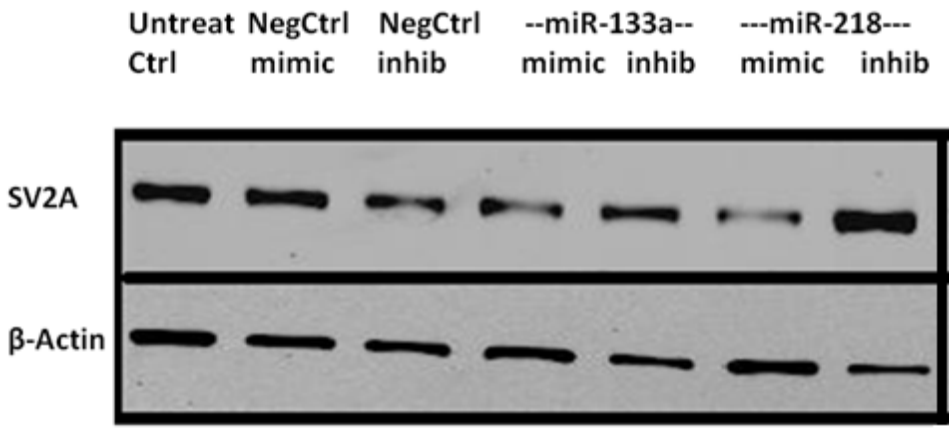


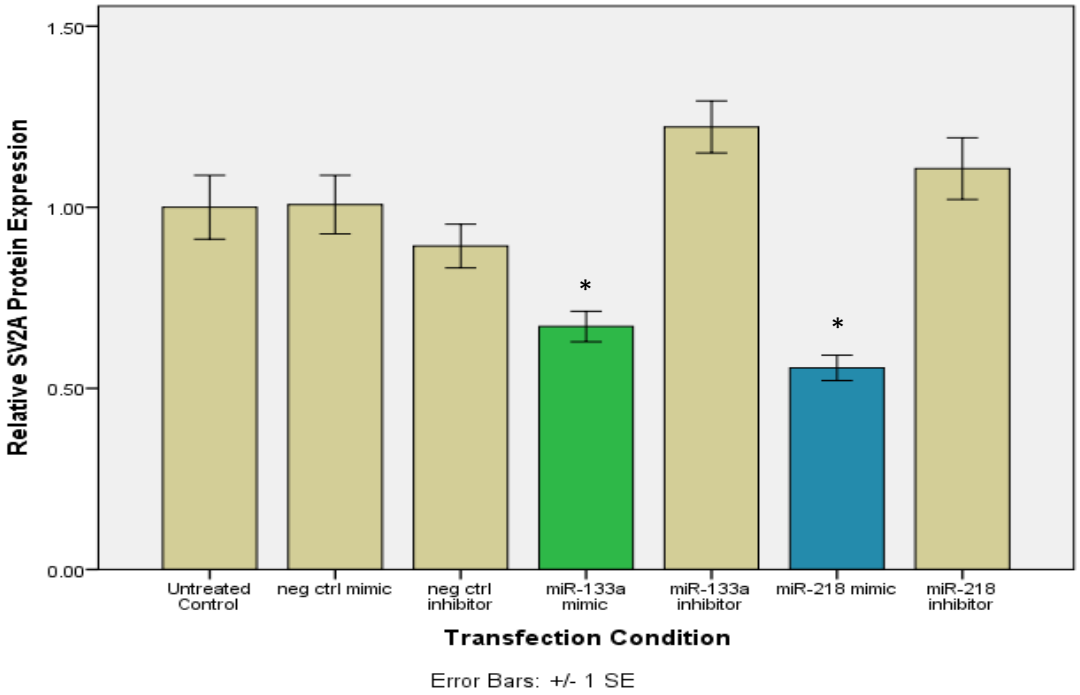
Figure 1.6. Transfection with miR-133a and miR-218, but not miR-138, Decreases Endogenous SV2A mRNA Levels in SH-SY5Y Cells.

miR-133a, miR-138, and miR-218 mimics, as well as relevant controls, were transfected into SH-SY5Y cells as separate experimental conditions. After 24 hours, SV2A mRNA levels were assessed by RT-qPCR. Each experiment was repeated independently three times, and each condition was conducted in pentuplicate with exclusion of at most 1 measurement. Cells transfected with miR-133a and miR-218 mimics show a significant decrease in SV2A mRNA, whereas cells transfected with miR-138 do not show a decrease in SV2A mRNA. Error bars are two standard errors of the mean. *Statistical significance is based on one-way ANOVA with Tukey post-hoc test, p-value < 0.05 vs untreated control condition and mock transfection with negative control mimic.

Figure 1.7. Transfection with miR-133a and miR-218, but not miR-138, Decreases Endogenous SV2A Protein Levels in SH-SY5Y Cells

A. Transfection with miR-133a and miR-218 Decreases Endogenous SV2A Protein Levels in SH-SY5Y Cells





B. Transfection with miR-138 does not Decrease Endogenous SV2A Protein Levels in SH-SY5Y Cells

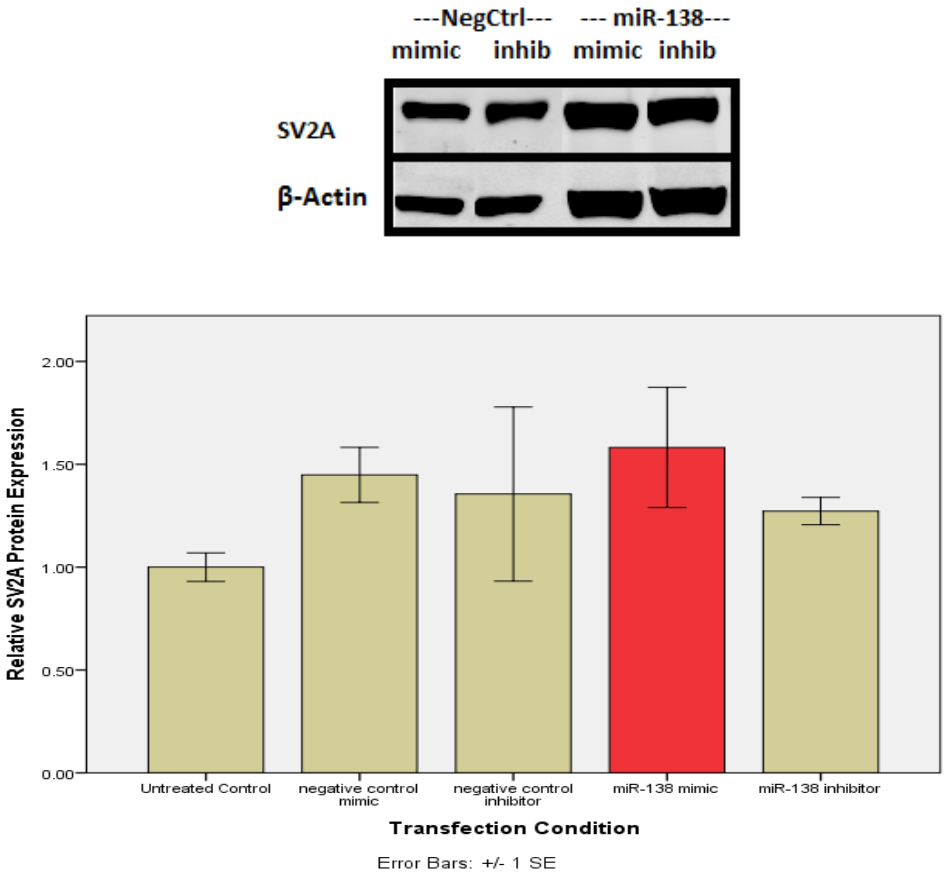
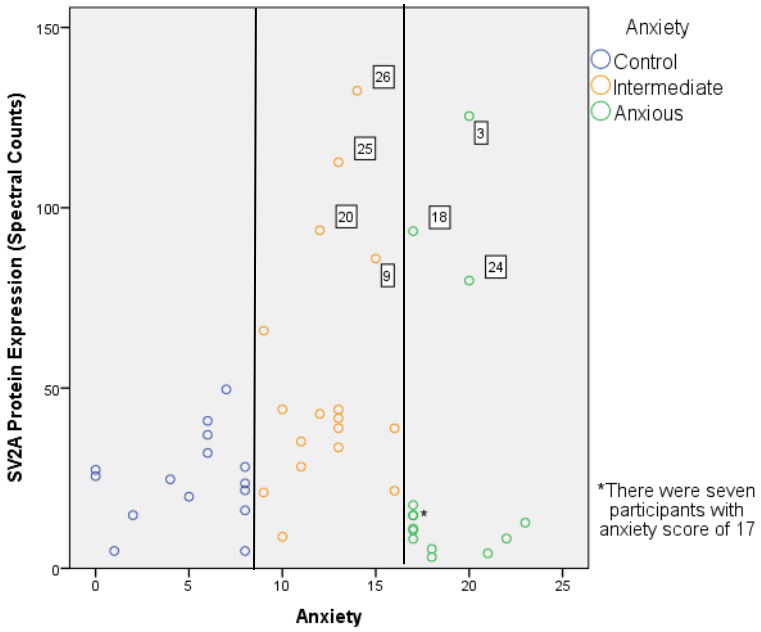


Figure 1.7. Transfection with miR-133a and miR-218, but not miR-138, Decreases Endogenous SV2A Protein Levels in SH-SY5Y Cells.

miR-133a and miR-218 mimics **(Panel A)** and miR-138 mimic **(Panel B)** were transfected into SH-SY5Y cells along with relevant controls as separate experimental conditions. After 48 hours, SV2A protein levels were assessed by quantitative immunoblotting. Each experiment was repeated independently three times, and each condition was conducted in quadruplicate with exclusion of at most 1 measurement. Cells transfected with miR-133a and miR-218 mimics show a significant decrease in SV2A protein levels, whereas cells transfected with miR-138 do not show a decrease in SV2A protein levels. The blot images are from representative immunoblots. Error bars are standard error of the mean. *Statistical significance is based on one-way ANOVA with Tukey post-hoc test, p-value < 0.05 vs untreated control condition and mock transfection with negative control mimic.

Figure 1.8. High Anxiety Scores are Associated with both high and low levels of SV2A Protein Expression

A. SV2A Protein Expression as a Function of Anxiety Score



B. SV2A Protein Expression as a Function of Anxiety Score in Cohorts 1 and 2

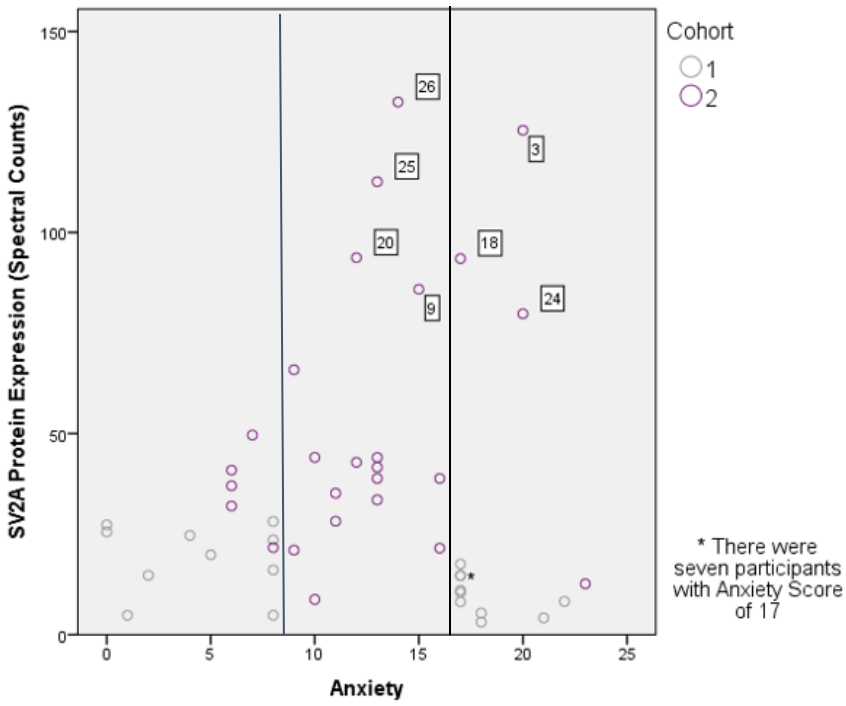
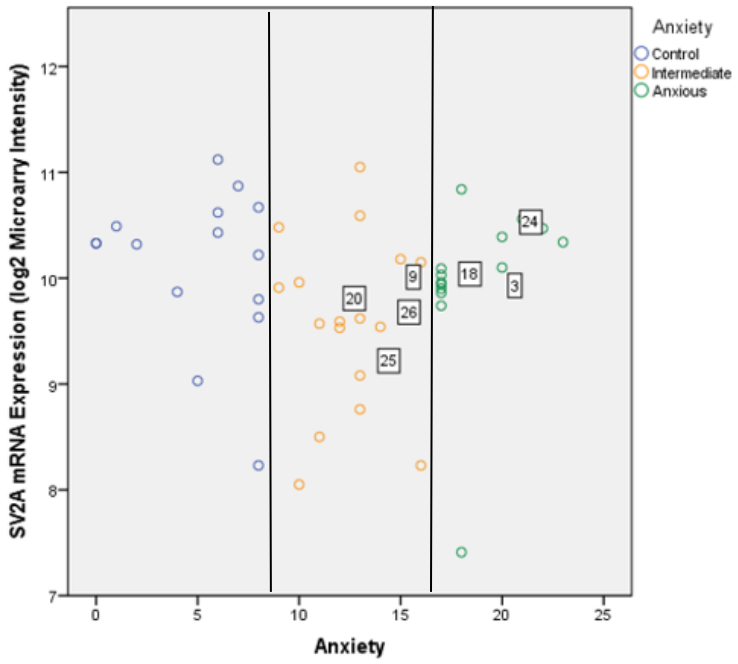


Figure 1.8. High Anxiety Scores are Associated with both high and low levels of SV2A Protein Expression.

A). Genome-wide proteomics analysis for additional samples was conducted with Mass Spectrometry (MudPit). The inverse relationship between SV2A protein expression and anxiety seen in Cohort 1 was not replicated in the enlarged cohort (Pearson R for correlation between SV2A protein expression and anxiety score = -0.067 with p=0.657). However, visual inspection of the above graph suggests that both increased and decreased SV2A expression is associated with anxiety. Individual differences from mean SV2A expression in the enlarged cohort correlate with anxiety score such that larger deviations from the mean correlate with higher anxiety scores (Pearson correlation coefficient= 0.37 with p=0.01 for the correlation between anxiety score and deviation from mean SV2A expression). The seven participants that show increased SV2A expression with increased anxiety score were identified by participant ID number and tracked in further analyses in order to determine whether they differ from the rest of the sample in a significant way. For improved visual analysis, sample participants were divided into three groups with "control" individuals = anxiety scores < 9, "intermediate" individuals = anxiety scores between 9 and 16, and "anxious" individuals = anxiety scores >16. **B).** SV2A protein expression was plotted as a function of anxiety score such that samples that make up the first cohort (Cohort 1 n=20) can be distinguished from samples from the second cohort (Cohort 2 n=26). Samples from both Cohorts 1 and 2 make up the enlarged cohort (n=46).

Figure 1.9. SV2A mRNA Expression does not Correlate with Anxiety Score

A. SV2A mRNA Expression as a Function of Anxiety Score



B. SV2A mRNA Expression as a Function of Anxiety Score in Cohorts 1 and 2

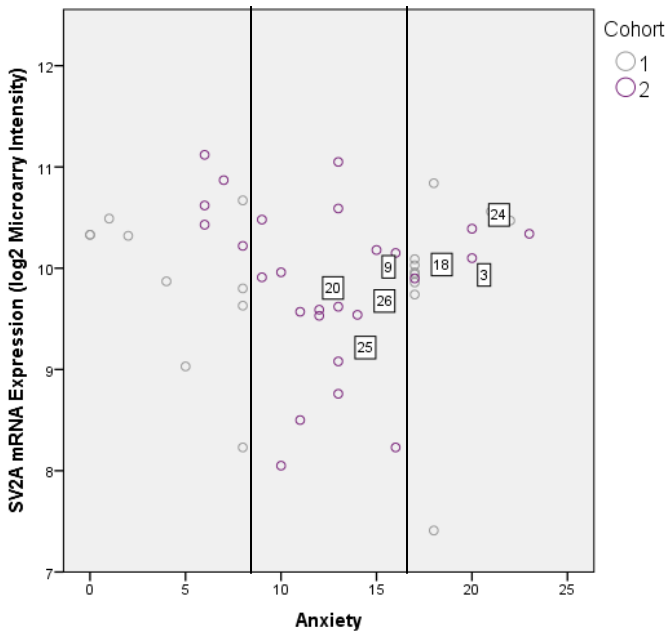
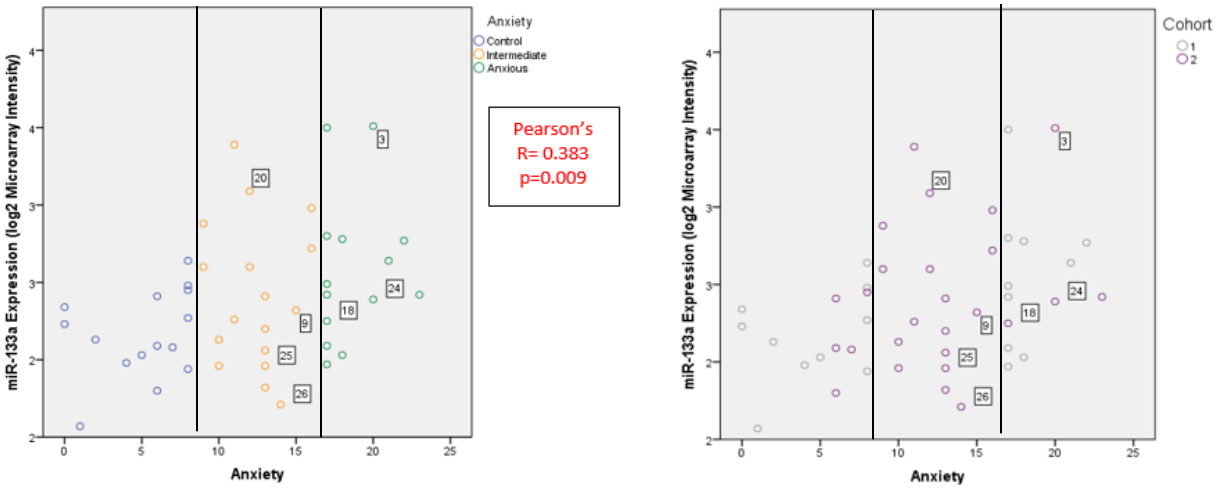


Figure 1.9. SV2A mRNA Expression does not Correlate with Anxiety Score.

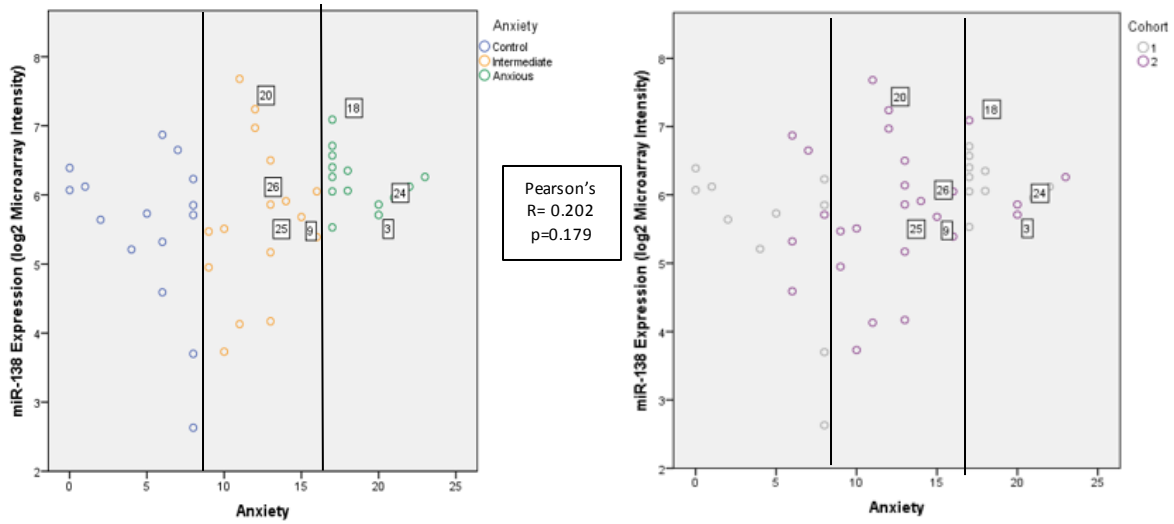
A). Genome-wide mRNA expression analysis for additional samples was conducted with Affymetrix microarrays. There was no significant correlation between SV2A mRNA expression and anxiety score (Pearson's R= -0.088, p=0.559), nor was there a significant correlation between individual differences from mean SV2A expression and anxiety score (Pearson's R= -0.122, p=0.420). The seven participants with increased SV2A protein expression and increased anxiety scores are identified by participant ID number in the graph and do not show an aberrant pattern of SV2A mRNA expression compared with the rest of the sample. **B).** SV2A mRNA expression was plotted as a function of anxiety score such that samples that make up the first cohort (Cohort 1 n=20) can be distinguished from samples from the second cohort (Cohort 2 n=26). Samples from Cohorts 1 and 2 make up the enlarged cohort (n=46).

Figure 1.10. miR-133a and miR-218 Expression, but not miR-138 Expression, Increase with Anxiety Score in the Enlarged Cohort

A. miR-133a Expression as a Function of Anxiety Score



B. miR-138 Expression as a Function of Anxiety Score



C. miR-218 Expression as a Function of Anxiety Score

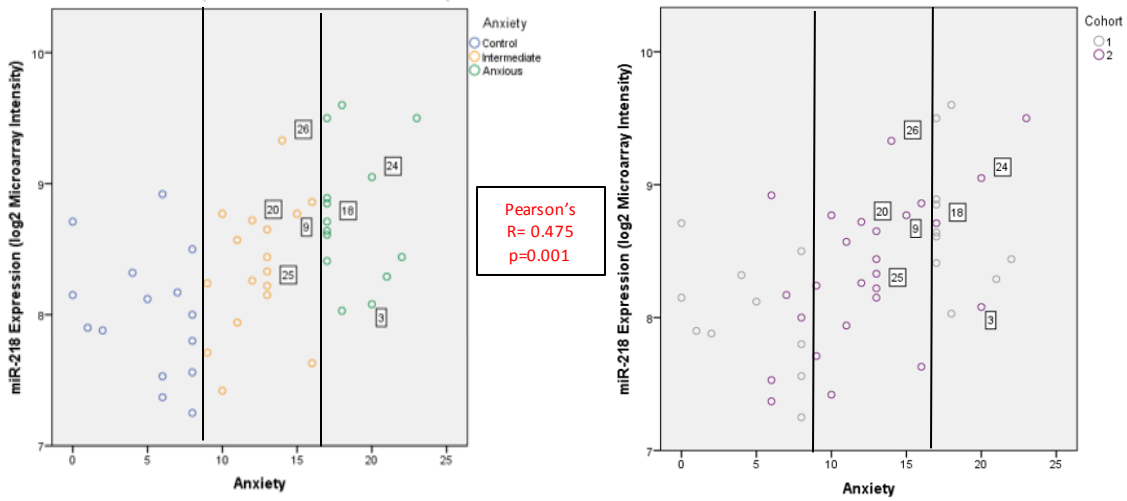


Figure 1.10. miR-133a and miR-218 Expression, but not miR-138 Expression, Increase with Anxiety Score in the Enlarged Cohort.

Genome-wide miRNA profiling on amygdala samples was conducted by microarray. miR-133a **(Panel A)** and miR-218 **(Panel B)** expression show a positive correlation with anxiety score (Pearson's R=0.383 and 0.475 respectively, both with $p<0.05$), whereas miR-138 **(Panel C)** does not (Pearson's R=0.202 and $p>0.05$). The seven individuals with high anxiety scores and high SV2A protein levels are tracked by participant ID number in the graph and several participants show opposite patterns of miR-133a/miR-218 expression. For example, Participant #3 shows a higher level of miR-133a and a lower level of miR-218; Participant #24 shows a lower level of miR-133 and a higher level of miR-218, Participant #25 shows a lower level of miR-133a and a higher level of miR-218. Participant #26 shows a lower level of miR-133a and a higher level of miR-218.

Figure 1.11. SV2A Protein Expression is not Correlated with SV2A mRNA Expression

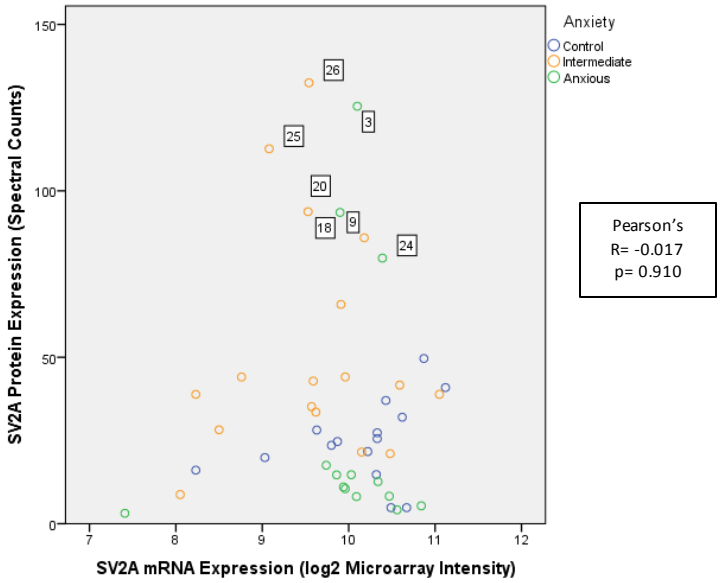


Figure 1.11. SV2A Protein Expression is not Correlated with SV2A mRNA Expression.

SV2A protein and mRNA levels are not correlated in the enlarged cohort, and the seven individuals with increased anxiety scores and increased SV2A protein expression (indicated by participant ID number) do not show aberrantly high SV2A mRNA levels.

Figure 1.12. SV2A Protein Expression is not Correlated with the Expression of Individual miRNAs (miR-133a, miR-138, or miR-218).

A.

B.

C.

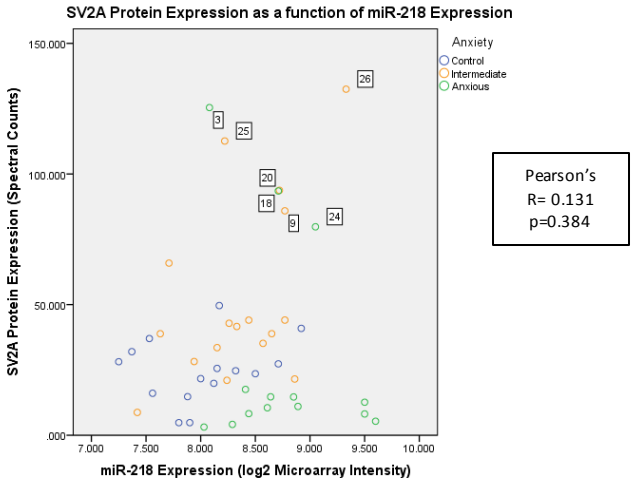


Figure 1.12. SV2A Protein Expression is not Correlated with the Expression of Individual miRNAs (miR-133a, miR-138, or miR-218).

SV2A protein expression is not significantly correlated with the expression of miR-133a **(Panel A)**, miR-138 **(Panel B)**, or miR-218 **(Panel C)**. The seven individuals with increased anxiety scores and increased SV2A protein expression (identified by participant ID number) do not show aberrant expression of the above-mentioned miRNAs when compared to the remaining sample.

Figure 1.13. miR-138 is positively correlated with miR-218 Expression

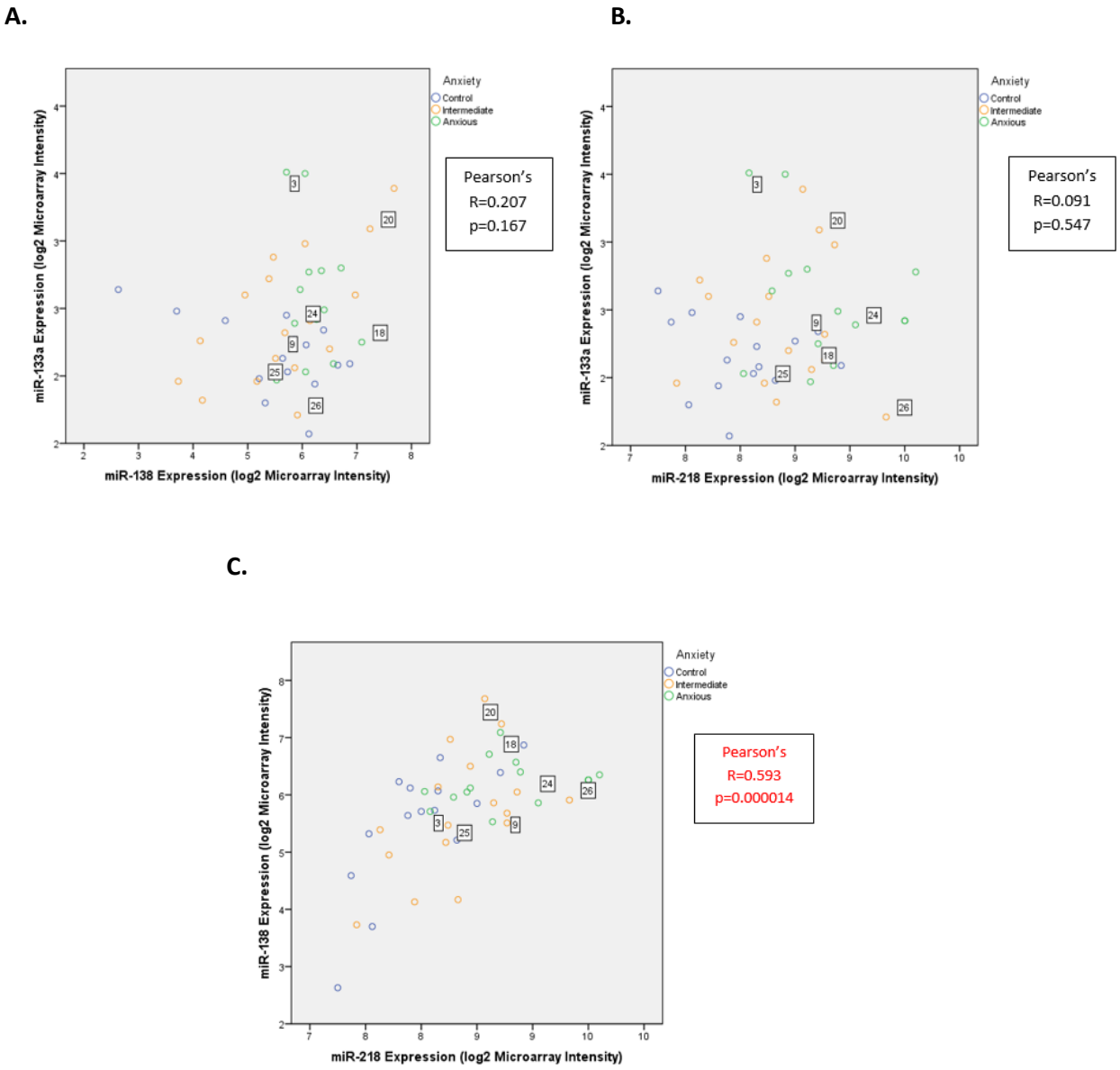
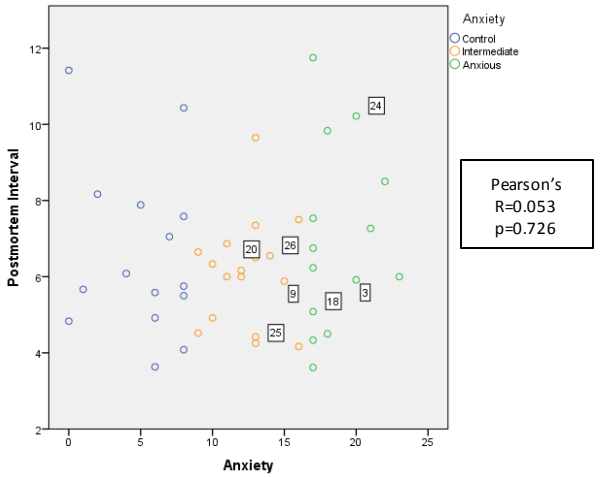


Figure 1.13. miR-138 is positively correlated with miR-218 Expression

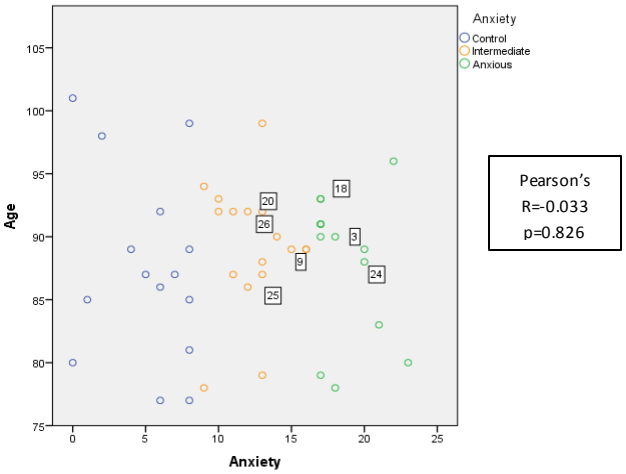
miR-133a expression is not correlated with either miR-138 **(Panel A)** or miR-218 **(Panel B)** expression. miR-138 expression correlates positively with miR-218 expression **(Panel C)** (Pearson's R=0.593 p=0.000014), suggesting possible regulation by a common upstream factor.

Figure 1.14. Potential Confounding Factors as a Function of Anxiety Score.

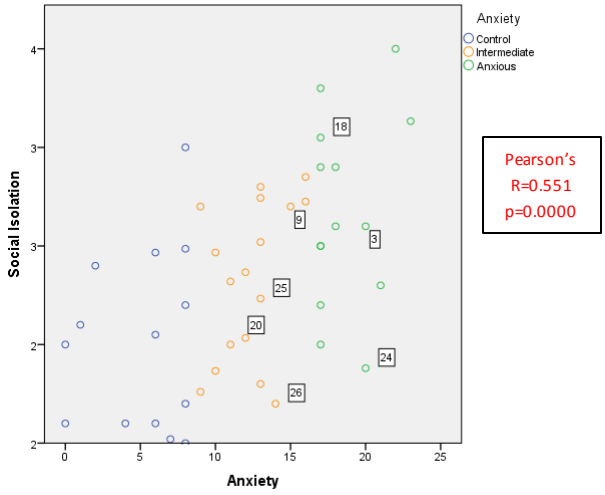
A.



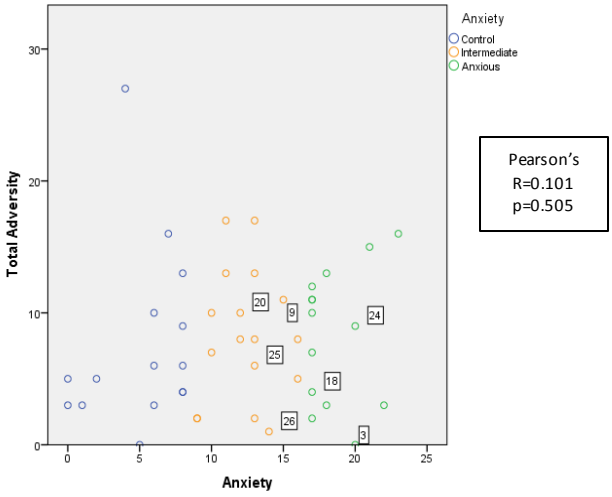
B.



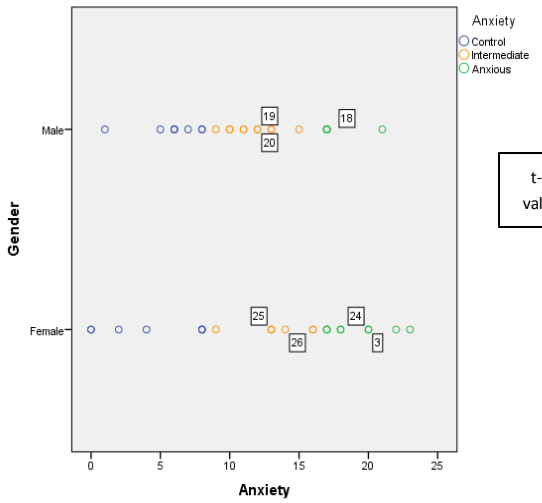
C.



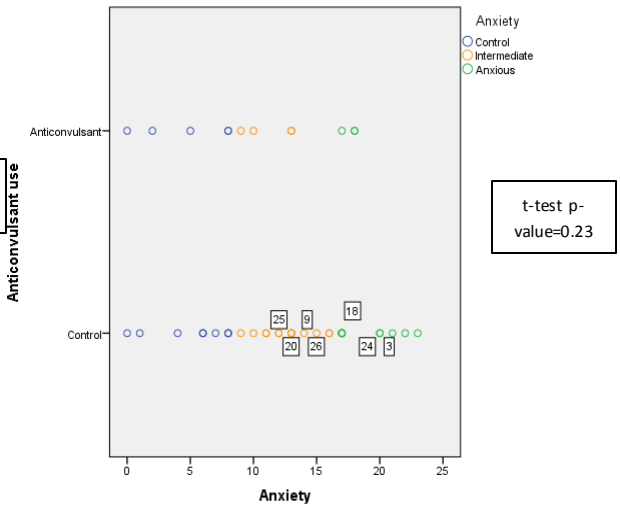
D.



E.



F.



G.

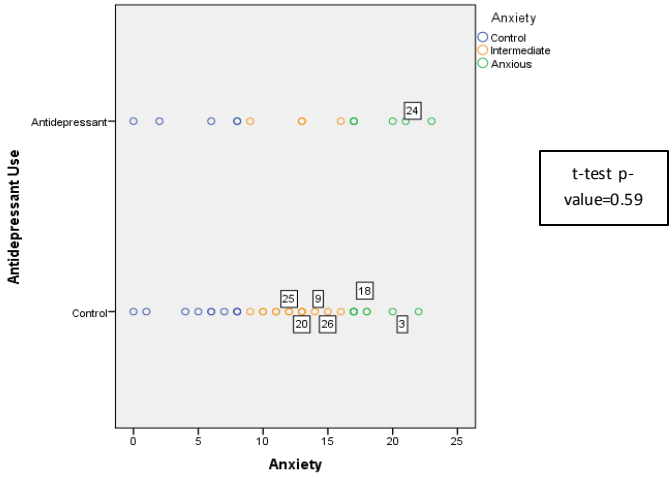
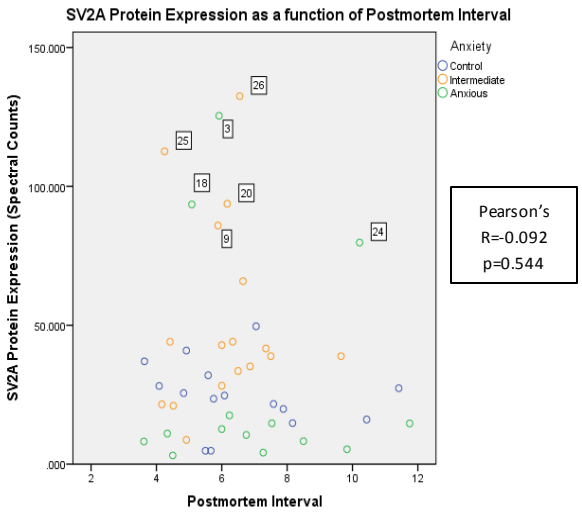


Figure 1.14. Potential Confounding Factors as a Function of Anxiety Score.

Anxiety score did not show significant correlation with postmortem interval **(Panel A)**, age **(Panel B)**, or total adversity **(Panel D)**, but did show significant correlation with social isolation **(Panel C)** (Pearson's R=0.551 p=0.000086). Anxiety score was not associated with gender **(Panel E),** anticonvulsant use **(Panel F)**, or antidepressant use **(Panel G)**.

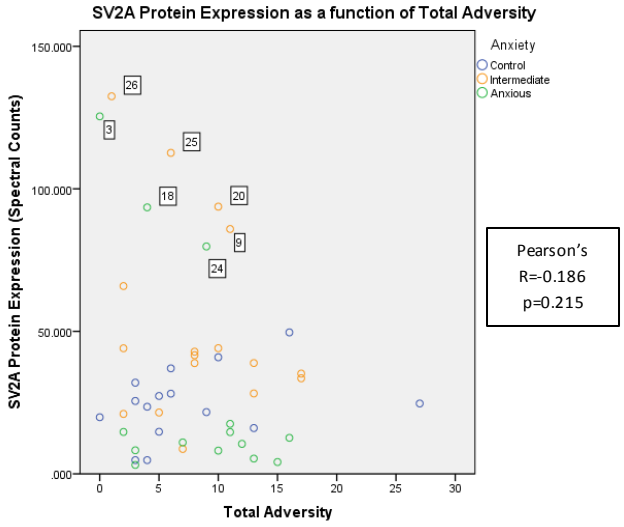
Figure 1.15. SV2A Protein Expression as a Function of Potential Confounding Factors

A.

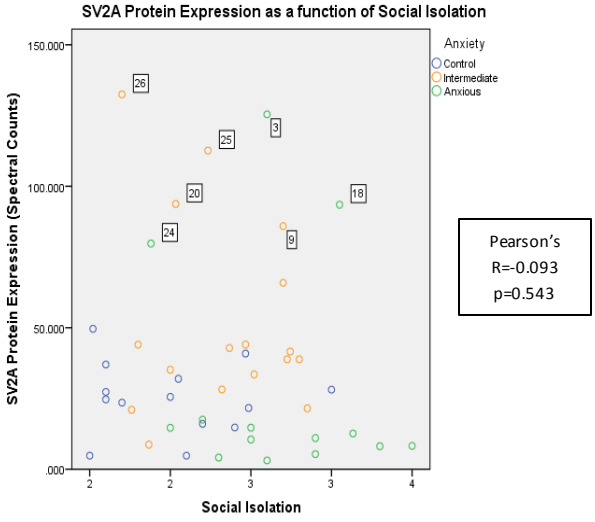


B.

C.



D.



E.

F.

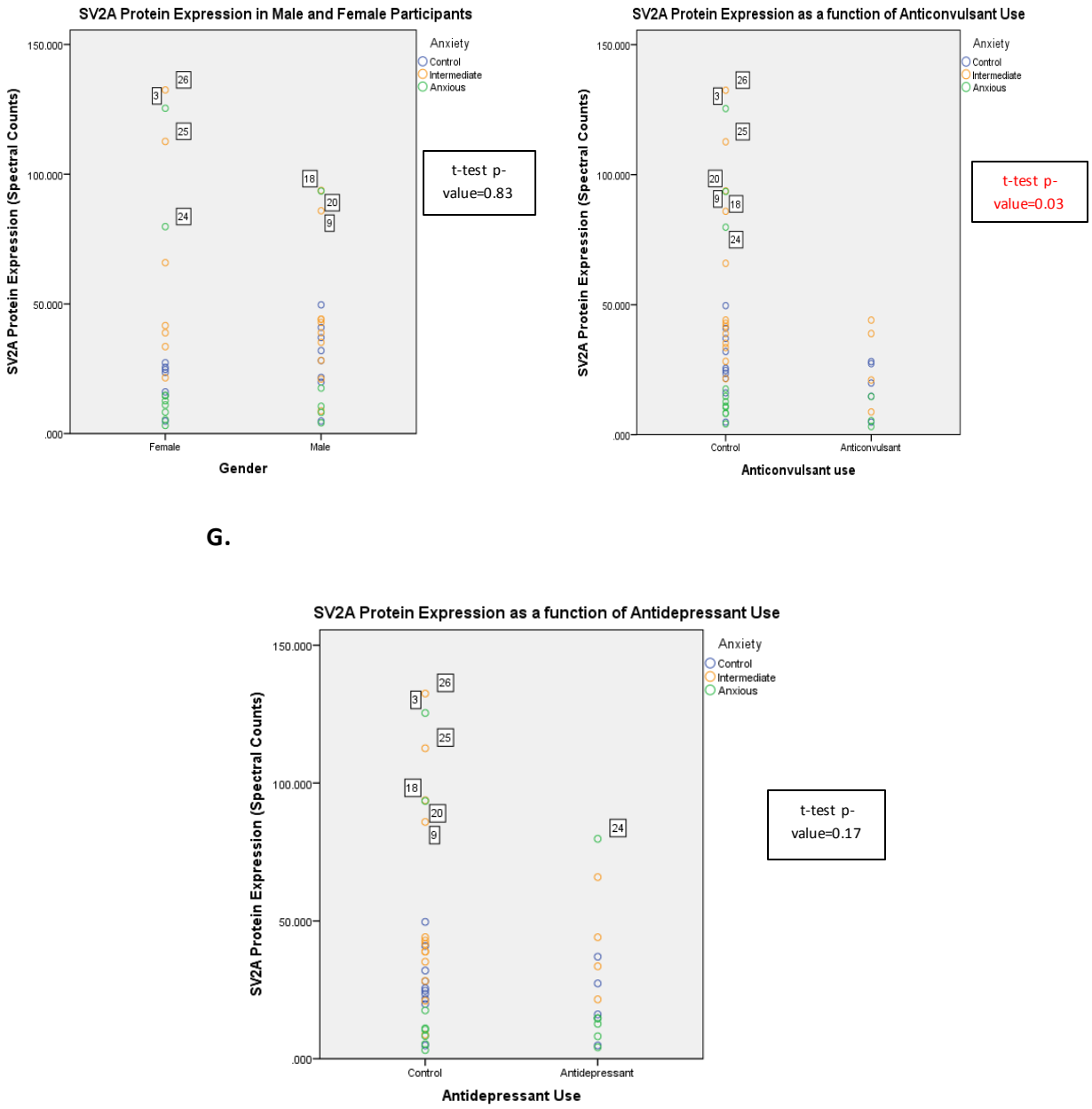
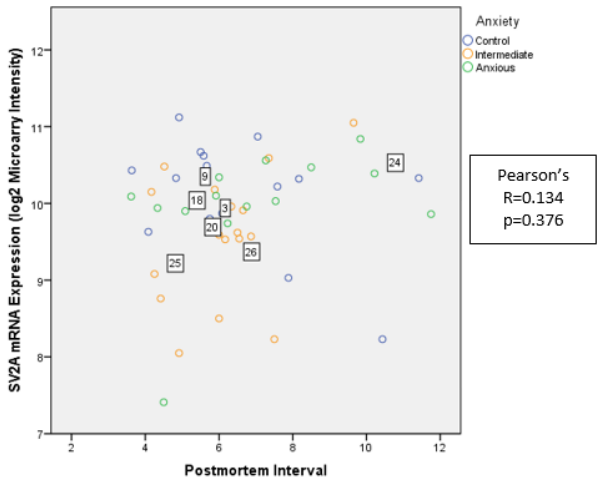


Figure 1.15. SV2A Protein Expression as a Function of Potential Confounding Factors.

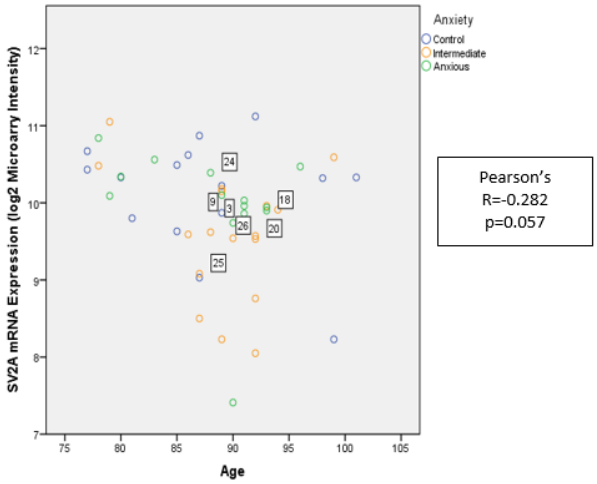
SV2A protein expression did not significantly correlate with postmortem interval **(Panel A)**, age **(Panel B)**, total adversity **(Panel C)**, or social isolation **(Panel D).** SV2A protein expression was not associated with gender **(Panel E)** or antidepressant **(Panel G)** use, but it was significantly associated with anticonvulsant use **(Panel F)** (t-test p-value=0.03). The seven individuals with increased anxiety scores and increased SV2A protein expression are identified by participant ID number and did not show aberrant values for any of the above-mentioned factors.

Figure 1.16. SV2A mRNA Expression as a Function of Potential Confounding Factors

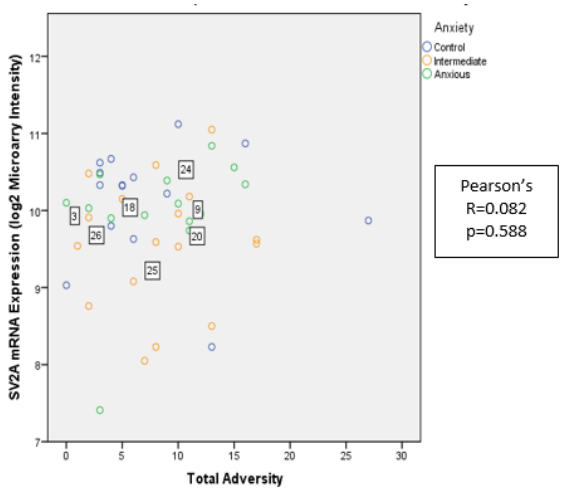
A.



B.



C.



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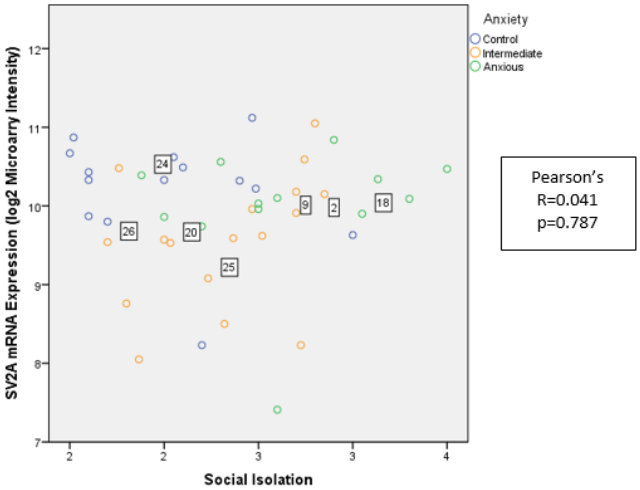
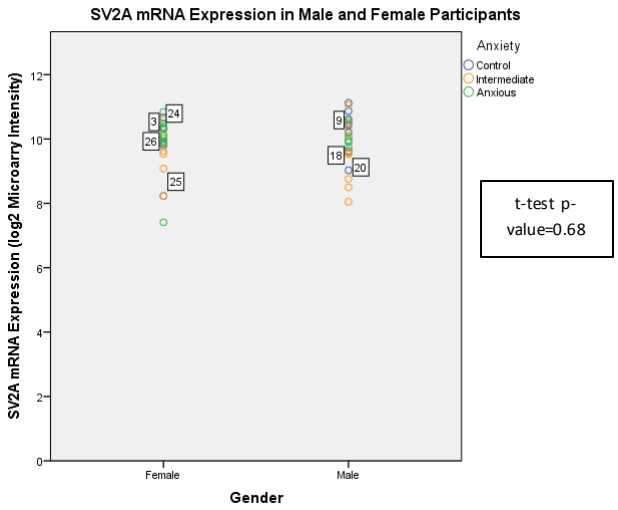
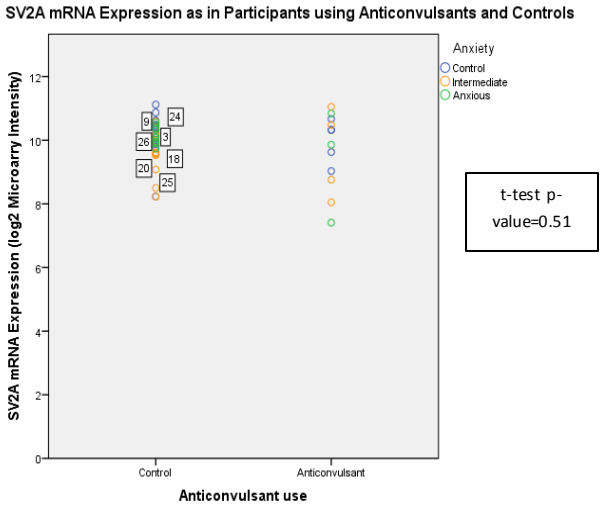


E.



F.



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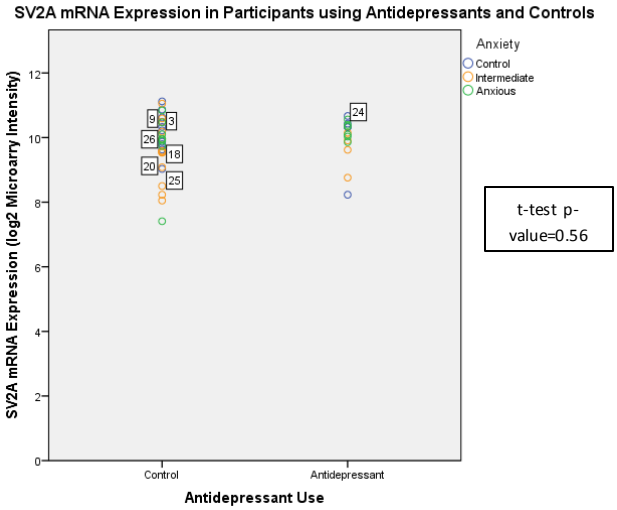
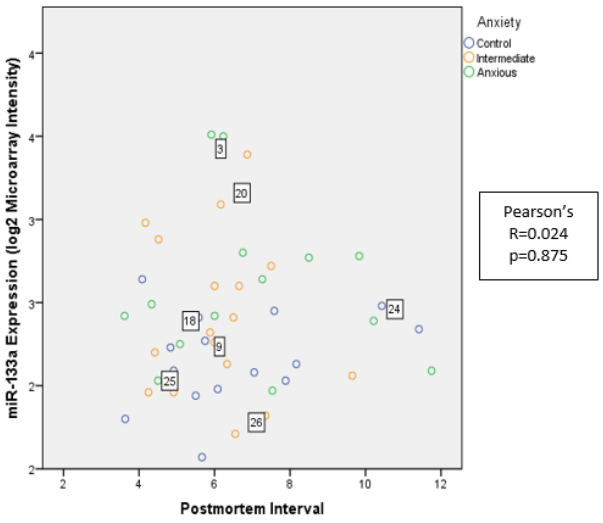


Figure 1.16. SV2A mRNA Expression as a Function of Potential Confounding Factors.

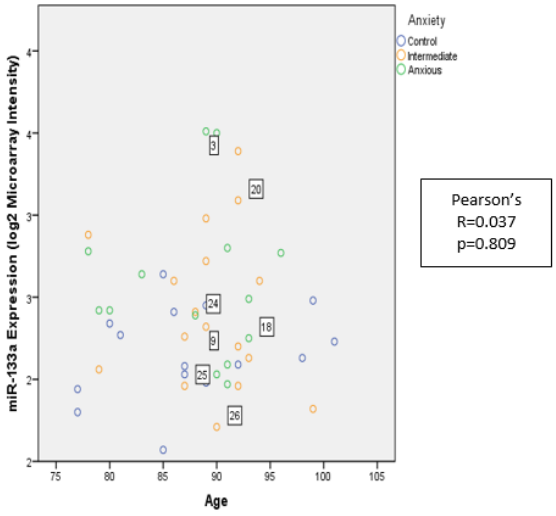
SV2A mRNA expression did not significantly correlate with postmortem interval **(Panel A)**, age **(Panel B)**, total adversity **(Panel C)**, or social isolation **(Panel D)**. SV2A expression was not associated with gender **(Panel E)**, anticonvulsant use **(Panel F)**, or antidepressant use **(Panel G)**. The seven individuals with increased anxiety scores and increased SV2A protein expression were identified by participant ID number and did not show aberrant values for any of the above-mentioned factors.

Figure 1.17. miR-133a Expression as a Function of Potential Confounding Factors

A.



B.



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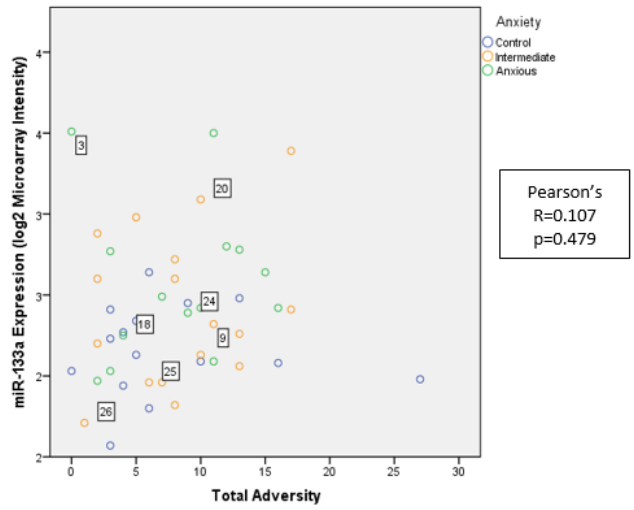
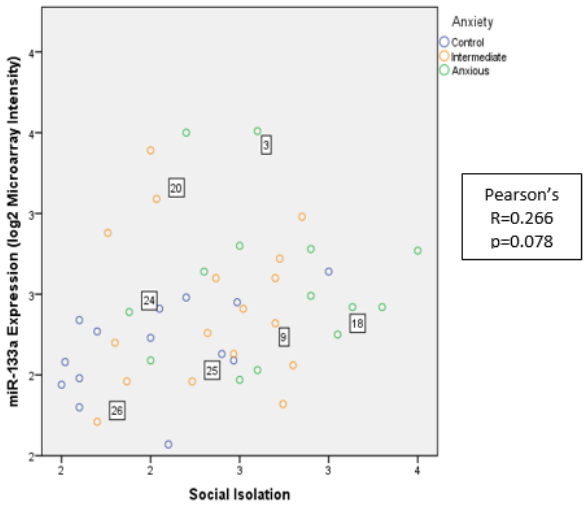
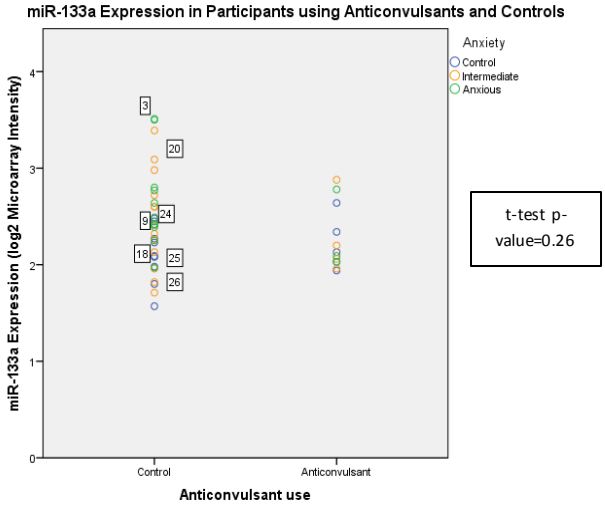


D.



E.

F.



G.

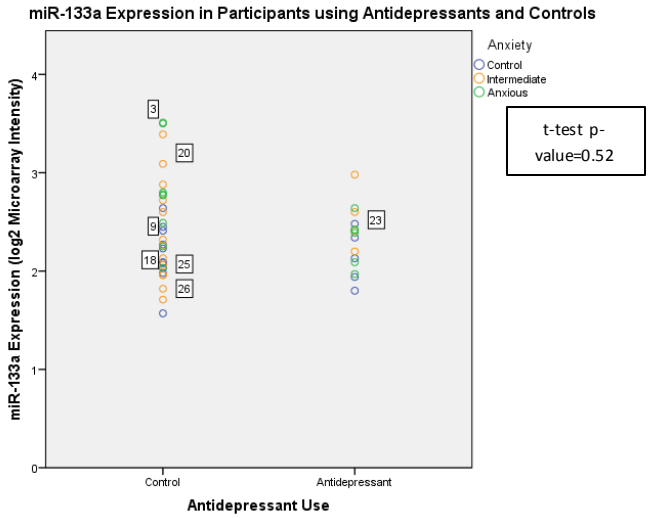
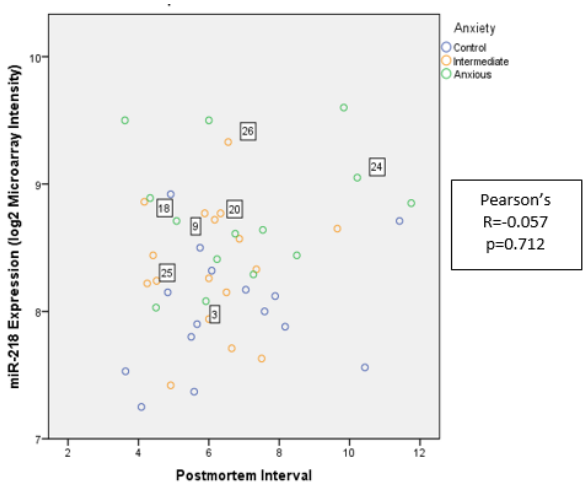


Figure 1.17. miR-133a Expression as a Function of Potential Confounding Factors.

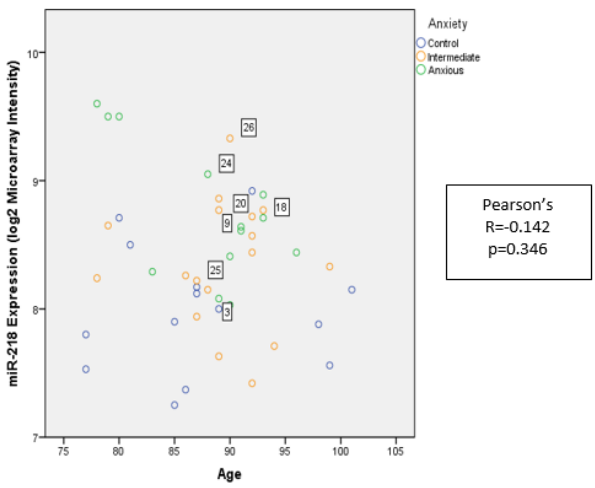
miR-133a expression did not significantly correlate with postmortem interval **(Panel A)**, age **(Panel B)**, total adversity **(Panel C)**, or social isolation **(Panel D)**. miR-133a expression was not associated with gender **(Panel E)**, anticonvulsant use **(Panel F)**, or antidepressant use **(Panel G)**. The seven individuals with increased anxiety scores and increased SV2A protein expression were identified by participant ID number and did not show aberrant values for any of the above-mentioned factors.

Figure 1.18. miR-218 Protein Expression as a Function of Potential Confounding Factors

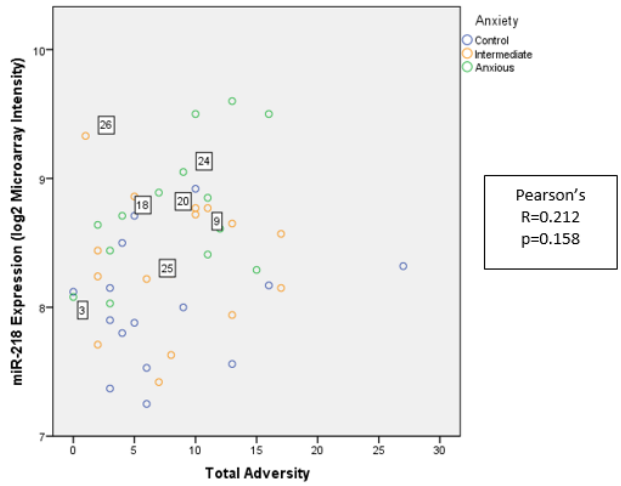
A.



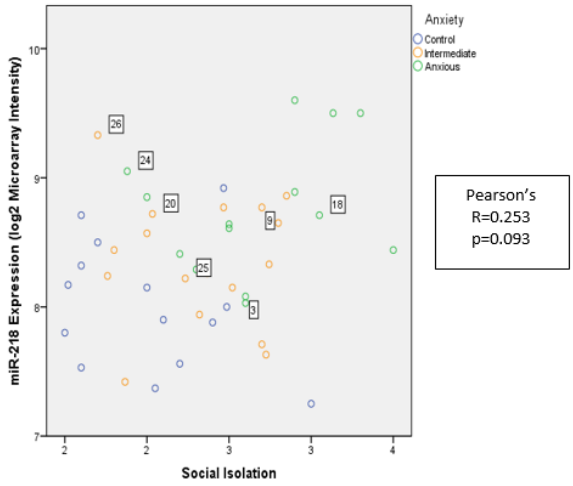
B.



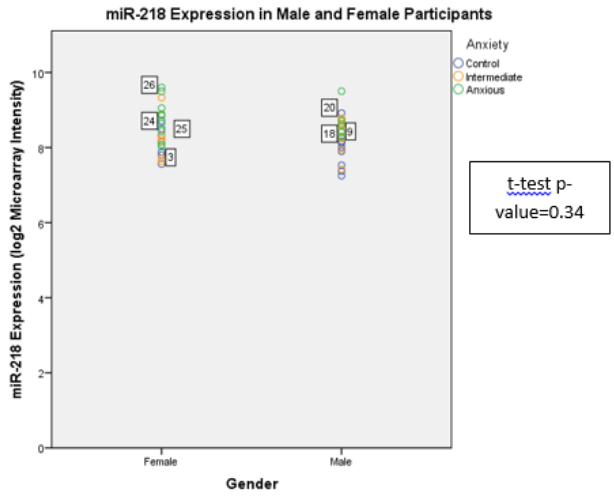
C.



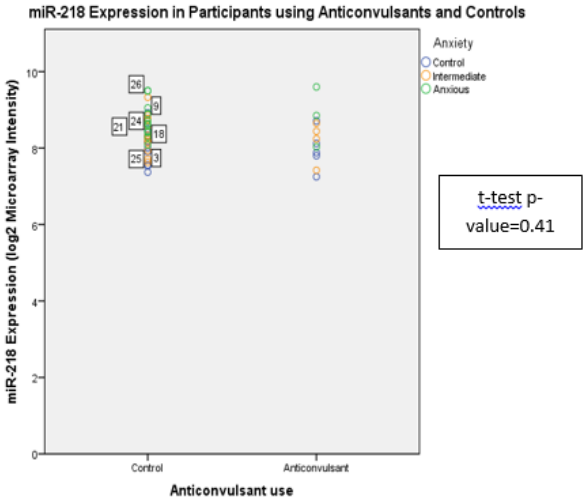
D.



E.



F.



G.

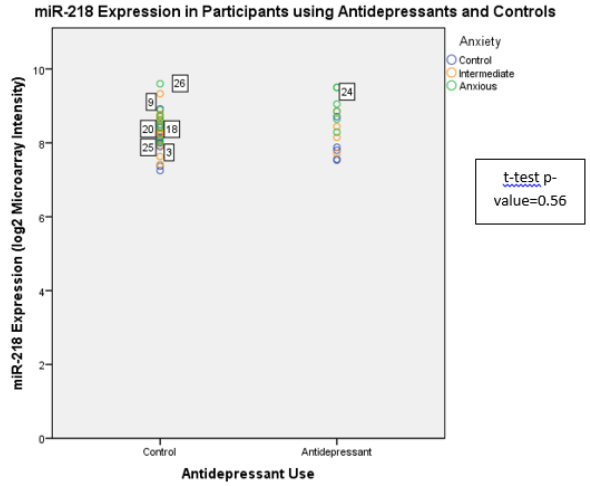
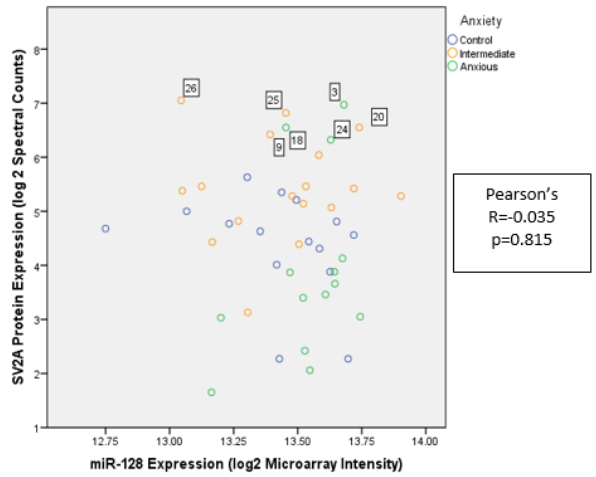


Figure 1.18. miR-218 Expression as a Function of Potential Confounding Factors.

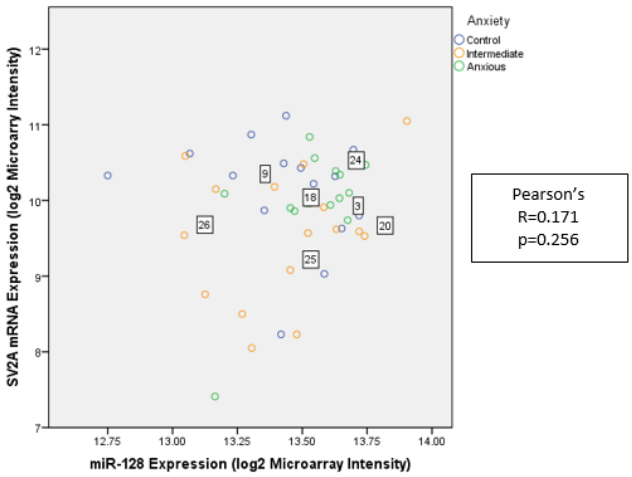
miR-218 expression did not significantly correlate with postmortem interval **(Panel A)**, age **(Panel B)**, total adversity **(Panel C)**, or social isolation **(Panel D)**. miR-218 expression was not associated with gender **(Panel E)**, anticonvulsant use **(Panel F)**, or antidepressant use **(Panel G)**. The seven individuals with increased anxiety scores and increased SV2A protein expression did not show aberrant values for any of the above-mentioned factors.

Figure 1.19. SV2A Protein and mRNA Expression as a function of Targetscan-Predicted miRNA Expression

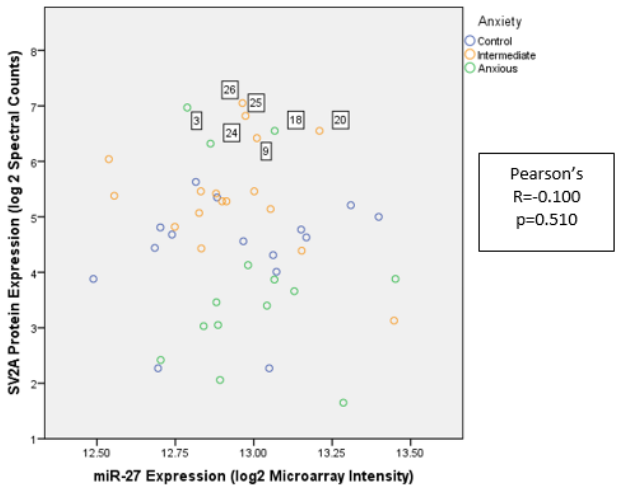
A.



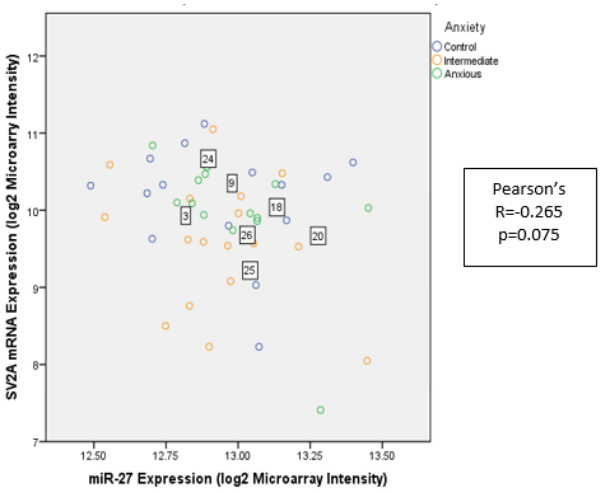
B.



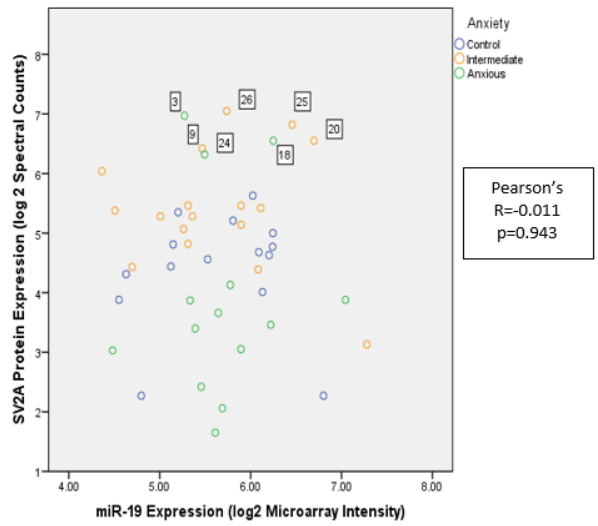
C.



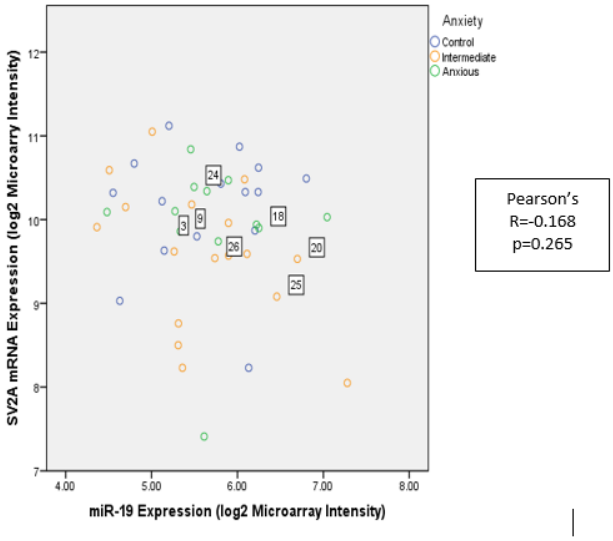
D.



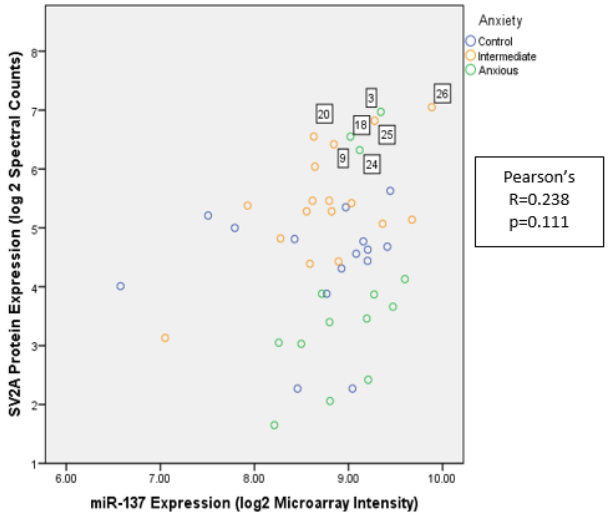
E.


Anxiety
Control
Intermediate
Anxious
SV2A Protein Expression (log 2 Spectral Counts)
miR-19 Expression (log2 Microarray Intensity)
Pearson's
R=-0.011
p=0.943

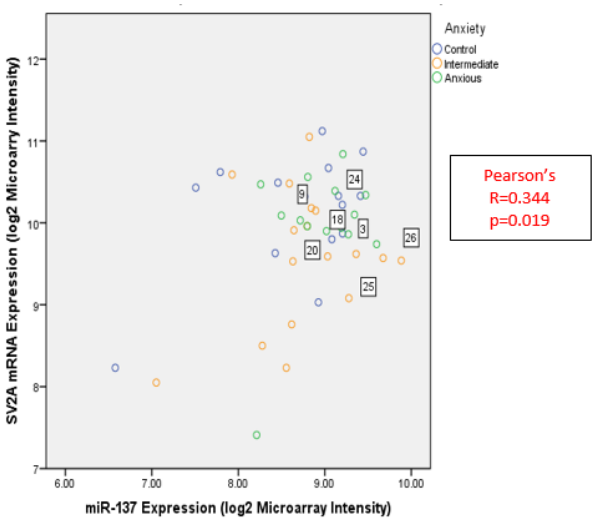
F.


Anxiety
Control
Intermediate
Anxious
SV2A mRNA Expression (log2 Microarry Intensity)
miR-19 Expression (log2 Microarray Intensity)
Pearson's
R=-0.168
p=0.265

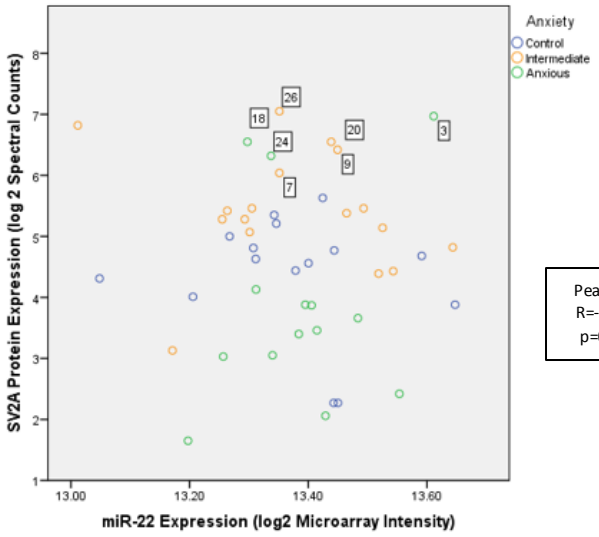
G.


Anxiety
Control
Intermediate
Anxious
SV2A Protein Expression (log 2 Spectral Counts)
miR-137 Expression (log2 Microarray Intensity)
Pearson's
R=0.238
p=0.111

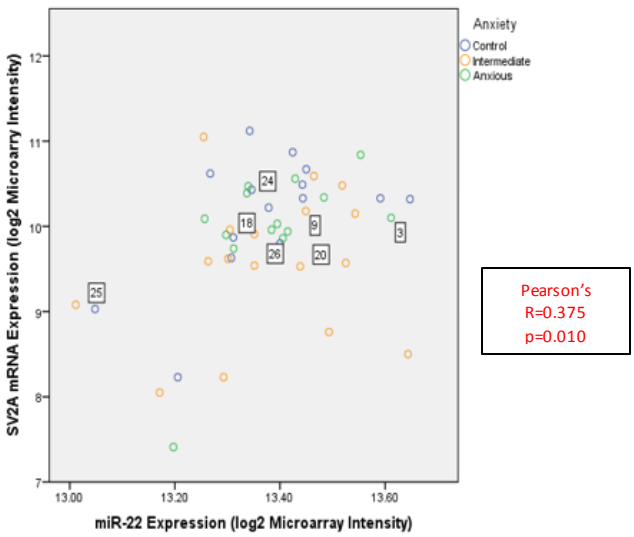
H.


Anxiety
Control
Intermediate
Anxious
SV2A mRNA Expression (log2 Microarry Intensity)
miR-137 Expression (log2 Microarray Intensity)
Pearson's
R=0.344
p=0.019

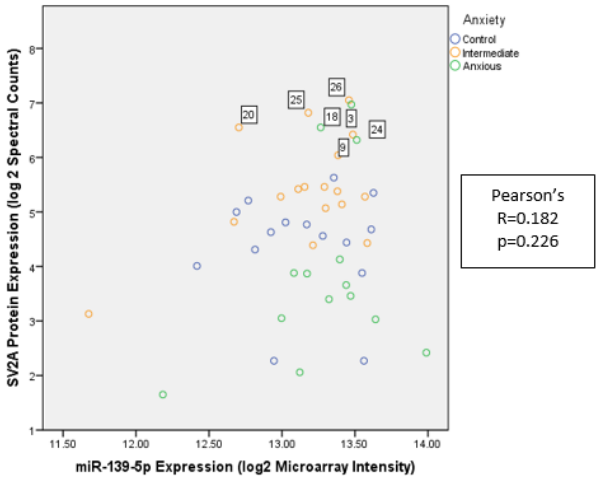
I.


Anxiety
Control
Intermediate
Anxious
SV2A Protein Expression (log 2 Spectral Counts)
miR-22 Expression (log2 Microarray Intensity)
Pearson's
R=-0.042
p=0.781

J.


Anxiety
Control
Intermediate
Anxious
SV2A mRNA Expression (log2 Microarry Intensity)
miR-22 Expression (log2 Microarray Intensity)
Pearson's
R=0.375
p=0.010

K.


Anxiety
Control
Intermediate
Anxious
SV2A Protein Expression (log 2 Spectral Counts)
miR-139-5p Expression (log2 Microarray Intensity)
Pearson's
R=0.182
p=0.226

L.

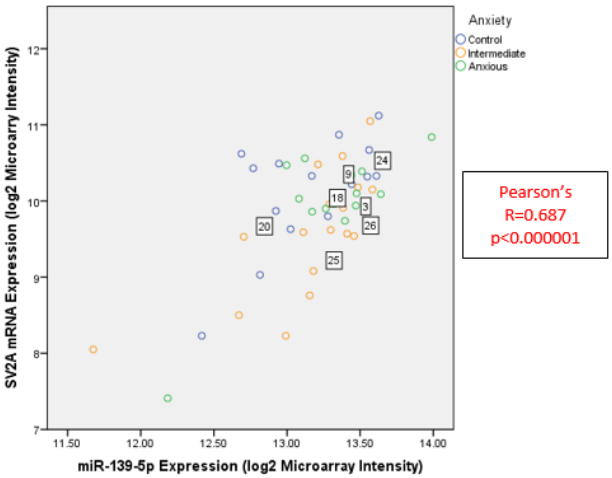
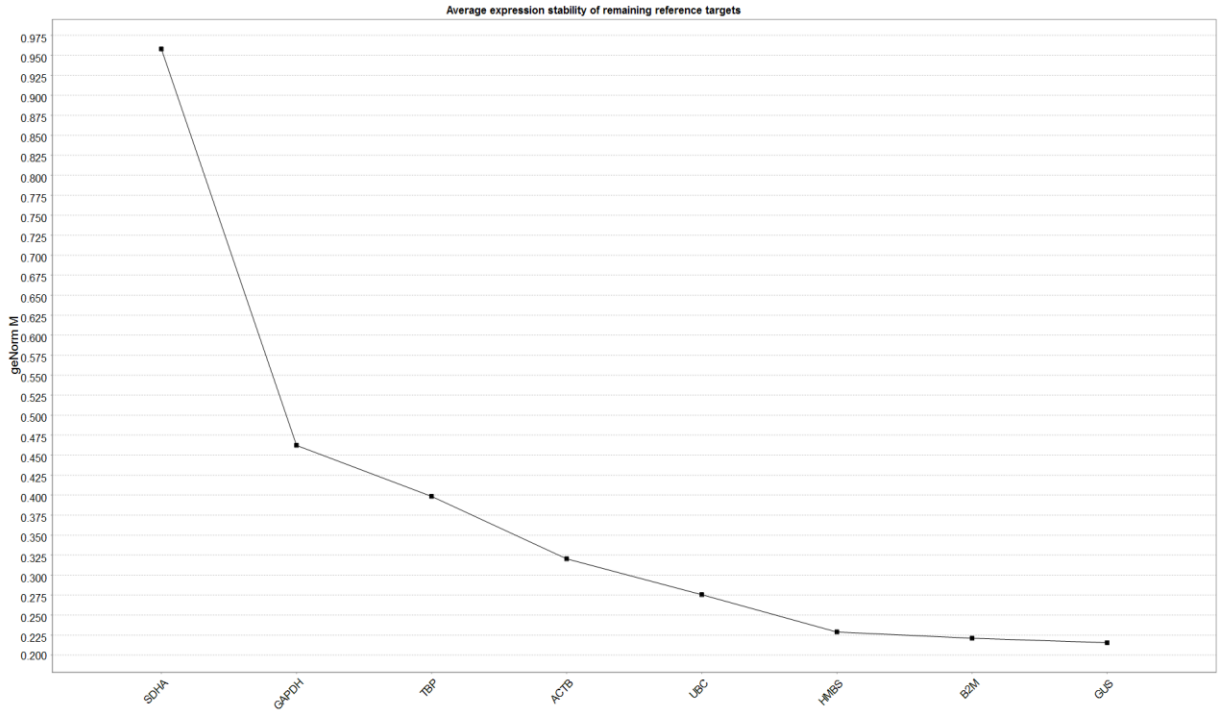

Anxiety
Control
Intermediate
Anxious
SV2A mRNA Expression (log2 Microarry Intensity)
miR-139-5p Expression (log2 Microarray Intensity)
Pearson's
R=0.687
p<0.000001

Figure 1.19. SV2A Protein and mRNA Expression as a function of Targetscan Predicted miRNA Expression.

There was a positive correlation between miR-139-5p levels and SV2A mRNA expression **(Panel K)** ($R=0.688$ $p<0.000001$). There was also a positive correlation between miR-22 levels and SV2A mRNA expression **(Panel I)** ($R=0.375$, $p=0.010$), as well as a positive correlation between miR-137 expression and SV2A mRNA levels **(Panel G)** ($R=0.344$ $p=0.019$). There was no correlation between levels of these miRNAs and SV2A protein expression **(Panels G, I, K).** Levels of additional miRNAs predicted to target SV2A by Targetscan, namely miR-128 (**Panels A and B)**, miR-27 (**Panels C and D)**, miR-19 **(Panels E and F)**, were not significantly correlated with SV2A mRNA or protein levels.

Figure 1.S1. B2M and GUS are the most stable control genes for RT-qPCR in SH-SY5Y cells

A. Average Expression Stability of Remaining Reference Targets



A. Determination of the Optimal Number of Control Genes for Normalization

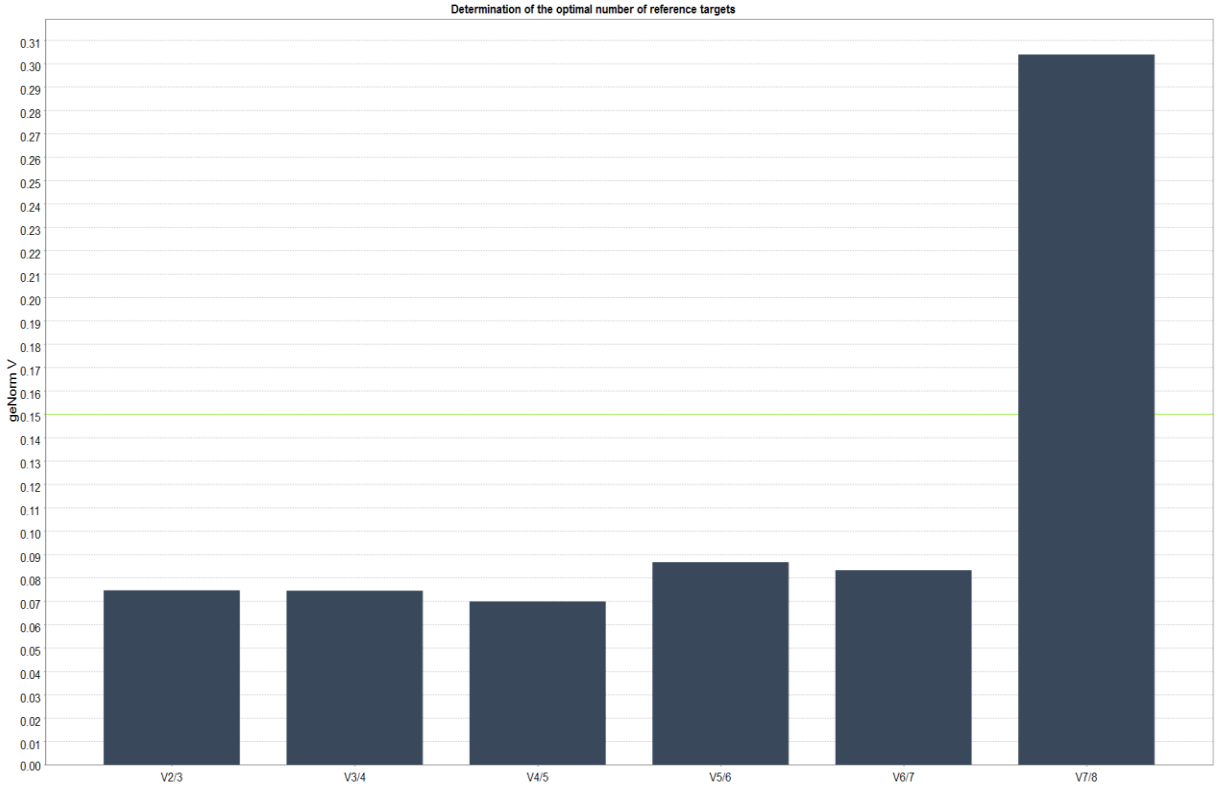


Figure 1.S1. B2M and GUS are the most stable control genes for RT-qPCR in SH-SY5Y cells

A). The internal control gene-stability measure M is the average pairwise variation of a particular gene with all other control genes. Genes with the lowest M values are the most stable. Assuming that the control genes are not co-regulated, stepwise exclusion of the gene with the greatest M value results in the combination of two constitutively expressed housekeeping genes that have the most stable expression in tested samples [162]. B2M and GUS show the most stable expression in SH-SY5Y cells. **B).** Pairwise variation (*Vn*/*n*+1) analysis between the normalization factors NF*n* and NF*n*+1 is done to determine the number of control genes required for accurate normalization. The optimal number of genes for normalization is found below the geNorm Variation (V) threshold of 0.15. Thereby, two genes are sufficient for normalization in this experiment since the addition of a third gene does not contribute to a large variation V. A large variation means that the added gene has a significant effect and should preferably be included for calculation of a reliable normalization factor [162].

TABLES

Table 1.1. Trait Anxiety Phenotype Assessment

Codebook variables	Label of variable	Allowable Codes
Neo1wor (F)	I am not a worrier	1 = Strongly disagree 2 = Disagree 3 = Neutral 4 = Agree 5 = Strongly agree
Neo7frig	I am easily frightened	
Neo13anx (F)	I rarely feel fearful or anxious	
Neo19jit	I often feel tense or jittery	
Neo25fut (F)	I am seldom apprehensive about the future	
Neo31wor	I often worry about the things that might go wrong	
Neo37fer (F)	I have fewer fears than most people	
Neo43tht	Frightening thoughts sometimes come into my head	

Table 1.1. Trait Anxiety Phenotype Assessment

The trait anxiety variable is the sum of the codebook variables found in the table above (taken from the NEO Personality Questionnaire). F instructs to flip the variable, for example, 5 would be recoded to 1 and 4 to 2, etc. Anxious individuals scored in the top quartile with anxiety scores greater than 16 whereas control individuals scored in the bottom quartile with anxiety scores below 9.

Table 1.2. Sample Information, Postmortem Amygdala Cohort 1

	Anxious	Control	T-test	Chi Square
Sample Size	10	10		
Anxiety (mean score/sd)	18.1 (1.8)	4.4 (3.5)	p < 2 e-9	
Gender (Male)	3	4		ns
Age at Death (years/sd)	88.2 (6.0)	88.3 (8.5)	ns	
PMI (hours/sd)	7.0 (2.6)	7.0 (2.4)	ns	
Total Adversity (score/sd)	7.0 (2.4)	8.7 (4.6)	ns	
Social Isolation (score/sd)	2.7 (0.5)	2.0 (0.5)	p < 0.001	
Antidepressant use	4	4		ns
Anticonvulsant use	3	5		ns

Table 1.2. Sample Information, Postmortem Amygdala Cohort 1

Anxious participant and control groups did not differ significantly in gender composition, age at death, postmortem interval (PMI), total adversity, antidepressant use, or anticonvulsant use. They did differ in anxiety scores and social isolation. sd= standard deviation, ns= non-significant p-value > 0.05 for Student's T-test or Pearson Chi Square where applicable.

Chapter 2: The Role of microRNA in State Anxiety

INTRODUCTION

The preservation of a dynamic equilibrium with the environment is key to an organism's survival, and homeostasis requires maintenance at molecular, cellular, physiological, and behavioral levels [221]. Stress threatens this equilibrium, and an organism's survival is dependent on successful adaptation that includes an adequate and controlled stress response [221]. The hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) are key to the stress response and are largely responsible for translating stressful stimuli into biological signals that aim to restore homeostasis by regulating gene transcription [221]. The focus of this study is on the gene expression response during state anxiety induced by acute psychosocial stress with a special emphasis on miRNA regulation.

The Hypothalamic-Pituitary-Adrenal (HPA) Axis

The hypothalamus is found at the base of the brain, beneath the third ventricle and above the pituitary gland and optic chiasm [222]. It functions as the endocrine system's coordinating center and is instrumental to the consolidation of signals from upper cortical inputs, autonomic function, environmental cues such as light and temperature, as well as peripheral endocrine feedback [222]. The hypothalamus delivers defined signals to the pituitary gland, which in turn releases hormones that affect most of the body's endocrine systems [222]. Corticotropin-releasing hormone (CRH) is synthesized and released by the hypothalamus and in turn acts on the pituitary gland to induce the release of corticotropin (ACTH); CRH is also synthesized by the

pituitary's corticotrophs and functions in both an autocrine and paracrine manner to regulate ACTH secretion ([223]; reviewed in [224]). ACTH stimulates the adrenal cortex to secrete glucocorticoids such as cortisol. The production of CRH is regulated by the circadian pacemaker, stress, as well as glucocorticoid negative feedback, which takes place at the level of the hypothalamus and the pituitary, and potentially at higher centers such as the hippocampus [224].

Glucocorticoids exert their effects on immune cells by binding to cytosolic receptors, translocating into the nucleus along with the receptor, and binding to glucocorticoid response elements on gene promoters, resulting in regulation of gene expression. In this way, activated glucocorticoid receptors exert their anti-inflammatory effects in response to stress [225].

Sympathetic Nervous System (SNS)

The sympathetic nervous system consists of preganglionic sympathetic neurons that stem from the thoracolumbar region of the spinal cord and synapse on a postganglionic neuron in a nearby ganglion [226]. The postganglionic neurons in turn extend to other parts of the body and exert their effects through secretion of norephinepherine (with the exception of sweat glands, which release acetylcholine), which functions to activate adrenergic receptors in peripheral tissues. Preganglionic neurons also synapse on chromaffin cells of the adrenal medulla and stimulate the release of catecholamines, namely norepinephrine and epinephrine, into the circulation [227].

Both primary and secondary lymphoid organs are innervated by the SNS, and catecholamines exert their effects on immune cells by binding to alpha(α)- and beta(β)-adrenergic receptors. Adrenergic receptor activation in the absence of immunological stimuli leads to an increase in the production of pro-inflammatory cytokines by various cell types [225].

The stress response is thought to occur in two stages: the first stage consists of the SNS-based short latency catecholamine component, and the second stage consists of the HPA-rooted slower acting glucocorticoid component [228-230]. In this way, glucocorticoids can function to inhibit inflammatory cells activated by the SNS.

The Trier Social Stress Test (TSST)

The TSST is a standard laboratory paradigm used to reliably induce psychosocial stress. It consists of an anticipation period of 5-10 minutes and a test period during which participants deliver a free speech and perform mental arithmetic in front of a non-responsive audience. The paradigm has been found to induce significant activation of the HPA axis and concentration changes in ACTH, cortisol (serum and saliva), growth hormone, and prolactin. It has also been shown to lead to activation of the SNS with increased levels of catecholamines observed within 1 minute of starting the free speech task [221], along with significant increases in heart rate. The TSST induces psychosocial stress through exposure to social-evaluative threat, as a friendly version of the TSST with a warm and responsive audience did not activate the HPA axis [231].

Stress and inflammation

To date, inflammation has been shown to play a role in the development of diabetes, cancer, cardiovascular disease, and neurodegenerative disorders. There is also increasing evidence that inflammation is involved in the development of neuropsychiatric diseases such as major depression, and pharmacological interventions targeting inflammation may be key to preventing illness and thwarting its development and progression [232].

Studies of depression and inflammation have noted increased inflammatory markers in individuals with major depression with increases in peripheral blood levels of cytokines such as interleukin (IL)-6 tumor and necrosis factor-alpha (TNF-α), and the acute-phase reactant c-reactive protein ([233, 234]; reviewed in [232]). Increases in inflammatory cellular adhesion molecules and peripheral blood chemokines, as well as stress-induced nuclear factor-κB (NF-κB), have also been seen in depressive patients [232]. Animal and human studies have noted depressive-like behavior as a result of the administration of inflammatory cytokines or cytokine inducers [232]. For example, endotoxin administration and typhoid vaccination have resulted in behavioral changes in humans that consist of depressed mood, cognitive dysfunction, and fatigue, while chronic administration of the inflammatory cytokine interferon-alpha (IFN-α) has also been shown to lead to symptoms of depression (reviewed in [232]).

Psychological stress is associated with increased atherosclerosis, a relationship that is mainly mediated through altered immune reactions that influence vascular endothelial function as well as the recruitment of circulating monocytes and their conversion to foam cells [235]. It is thought that stress-induced expression of pro- and

anti-inflammatory cytokines by catecholamines and corticosteroids may be involved in mediating these processes [235].

Thereby, current research suggests that the inflammatory response is activated by both acute and chronic psychosocial stress and constitutes a potential link between stress and disease as it relates to the immune system [232]. Better understanding of the bidirectional communication between the neuroendocrine and immune systems can lead to novel treatment strategies for a number of inflammation-based disorders [235].

Psychosocial stress, NF-κB, and inflammation

NF-κB activation constitutes a potential mechanism converting psychosocial stress into cellular activation [236]. NF-κB transcription factors are formed of homo- or heterodimers consisting of the following subunits: p65 (RelA), c-Rel, RelB, p100/p52 and p105/p50 ([237]; reviewed in [238]). NF-κB is found in the cytoplasm bound to inhibitor IκB proteins, which function to prevent NF-κB's nuclear translocation [239]. Activation of IκB kinases leads to IκB phosphorylation and subsequent polyubiquitination and proteosomal degradation (reviewed in [238]). This dissociation enables exposition of NF-κB's nuclear localization sequence, subsequent nuclear translocation, and transcriptional activation through binding to DNA promoter elements [238]. NF-κB activation leads to increased expression of pro-inflammatory genes encoding cytokines, enzymes, and adhesion molecules [230]. Pro-inflammatory cytokines such as interleukin (IL)-1β and TNF-α in turn activate NF-κB, thereby perpetuating local inflammatory responses ([230]; reviewed in [221]).

SNS activation in response to acute psychosocial stress in the form of the TSST has been associated with an increase in activation of the NF-κB complex consisting of the NF-κB p50/p65 heterodimer in peripheral blood mononuclear cells (PBMCs) of study participants 10 minutes following induction of psychosocial stress, thereby serving as a potential link between psychosocial stress and mononuclear cell activation [236]. Sixty minutes following the stressor, NF-κB binding activity returns to baseline levels. *In vitro* experiments in human monocytic (THP-1) cells showed that physiologic levels of norepinepherine result in a functional increase in NF-κB binding activity and a consequent increase in NF-κB regulated IL-6 transcription [236]. Administration of α_1- and β-adrenergic antagonists disrupts this increase in NF-κB binding activity, suggesting that this process is mediated by norepinephrine acting on α_1- and β-adrenergic receptors [236]. NF-κB activation is thought to contribute to the pathophysiology of diabetes mellitus, cardiovascular disease, and atherosclerosis (reviewed in [236]), and its psychosocial stress-dependent activation may represent one mechanism by which this occurs [236].

Major depression and a history of early life stress amplify the inflammatory response to acute psychosocial stress, as patients with major depression and a history of early life stress showed greater increases in NF-κB DNA binding in PBMCs and an increased plasma IL-6 response during the TSST compared to non-depressed controls [240]. Acute psychosocial stress induced by the TSST also results in an increase in IL-6 in non-depressed individuals with a history of early life stress [241]. These findings suggest that early life stress (and/or childhood maltreatment) and depression may contribute to the higher inflammation seen both at baseline (in other studies) and

following acute psychosocial stress and might contribute to the medical sequelae of depression and early-life adversity [242].

Glucocorticoids

Glucocorticoids have anti-inflammatory action, part of which is due to their activity as potent NF-κB inhibitors. Cortisol binds to the cytoplasmic glucocorticoid receptor/heat shock protein complex, resulting in dissociation of the heat shock protein and subsequent nuclear translocation of the cortisol-glucocorticoid receptor complex. In the nucleus, this complex serves to activate target genes by binding to glucocorticoid-responsive elements. This results in decreased transcription of pro-inflammatory genes such as IL-1β and TNF-α and increased transcription of genes such as those encoding IκB, which serves an inhibitor function by binding to NF-κB in the cytoplasm. Glucocorticoids can also bind directly to activated NF-κB to prevent its nuclear binding, further limiting its inflammatory effects [221].

Cortisol's anti-inflammatory effects would imply that its contribution to stress-evoked disease would be one of immune suppression. According to such a model, the stress-induced increase in cortisol secretion should function to improve the symptoms associated with diseases that have an inflammatory component, but this is not the case [243]. To resolve this paradox, an alternative hypothesis that describes cellular resistance to cortisol-mediated signaling has gained popularity. It states that chronic stressors cause sustained cortisol elevations that result in compensatory downregulation of glucocorticoid receptor activity in immune cells over time, thereby limiting the ability of cortisol to chronically dampen immune responses. In cells that

experience tight regulation by cortisol, such as monocytes, this downregulation acts to disengage tonic inhibition of NF-κB and other pro-inflammatory transcriptional control pathways. Over time, this process results in mild low-grade inflammation promoted by monocytes that are no longer regulated by cortisol. This persistent inflammation is thought to contribute to inflammation-related diseases linked to stress, such as infectious, autoimmune, and cardiac disorders [243].

PBMC Gene Expression and Stress

Previous studies have demonstrated changes in PBMC gene expression in individuals undergoing chronic or acute stress. Widespread changes in the transcriptome of circulating leukocytes were observed within 30-120 minutes following acute stress [244] in the context of the TSST with demonstrated changes in cell cycle, cell signaling, adhesion, and immune pathways, among others [244]. Changes in gene expression were also observed in the context of academic presentation stress [245]. Studies of chronic loneliness and threat of social loss also demonstrate an increase in NF-κB mediated gene expression along with concurrent decrease in genes with anti-inflammatory glucocorticoid response elements [243, 246].

In response to stresses, cells work to either restore or reprogram their gene expression patterns in an effort to maintain homeostasis. This process is in part mediated through miRNA, particularly through modulation of the amount of miRNA, number of mRNA targets, or the activity/mode of action of miRNA-protein complexes [247], leading to changes in the specificity, timing, and concentration of gene products expressed upon exposure to stressors [247]. Changes in miRNA expression have been

observed during psychological stress; for example, brief academic stress has been shown to lead to changes in miR-21 and let-7b levels in blood [248]. Both of these miRNAs are associated with cancer [248]. However, no studies to date have investigated genome-wide miRNA expression changes in response to acute psychosocial stress and how they relate to changes in mRNA expression in key inflammatory pathways.

Present Study

The purpose of this study was to examine global changes in miRNA levels in PBMCs in response to acute psychosocial stress induced by the TSST in healthy volunteers and to examine individual differences in patterns of miRNA gene expression. We were also interested in potential miRNA regulation of NF-κB complex genes and aimed to more closely investigate a potential link between miR-9 and acute psychosocial stress. miR-9 has been shown to fine-tune NF-κB levels in human polymorphonuclear neutrophils (PMN) and monocytes by targeting the 3' untranslated region (3'UTR) of the NFKB1 gene to decrease levels of the p50 protein of the NF-κB complex [249]. miRNA profiling by microarray showed a decrease in miR-9 levels in PBMCs during the course of the TSST in our sample, and so mRNA microarray data were screened for changes in NFKB1 transcript levels in each participant to determine whether a decrease in miR-9 expression might correlate with an increase in NFKB1 mRNA levels.

Since TSST participants with major depression and early life stress have been shown to experience greater NF-κB activation than controls [240, 241], we aimed to

determine whether miR-9 and NFKB1 gene expression during the course of the TSST are affected by the sum scores of a series of psychological variables that include trait anxiety, depression, chronic stress, childhood trauma, and levels of state anxiety in anticipation of the stress paradigm.

MATERIALS AND METHODS

Participants

Participants were Caucasian and recruited via flyers posted on the Stony Brook University campus and through advertisements in local newspapers in surrounding communities in Long Island, NY, USA. Study exclusion criteria include: age over 65 years, significant current or prior presence of psychiatric illness, presence of substance or alcohol abuse in the past six months, hormonal, mood-altering, or psychoactive drug medication, and smoking. Participants were pre-screened for exclusion criteria by phone. Thirty-six participants were part of this study which was approved by the Institutional Review Board (IRB) and Committee on Research Involving Human Subjects (CORIHS) of Stony Brook University, Stony Brook, USA.

Psychosocial Stress Procedure – TSST

All participants were scheduled for a four hour weekday laboratory session conducted at the General Clinical Research Center (GCRC) at the Stony Brook University Hospital from 2:00 pm to 6:00 pm. Upon arrival, participants were informed about the study, completed the consent procedure, and underwent insertion of an intravenous catheter into the arm for the first blood draw. Participants then rested for a period of at least 45 minutes before they were introduced to the psychosocial stress paradigm. The Trier Social Stress Test (TSST) is a standardized social stress protocol that was used to induce psychosocial stress that been found to elicit a strong and reliable physiological response to laboratory stress [250]. Briefly, the standardized

laboratory stressor consists of a 5-min preparation period, a 5-min free speech task during which participants give a mock job interview, and a 5-min mental arithmetic task wherein participants perform serial subtraction by thirteen in front of a non-responsive audience (Figure 2.1).

Biological Measures: Cortisol

In order to assess cortisol levels, ten salivary samples were obtained from each participant throughout the duration of the study. Samples were collected using Salivettes® (Sarstedt) at arrival, 2 minutes before beginning the TSST, and again at 2, 10, 20, 30, 45, 60, 90 and 105 minutes post stress task. Participants were instructed to gently chew on the cotton swab for approximately one minute during each collection. Cotton swabs were then transferred to plastic containers and immediately stored at -20^{o}C until shipment to the analyzing laboratory (N. Rohleder, Brandeis University, Waltham, MA). Upon thawing, salivettes were centrifuged at 2,000 x g for 10 min, which resulted in a clear supernatant of low viscosity. Salivary cortisol concentrations were measured using a commercially available chemiluminescence immunoassay (CLIA; RE62019) with a sensitivity of 0.16 ng/ml (IBL International). Inter-assay variability was 3.3%, and intra-assay variability was 3.4%. Salivary cortisol concentrations have been shown to highly correlate with serum cortisol concentrations, making salivary cortisol a reliable indicator of HPA activity [251]. Participants did not intake anything per oral with the exception of water for 3 hours before beginning the study session.

Cortisol response to the stress paradigm was assessed by subtracting the cortisol concentration two minutes before the TSST from the cortisol concentration 20

minutes following the stressor. K-means clustering was used to group participants into "Cortisol Responder" and "Cortisol Non-Responder" categories to identify individuals who had a robust cortisol response to the task.

Biological Measures: Blood Collection

Directly upon arrival and following the informed consent procedure, an intravenous (IV) catheter was inserted into each participant's arm for the first blood draw (Time Point A). Participants then rested for a period of at least 45 minutes before they were introduced to the psychosocial stress paradigm. The second blood draw occurred 45 minutes after the stress paradigm (Time Point B) and the third blood draw occurred 105 minutes post stress (Time Point C). Ten ml of blood was collected at each of the three time points into two 6 ml K3E ethylenediaminetetraacetic acid (EDTA) K3 tubes (Vacuette^{R} Greiner Bio-one). Blood was immediately processed according to the Leucosep® Instruction Manual (Grenier Bio-one), and cell pellets were frozen and stored at -80^{o}C.

Psychological Assessments

Before and after the TSST, participants completed various self-rating scale questionnaires including the Beck Depression Inventory (BDI) [252], the Beck Anxiety Inventory (BAI) [253], the Trier Inventory for the Assessment of Chronic Stress (TiCS) [254], the Childhood Trauma Questionnaire (CTQ) [255] as well as the state anxiety component of the State-Trait Anxiety Inventory (STAI) [256] to assess state anxiety

before beginning the TSST. Participants also underwent a structured interview for the assessment of stressful life events (SLE).

RNA Isolation, Quantification, Quality Control

Total RNA was extracted from PBMCs at each time point using the Qiagen All-Prep kit with company-recommended modification to extract small RNA (Qiagen). RNA quantity was assessed using a Nanodrop Technologies ND-1000 instrument (NanoDrop Technologies). Samples with an A260/230 ratio below 1.5 were desalted until a ratio greater than 1.5 was observed. Total RNA quality was measured using the Agilent RNA 6000 Pico kit and the Agilent Bioanalyzer 2100A (Agilent Technologies), while miRNA content was resolved using the Small RNA Kit (Agilent).

miRNA Microarray Profiling

miRNA microarray profiling was performed at the Microarray Core Facility of Cold Spring Harbor Laboratory using the Affymetrix GeneChip miRNA 2.0 Array according to the manufacturer's protocol. Briefly, 400 ng of total RNA was labelled using the 3DNA Array Detection FlashTag™ Biotin HSR Kit following the manufacturer's recommendations (Genisphere). The Affymetrix GeneChip® Hybridization, Wash, and Stain Kit was used for RNA hybridization to the arrays and for standard Affymetrix array cassette staining and washing according to the manufacturer's instructions. Arrays were scanned on the Affymetrix GeneChip Scanner 3000, and feature extraction was conducted using Affymetrix Command Console software. Microarray background correction, quantile normalization, probe set summarization, and log2 transformation

were performed with the miRNA QC Tool Version 1.1.1.0 (Affymetrix). Three samples were identified as potential outliers with miRNA expression values that differed from remaining samples and were re-run on the miRNA 2.0 arrays. The run with higher correlation with other subjects in the same group was selected for further analysis.

mRNA Microarray Profiling

mRNA microarray profiling was performed at the ShanghaiBio Corporation using Affymetrix Human Gene ST 1.0 Arrays according to the manufacturer's protocol. One sample (Participant #1 at Time Point A) was excluded from the analysis as there was insufficient RNA to run on the microarray. Briefly, 500 ng of total RNA was used to synthesize cDNA by reverse transcription using random hexamers tagged with a T7 promotor primer sequence. The double-stranded cDNA was subsequently used as a template and amplified by T7RNA polymerase producing, several copies of antisense complementary RNA (cRNA). In the second cycle of cDNA synthesis, random hexamers were used to prime reverse transcription of the cRNA from the first cycle to produce single-stranded DNA in the sense orientation. The DNA was fragmented with a combination of uracil DNA glycosylase (UDG) and apurinic/apryimidinic endonuclease (APE1). DNA was labeled by terminal deoxynuclotidyltransferase (TdT), and hybridization was performed according to the manufacturer's protocol. The arrays were subsequently washed, stained, and scanned according to standard Affymetrix protocols. Images were processed using Affymetrix Microarray Suite 5.0 Expression Console, and image quality was subsequently assessed. Quantitative image files were loaded into GenePattern software and normalized using the Robust Multi-Array Average (RMA) method with quantile normalization. Expression values were log2 transformed for further

analysis. For the purpose of this study, the data from the mRNA arrays was used only to examine the expression of the NFKB1 gene. Analysis of genome-wide mRNA expression patterns and integration with global miRNA expression will be performed at a future date.

Statistical Analysis of miRNA Microarray Data

Time-course microarray data analysis was conducted using the Linear Models for Microarray Data (LIMMA) Bioconductor package [257]. Linear models were fitted in order to distinguish differentially expressed miRNAs between the three time points (A,B,C) on the Affymetrix platform. miRNAs showing a $|FC|>1.2$ and p value < 0.01 for at least one of the time point comparisons (B to A, C to A, B to C) were selected for further analysis. The heatmap image was generated using the GenePattern Image Creator. Outlier identification was conducted using Extreme Values Analysis (EVA) [258]. EVA was applied under a control:experimental design, wherein time point A was treated as the control group. The mean and standard deviation (SD) of log2-transformed expression levels was computed for all probe sets at each time point. An "extreme value" was defined as a value that is ± 3 fold greater than the mean expression level of the specific probe set in the control group that also lies outside 1.5 SD's of the control group. The heatmap was generated with R using the Bioconductor package.

Functional Profiling

Functional profiling of miRNA expression data was performed by the computational gene network prediction tool Ingenuity Pathway Analysis version 9.0

(IPA, Ingenuity® Systems) using human orthologues of all differentially expressed miRNAs (LIMMA $p<0.01$ and $|FC|>1.2$) that readily mapped to IPA. A miRNA target filter analysis was conducted to identify potential mRNA targets of differentially expressed miRNAs. A core analysis was run on the set of differentially expressed miRNAs and mRNA targets with a focus on identifying genes in the following pathways: Cellular Immune Response, Cellular Stress and Injury, Cytokine Signaling, Humoral Immune Response, and Cardiovascular Signaling.

Correlation of miR-9 and NFKB1 Expression with Psychological Sum Scores

Baseline miR-9 and NFKB1 expression, as well as changes in miR-9 and NFKB1 expression over the course of the study session, were correlated with sum scores of the Childhood Trauma Questionnaire (CTQ), the Beck Depression Inventory (BDI), Beck Anxiety Inventory (BAI), the Trier Inventory for the Assessment of Chronic Stress (TiCS) and the State-Trait Anxiety Questionnaire (STAI), assessing state anxiety prior to commencement of the TSST. Pearson's correlation coefficient was used as a correlation metric. Analyses were conducted using IBM SPSS version 21.0.

In addition to the Pearson correlation coefficient, two types of linear models were used to examine possible effects of psychological variables on miR-9 expression in response to the TSST. These are stepwise linear regression analysis and repeated measures ANOVA.

Stepwise Linear Regression Analysis

Stepwise linear regression analysis was done to determine whether the baseline miR-9 expression and changes in miR-9 expression at each of the three time points was dependent on sum scores for the above-mentioned psychiatric variables and to determine how much of this dependence is due to a particular psychological variable adjusting for the presence of others. Three separate models investigated the fold change between Time Point B and Time Point A (B/A), Time Point C and Time Point B (C/B), and Time Point C and Time Point A (C/A) as dependent variables and the psychological variable sum scores for the BDI, BAI, TICS, CTQ, and STAI state anxiety before beginning the TSST as covariates in a stepwise regression procedure that aimed to generate the most parsimonious model with relevant variables. Baseline miR-9 expression was also analyzed using a similar approach.

Repeated Measures ANOVA

Repeated measures analysis of variance (ANOVA) was conducted using SPSS version 21.0 to assess the effect of the before-mentioned psychological variable sum scores on miR-9 expression over the course of Time Points A, B, and C during the study session. The dependent variable (miR-9 expression) was represented by the three measurement times, and the sum scores of the CTQ, BDI, BAI, TiCS, and STAI state anxiety before beginning the TSST were treated as covariates. A similar repeated measures ANOVA model was also conducted to assess changes in NFKB1 transcript expression over Time Points A, B, and C.

RESULTS

Participant Characteristics and Cortisol Responders

Participant characteristics are listed in Table 2.1. Twelve participants were classified as Cortisol Responders using K-means clustering with a mean cortisol increase of 13.60 nmol/l (+/-5.77), whereas 24 participants were Cortisol Non-Responders with a mean cortisol increase of 0.68 nmol/l (+/- 4.77) (Figure 2.2). Cortisol Responders were significantly older compared to Non-Responders, although there was considerable overlap between the two groups with a mean age of 43.2 years (+/- 19.3) for Cortisol Responders and 28.33 years (+/-11.9) for Non-Responders (t-test p=0.007). There was no significant difference in psychological variables of interest, as the BDI, BAI, TiCS, and CTQ sum scores were not significantly different between the two groups and there was no significant difference in the number of stressful life events between the two groups. Subsequent gene expression analyses were conducted on cortisol responders these individuals experienced a measurable physiological stress response to the TSST.

Gene Expression Analysis - miR-9 and NFKB1

Thirty-three miRNA genes showed a significant change in expression in Cortisol Responders over the course of the TSST (Time Points A, B, and C) and are depicted in Figure 2.3. Pathway analysis suggested that these miRNAs play a role in pathways associated with infection, the immune response, glucocorticoid signaling, cancer, and cardiovascular disease, among others (Figure 2.4). Since previous studies of the TSST

observed an increase in NF-κB binding activity in response to the stressor [236, 240], we aimed to determine whether any of the miRNAs differentially expressed in our study target NF-κB proteins. We found that miR-9, which decreased in expression during the course of the study period (Figure 2.5), has been experimentally shown to target the NFKB1 gene, which encodes the p105 protein that is in turn processed into the p50 protein of the NF-κB complex [249, 259]. We hypothesized that decreases in miR-9 expression could correspond to possible increases in NFKB1 mRNA expression following the stressor. However, mRNA expression analysis showed no change in NFKB1 expression during the course of the TSST (repeated measures ANOVA F=0.276 p=0.761) (Figure 2.6).

miR-9 and NFkB1 Expression and Psychological Measures

Baseline miR-9 levels were inversely correlated with CTQ sum scores (Pearson's R= -0.648 and p=0.023) (Table 2.2 and Figure 2.7A). This relationship was also significant in a stepwise linear regression model of baseline miR-9 expression wherein CTQ sum score was the only significant variable included in the model with p=0.023 and R^2=0.420. All other psychological variables were excluded, as they did not contribute to the model's predictive value. Repeated measures ANOVA showed a significant difference in miR-9 expression over time in Cortisol Responders (F=4.91, p=0.018); however, the CTQ score had no effect on miR-9 expression over time in this model (F=2.3 p=1.61).

Nevertheless, CTQ sum score was positively correlated with fold change in miR-9 expression between Time Point B and Time Point A (B/A) (Pearson's R=0.674

p=0.016) (Figure 2.7C), but this correlation was marginally insignificant in a stepwise linear regression model of the fold change B/A as a function of CTQ, BDI, BAI, TiCS, and STAI state anxiety before TSST (significance of CTQ variable p=0.054). The stepwise regression model excluded all of the above-mentioned psychological variables with the exception of STAI state anxiety before the TSST (p=0.011) to generate a model wherein fold change in miR-9 expression between Time Point B and Time Point A = 0.207 + 0.29 (STAI state anxiety before the TSST) with R^2=0.490. STAI state anxiety score before the TSST was positively correlated with B/A fold change in miR-9 expression (Pearson's R=0.700 p=0.011) (Figure 2.7E) and was marginally significant in its association with change in miR-9 expression over time in the repeated measures ANOVA (F=8.91 p=0.049).

There was a positive correlation between the fold change between Time Point B and Time Point A (B/A) and BDI sum score (Pearson's R= 0.640 and p=0.025) (Table 2.2, Figure 2.7D). However, repeated measures ANOVA suggested that BDI sum score had no effect on miR-9 expression over time (F=0.044, p=0.838), and the stepwise linear regression model did not suggest a significant association between BDI sum score and fold change B/A (p=0.135).

There was no significant correlation between baseline mir-9 expression, or changes in miR-9 expression and the Beck Anxiety Inventory (BAI) or the Trier Inventory for the Assessment of Chronic Stress (TiCS). Finally, changes in gene expression between Time Point C and Time Point B (C/B) and Time Point C and Time Point A (C/A) were not associated with any of the psychological measures tested. All variables were normally distributed according to the Shapiro-Wilk test (p>0.05).

Correlation results are summarized in Table 2.2, while ANOVA and stepwise linear regression results are summarized in Table 2.3.

There was no significant correlation between baseline NFKB1 expression or changes in NFKB1 expression and the Beck Depression Inventory (BDI), the Trier Inventory for the Assessment of Chronic Stress (TiCS), The Childhood Trauma Questionnaire (CTQ), or the State-Trait Anxiety Inventory (STAI) measure of state anxiety before the stress paradigm (Table 2.4). Repeated measures ANOVA did not show an effect of any of the above-mentioned variables on NFKB1 mRNA expression over Time Points A, B, and C.

Extreme Values Analysis (EVA)

Extreme Values Analysis identified two individuals (Participants #2 and #11) with differing expression patterns for a number of miRNA genes compared to the remaining sample of Cortisol Responders (Figure 2.8). These individuals differed from other cortisol responders on a number of psychological measures (Table 2.5).

Participant #2 had a cortisol response of 20.4 nmol/l, compared to the group mean of 13.6 nmol/l, along with a higher BAI sum score of 10 compared to the sample mean of 3.8. This individual had a CTQ sum score of 59 compared to the Responder mean of 39.3 along with a higher number of stressful life events (n=60) compared to the rest of the Cortisol Responders, who had an average of 43.2.

Participant #11 also differed from the rest of the Cortisol Responder group on a series of psychological measures. This individual had a TiCS sum score of 20 compared to the Responder mean of 10.33 and a CTQ sum score of 57 compared to

the Responder mean of 39.33. This person also reported a greater number of stressful life events (71 vs. group mean of 43.22).

DISCUSSION

This study aimed to examine miRNA gene expression changes in response to the TSST, a standard laboratory paradigm inducing acute psychosocial stress in healthy volunteers free of psychopathology. This study aimed to complement previous studies that showed mRNA expression changes in response to the TSST in an effort to provide a glimpse into the molecular basis of the stress response and insight into the mechanism by which stress poses a risk factor for inflammation-based diseases.

Analysis was conducted on individuals with a robust cortisol response to the stress task (Cortisol Responders). We chose to profile only Cortisol Responders as the observed increase in cortisol levels indicated that they experienced an objective stress response to the task. It is possible that Cortisol Non-Responders did not experience stress as a result of the task. There are additional reasons for differences in cortisol response - for example, the serum cortisol response is blunted in obese persons [260]. Also, individuals who have experienced childhood maltreatment have blunted HPA axis reactivity in adulthood [261], although we did not see a difference in CTQ sum score between Cortisol Responders and Non-Responders. In addition, individuals undergoing chronic severe stress show reduced HPA reactivity [262], but we did not see a difference in chronic stress between the two groups measured by the TiCS. Individuals with severe depressive symptoms experience a blunted cortisol stress response [263], and persons with panic disorder also exhibit blunted cortisol reactivity to the TSST [264]. Notably, there was no difference in depression or anxiety measures between the

Cortisol Responders and Non-Responders, and participants were screened to be free of psychopathology as part of the study exclusion criteria.

According to Kudielka et al, comparable HPA responses to the TSST are observed regardless of the time of the day that the session takes place in spite of the natural, diurnal fluctuation in cortisol levels. The group determined that there are no significant differences in increases in stress-related free salivary cortisol or in net increases in total plasma cortisol and ACTH in spite of differences in pre-stress baseline cortisol levels [265]. Thereby, it is unlikely that differences in cortisol response among subjects are related to the time of day the session took place.

Cortisol Responders were also prioritized over Non-Responders because participants without a stress-dependent increase in catecholamines, ACTH, and cortisol did not show an increase in NF-κB binding activity in response to the TSST in previous studies, suggesting that NF-κB activation is dependent on an acute physiological response to psychosocial stress [236]. Thereby, we chose to focus on Cortisol Responders for stress-induced gene expression analysis, because we cannot rule out the notion that some individuals who did not show a cortisol response were simply not stressed by the task. Future analyses aim to examine expression of specific genes of interest in Cortisol Non-Responders to compare with that seen in Cortisol Responders.

Interestingly, the Cortisol Responders tended to be older than the Non-Responders. It is possible that older individuals tended to experience greater stress in the public speaking task compared to younger participants, because younger participants were college students who are accustomed to this type of stressor in every-day life.

Our data showed changes in the expression of 33 miRNAs over the course of the TSST and pathway analysis suggested that these miRNAs and their target genes are involved in inflammatory, immune, and infectious pathways as well as pathways related to cancer and cardiovascular disease. Since previous studies of acute psychosocial stress induced by the TSST demonstrated an increase in NF-κB activation that was especially pronounced in individuals with a history of depression and childhood mistreatment [240], we investigated whether any of the differentially expressed miRNAs in our study target NF-κB proteins. miR-9 targets the NFKB1 gene that encodes the p105 protein, which is processed into the p50 subunit of the NF-κB complex [266]. Mean miR-9 levels decreased during the course of the study session in our sample of Cortisol Responders. Individuals with higher depressive symptoms (evidenced by higher BDI sum scores), higher childhood trauma (evidenced by higher CTQ sum scores), and greater state anxiety before beginning the TSST (evidenced by higher scores on the STAI state anxiety measure taken before beginning the paradigm), tended to have less of a decrease in miR-9 over the course of the study session and trended towards an increase in expression (fold change of miR-9 expression at Time Point B/Time Point A >1 with increasing scores on the above psychological measures). Individuals with lower scores on these psychological measures tended towards a decrease in miR-9 expression (fold change of miR-9 expression at Time Point B/Time Point A <1 with decreasing score on the above measures). In addition, baseline miR-9 levels were inversely correlated with CTQ sum score, suggesting that individuals with greater childhood trauma had lower levels of miR-9 before beginning the stress paradigm. Possible mechanisms behind the decreased baseline miR-9 expression in individuals

with depression and early life stress could be epigenetic in nature (for example, DNA and histone methylation).

Since our study population was a healthy community sample free of overt psychopathology, we could not determine whether further increases in sum scores of these particular measures would lead to significant and biologically meaningful increases in miR-9 expression. Coupled with the negative correlation between baseline miR-9 levels and CTQ sum score, these data potentially suggest a model wherein individuals with higher childhood trauma have lower basal levels of miR-9, potentially resulting in greater levels of p50 protein and consequently increased NF-κB reserve levels awaiting activation. Upon exposure to stress, increased miR-9 expression could result from a negative feedback loop observed between miR-9 and NF-κB, wherein increased NF-κB activity in response to the TSST (that is found in individuals with depressive symptoms and a history of childhood mistreatment) leads to an increase in miR-9 levels and a subsequent regulation of p50 protein expression. Bazzoni et al. observed this feedback loop in human neutrophils and monocytes, wherein NF-κB induces miR-9 expression, which in turn decreases levels of p50 protein in a negative feedback mechanism following NF-kB activation [266].

Individuals with lower scores on these psychological measures tended to show decreased miR-9 levels over the course of the study session. These individuals also had higher baseline miR-9 levels and likely lower p50 protein levels. It is likely that the decrease in miR-9 expression observed during the study session is part of the normal stress response that enables NF-kB activation in response to stress. Further studies in additional healthy participants and individuals with a history of childhood trauma and

depression would help to clarify these relationships as they are purely speculative at this time due to the small size of the study sample.

There was no change in NFKB1 mRNA levels during the course of the TSST, and baseline NFKB1 mRNA levels did not correlate with psychological variable sum scores. It is possible that the decrease that is seen in miR-9 levels is not sufficient to evoke a noticeable change in NFKB1 transcript levels, as miRNA targeting in each cell environment is dependent on multiple factors that include the relative abundance of the target mRNA. It is also possible that this particular biological system follows a mechanism of translational repression, and a decrease in the NFKB1 gene product (p105 and p50 proteins) would be seen on the protein level. Indeed, Bazzoni et al. observed translational repression of NKFB1 by miR-9 in human polymorphonuclear neutrophils and monocytes [266]. The authors observed that a concurrent increase in NFKB1 transcript and miR-9 expression did not lead to an observable increase in p50 protein levels in spite of increased mRNA levels [266].

Extreme values analysis identified two Cortisol Responders with miRNA expression patterns that differed from the rest of the group. One participant experienced a higher cortisol response along with greater BAI and CTQ scores and more stressful life events than the rest of the sample. The second participant experienced higher chronic stress along with increased childhood trauma measures. These results suggest that childhood trauma and stressful life events alter the gene expression signature in response to acute psychosocial stress. Future miRNA expression studies of clinical samples will be key to determining how these factors alter the stress response on a molecular level.

FUTURE DIRECTIONS

Currently, the expression of ten miRNAs showing the greatest expression differences over time is being confirmed through custom-designed TaqMan-based Low Density Arrays. In addition, changes in mRNA expression at Time Points A, B, and C will be identified via LIMMA and EVA. miRNA-mRNA target correlation analysis will be performed with IPA to integrate both data sets and to identify pathways that are potentially affected by the interaction of miRNA and mRNA in response to acute psychosocial stress.

In addition, we aim to increase the size of the study sample and to investigate the expression of specific stress-related miRNAs (such as miR-9) in a greater number of Cortisol Responders and to compare miR-9 expression to that seen in Non-Responders. miRNA gene expression will also be profiled in a clinical population with psychopathology to identify molecular differences in the stress response.

Future studies will also investigate the mechanism of the miR-9 decrease in psychosocial stress. Bierhaus et al. propose a model wherein noradrenergic stimulation leads to increased NF-κB activity in mice and in THP-1 monocytic cells stimulated with norepinepherine. Future experiments will aim to determine whether stimulation of THP-1 monocytic cells with norepinepherine leads to changes in expression of miR-9 and NFKB1. In order to test this hypothesis, THP-1 cells will be stimulated with increasing concentrations of norepinephrine as in Bierhaus et al. [236]. Total RNA (including miRNA) and protein will be collected and RT-qPCR will be used to determine changes in miR-9 and NFKB1 levels, while quantitative immunoblotting will be used to investigate changes in p105 and p50 protein levels.

This experiment will reveal whether the decrease in miR-9 expression could result from noradrenergic stimulation. One of the limitations of this experimental approach is that a decrease in miR-9 and corresponding increase in p105/p50 protein would not necessarily have functional significance for NF-κB activation. The effect of miR-9 inhibitors on NF-κB binding activity could be used in subsequent experiments in order to determine the specific contribution of this particular pathway to NF-κB activation.

FIGURES

Figure 2.1. TSST Paradigm

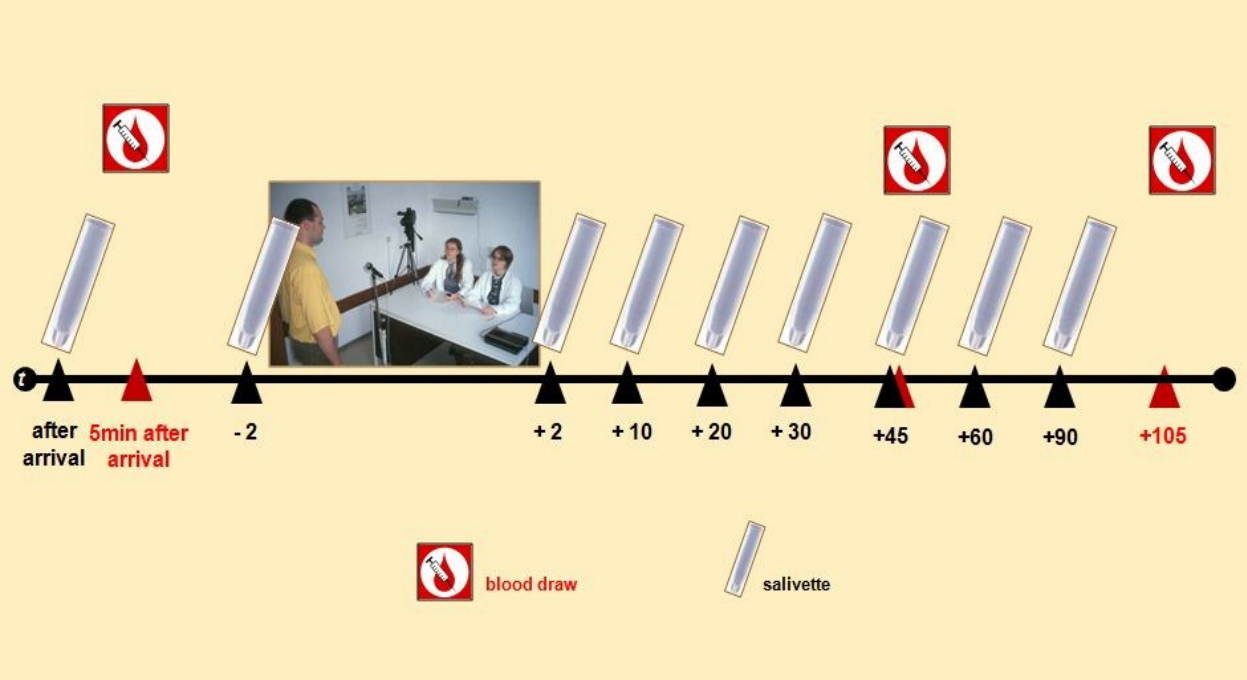


Figure 2.1. TSST Paradigm

The Trier Social Stress Test (TSST) is an established paradigm aimed to induce psychosocial stress through a 5 minute preparation period, a 5 minute public speaking task and a 5 minute mental arithmetic task in front of a non-responsive audience. Salivary cortisol levels were measured before the stress paradigm, shortly after the stressor, and for 90 minutes throughout the stress recovery period. An intravenous catheter was inserted five minutes after participant arrival, and the first blood draw took place following catheter insertion; two additional blood draws took place during the stress recovery period at 45 and 105 minutes following stress induction.

Figure 2.2. Cortisol Response to the TSST

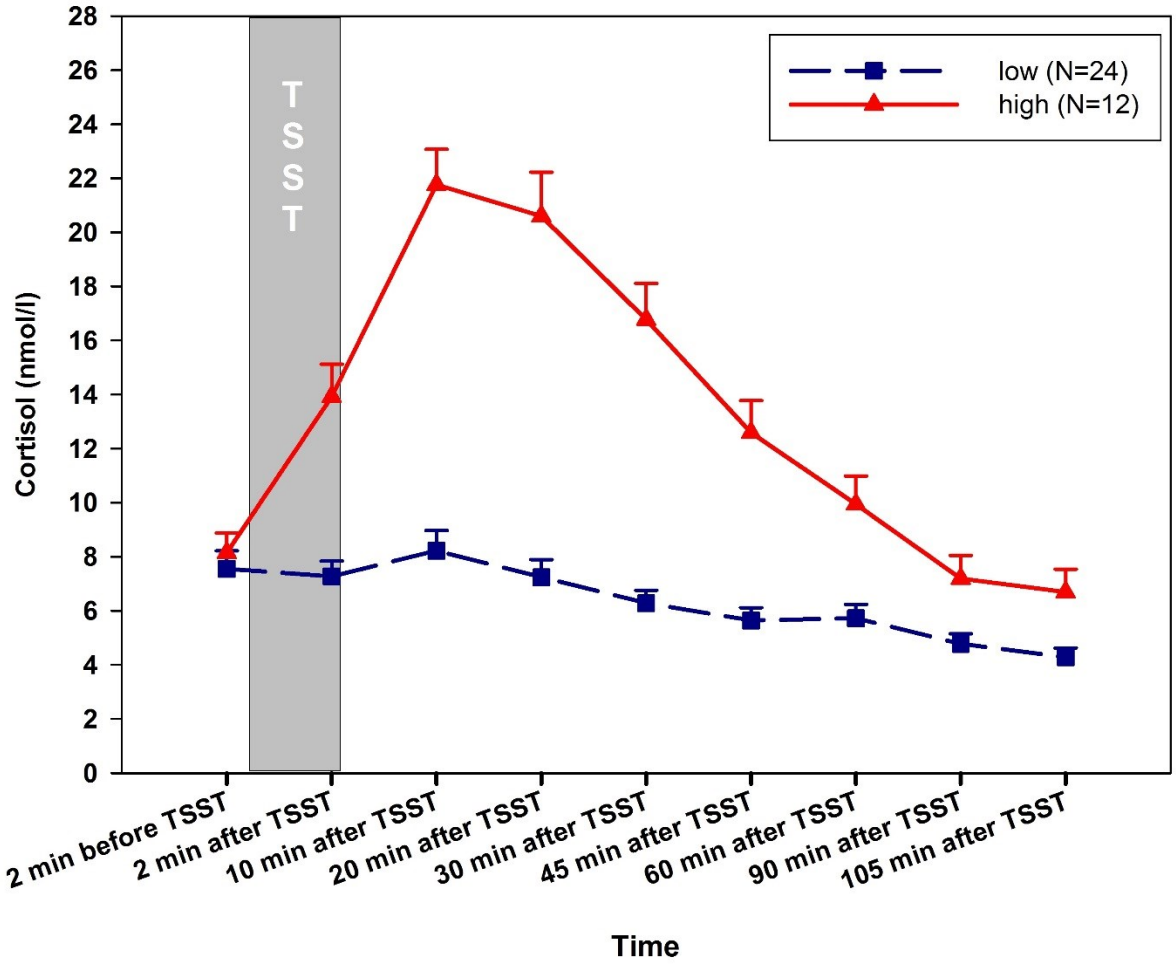


Figure 2.2 Cortisol Response to the TSST.

Salivary cortisol was measured throughout the course of the study. Cortisol response to the paradigm for each participant (n=36) was determined by subtracting the cortisol concentration two minutes before the TSST from the cortisol concentration 20 minutes following the TSST. K-means clustering was used to group participants into a "Cortisol Responder" category (n=12) with a high cortisol response and a "Cortisol Non-Responder" category (n=24) with a low cortisol response. Cortisol levels of Responders are shown in red and those of Non-Responders are shown in blue.

Figure 2.3 miRNAs Significant by LIMMA with |FC|>1.2 and p value < 0.01

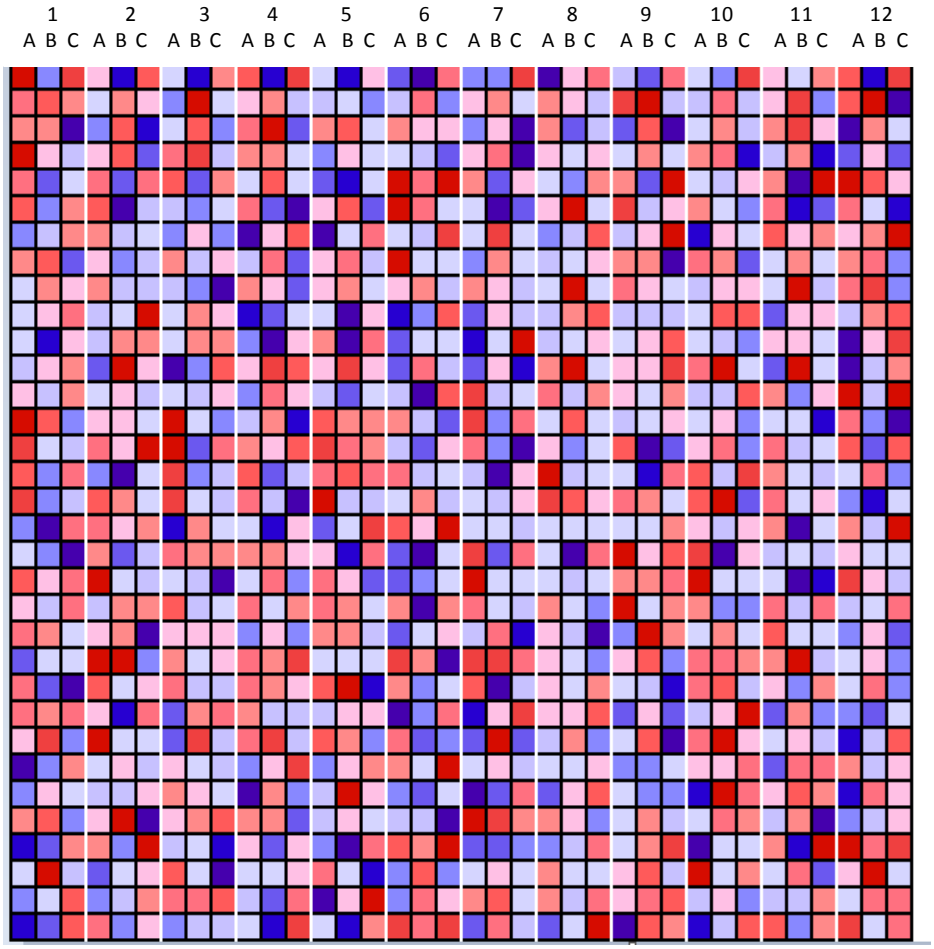


Figure 2.3. miRNAs Significant by LIMMA with |FC|>1.2 and p value < 0.01

Each row represents a miRNA probeset and each set of three columns represents one individual (labeled with participant ID number) at Time Points A, B, and C. Each sample was run once on one microarray at each Time Point. The expression value represented by each square is log2 of the microarray intensity value for the corresponding Time Point/individual for a given probeset. The largest gene expression values are displayed in red (hot), the smallest values in blue (cool), and intermediate values in shades of red (pink) or blue.

Figure 2.4. Genome-wide Analysis Results

Top Networks

ID	Associated Network Functions	Score
1	Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function	42
2	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry	38
3	Lymphoid Tissue Structure and Development, Organ Morphology, Tissue Morphology	38
4	Developmental Disorder, Cancer, Hematological Disease	36

Top Canonical Pathways

Name	p-value	Ratio
Molecular Mechanisms of Cancer	6.5E-67	111/381 (0.291)
Glucocorticoid Receptor Signaling	3.71E-51	85/294 (0.289)
Cardiac Hypertrophy Signaling	1.03E-47	77/247 (0.312)
Role of NFAT in Cardiac Hypertrophy	2.79E-47	70/209 (0.335)
B Cell Receptor Signaling	3.76E-46	65/171 (0.38)

Top Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Cancer	2.77E-52 - 1.35E-13	553
Infectious Disease	9.75E-41 - 1.03E-13	242
Hematological Disease	3.48E-40 - 6.37E-14	189
Cardiovascular Disease	3.91E-38 - 5.72E-14	218
Developmental Disorder	1.87E-35 - 7.10E-14	199

Molecular and Cellular Functions

Name	p-value	# Molecules
Cell Death and Survival	5.72E-82 - 1.33E-13	454
Cellular Growth and Proliferation	5.43E-71 - 1.52E-13	436
Gene Expression	5.22E-67 - 3.25E-15	337
Cellular Movement	1.52E-65 - 9.65E-14	316
Cellular Development	3.99E-63 - 1.52E-13	436

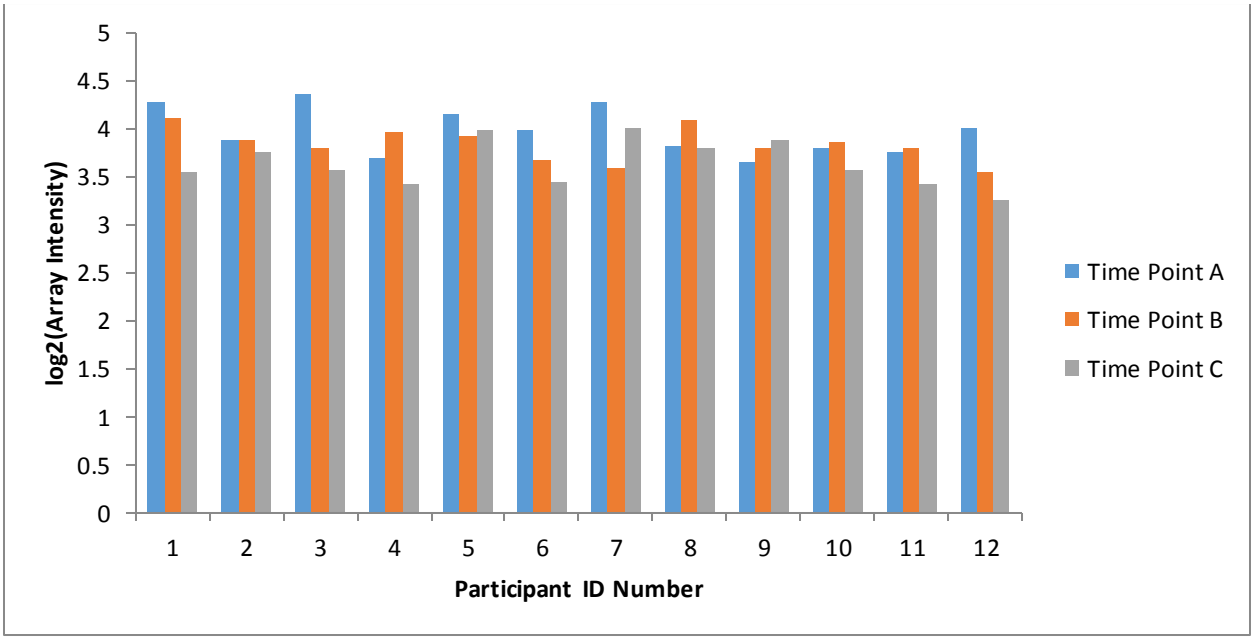
Physiological System Development and Function

Name	p-value	# Molecules
Organismal Survival	2.56E-71 - 5.18E-21	354
Tissue Morphology	1.03E-65 - 1.42E-13	319
Hematological System Development and Function	1.97E-55 - 1.52E-13	345
Cardiovascular System Development and Function	1.58E-43 - 1.51E-13	225
Hematopoiesis	3.57E-42 - 2.21E-14	207

Figure 2.4. Genome-wide Analysis Results

A miRNA target filter was run using IPA on the human orthologues of the miRNAs identified by LIMMA to be differentially expressed during the course of the stress paradigm. miRNAs and their mRNA targets were entered into a Core analysis that demonstrated involvement in immune and infectious pathways as well as glucocorticoid signaling, cardiovascular disease, and cancer, among others.

Figure 2.5. miR-9 Levels Decrease During the Course of the TSST

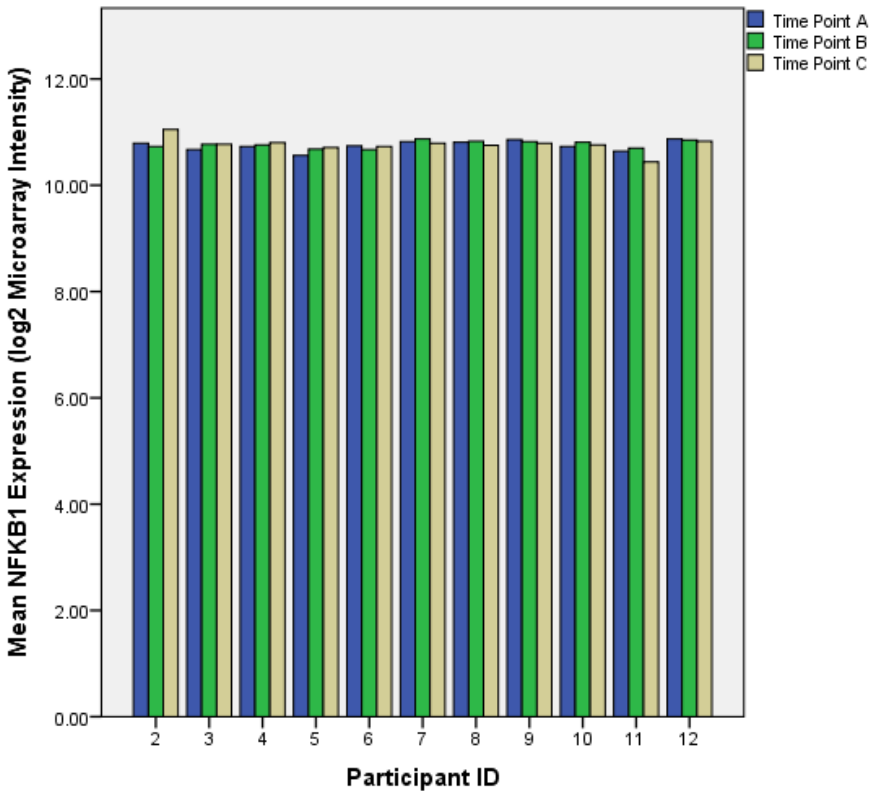


Targeted Gene	miRNA	Fold Change B vs A	Fold Change C vs A	Fold Change C vs. B	miRNA targeting
NFKB1	miR-9-5p	0.91	0.79	0.87	Experimentally Observed [266]

Figure 2.5. miR-9 Levels Decrease During the Course of the TSST

miR-9 levels were measured by microarray in Cortisol Responders. miR-9 levels decrease during the course of the TSST as shown by the Fold Change between Time Point B and Time Point A (0.91), Time Point C and Time Point A (0.79), and Time Point C and Time Point B (0.87).

Figure 2.6. There is No Change in NFKB1 mRNA Levels During the Course of the TSST



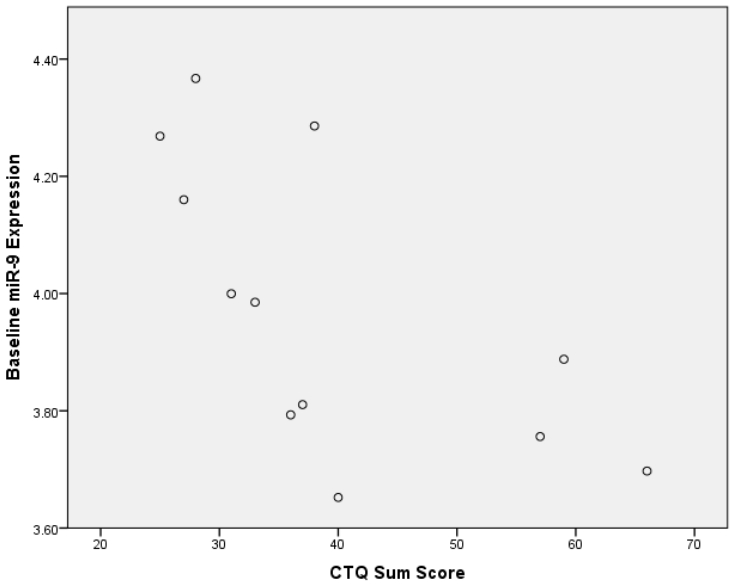
Targeted Gene	Fold Change B vs A	Fold Change C vs A	Fold Change C vs. B	miRNA targeting
NFkB1	1.01	1.00	1.00	Experimentally Observed to be targeted by miR-9 [249]

Figure 2.6. There is No Change in NFKB1 mRNA Levels During the Course of the TSST

NFKB1 mRNA levels, as measured by microarray, did not change during the course of the TSST as evidenced by a fold change of 1 observed between time points B and A, C and A, as well as C and B. (repeated measures ANOVA F=0.276 p=0.761).

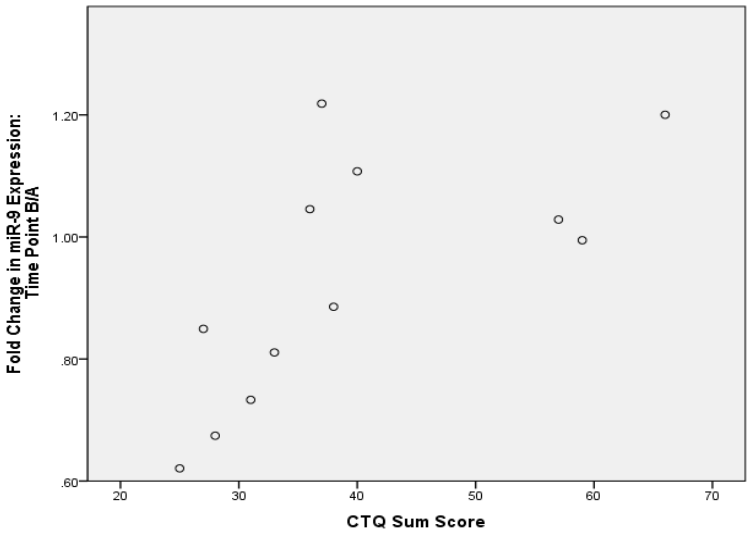
Figure 2.7. Correlation between miR-9 Levels and Psychological Measures

A. Baseline miR-9 Expression as a Function of CTQ Sum Score



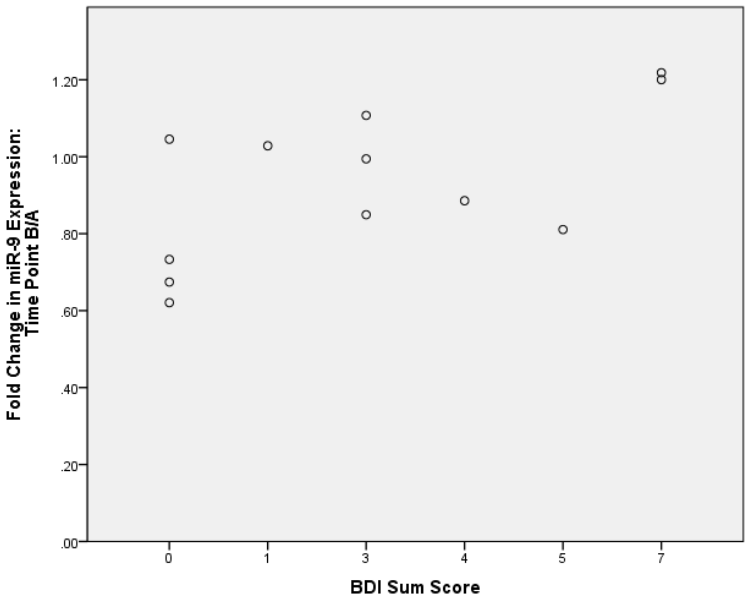
Pearson's R= -0.648
p=0.023

B. Fold Change in miR-9 Expression Between Time Points B and A as a Function of CTQ Sum Score



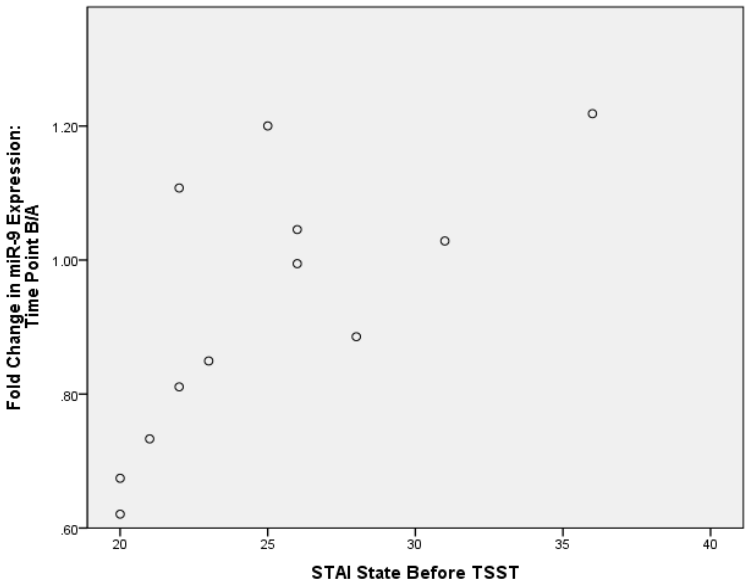
Pearson's R= 0.674
p=0.016

C. Fold Change in miR-9 Expression Between Time Points B and A as a Function of BDI Sum Score



Pearson's R= 0.640
p=0.025

D. Fold Change in miR-9 Expression Between Time Points B and A as a Function of STAI State Anxiety Before TSST



Pearson's R= 0.700
p=0.011

Figure 2.7. Correlation between miR-9 Levels and Psychological Measures

The above scatter plots depict bivariate relationships with significant Pearson's correlation coefficients. **Panel A** shows miR-9 expression as a function of CTQ sum score, **Panel B** shows the fold change in miR-9 expression between Time Points B and A as a function of CTQ sum score; **Panel C** shows the fold change in miR-9 expression between Time Points B and A as a function of BDI sum score; **Panel D** shows the fold change in miR-9 expression between Time Points B and A as a function of STAI state anxiety before the TSST.

Figure 2.8. Extreme Values Analysis (EVA)

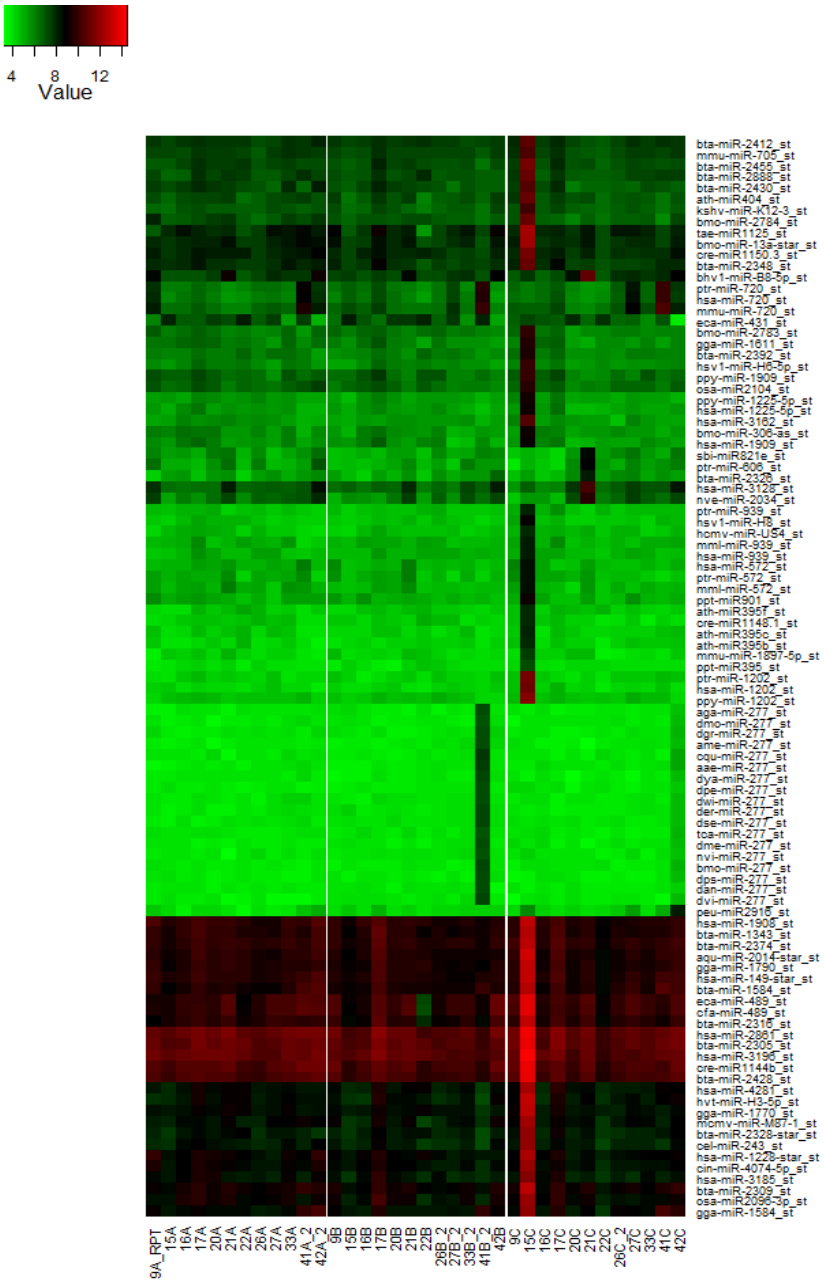


Figure 2.8. Extreme Values Analysis (EVA).

Each row represents a miRNA probeset identified by EVA analysis, and each column represents one individual at time A, B, or C. The expression value represented by each square is the log of the microarray intensity value for the corresponding time point/individual for a given probeset. The expression profile of Participant #11 differs from that of other participants at time point “B”. The expression profile of Participant #2 differs from that of other participants at time point “C”.

TABLES

Table 2.1. TSST Participant Characteristics

Sample	Cortisol Responders	Cortisol Non Responders	t-test *p* value
N	12	24	
Age (years)	43.17 (19.33)	28.33 (11.92)	.007
Cortisol Response (in nmol/l)	13.60 (5.77)	.68 (4.71)	.000
BDI (missing data of n=1)	6.43 (6.61)	2.36 (2.38)	.057
BAI	3.75 (3.05)	5.79 (4.61)	.175
TiCS	10.33 (7.24)	16.21 (9.56)	.07
CTQ	39.33 (13.86)	32.63 (8.45)	.080
SLE	43.22 (14.18)	33.04 (16.63)	.114

Table 2.1. TSST Participant Characteristics.

BAI: Beck Anxiety Questionnaire, Sum Score; BDI: Beck Depression Questionnaire, Sum Score; TiCS: Trier Inventory for the Assessment of Chronic Stress, Sum Score; CTQ: Childhood Trauma Questionnaire, Sum Score; SLE: Stressful Life Events (based on a structured interview). All values are mean (+/- standard deviation). Higher sum scores indicate increased symptom severity. There was a significant difference between Cortisol Responder and Cortisol Non-Responder groups in age and cortisol response (t-test p-value <0.05), while there was no significant difference in BAI, BDI, TiCS, or CTQ sum scores (t-test $p>0.05$), nor was there a significant difference in the number of stressful life events (t-test $p>0.05$).

Table 2.2. Correlation Table of miR-9 Expression and Psychological Variable Sum Scores

		Baseline miR-9 Expression	Fold Change miR-9 Expression: Time Point B/A	Fold Change miR-9 Expression: Time Point C/B	Fold Change miR-9 Expression: Time Point C/A
BDI	Pearson's R	-.363	.640	-.375	-.303
	Significance (2-tail)	.246	.025	.230	.338
BAI	Pearson's R	.100	.058	.037	-.147
	Significance (2-tail)	.758	.857	.910	.648
TiCS	Pearson's R	-.527	.392	-.104	-.339
	Significance (2-tail)	.078	.207	.748	.282
CTQ	Pearson's R	-.648	.674	-.477	-.287
	Significance (2-tail)	.023	.016	.117	.365
STAI State Anxiety Before TSST	Pearson's R	-.414	.700	-.478	-.306
	Significance (2-tail)	.181	.011	.116	.333

Table 2.2 Correlation Table of miR-9 Expression and Psychological Variable Sum Scores.

Baseline levels of miR-9 were significantly negatively correlated with Childhood Trauma Questionnaire (CTQ) sum score (Pearson's R=-0.648 p=0.023). There was also a significant positive correlation between sum scores of the Beck Depression inventory (BDI) and the fold change in miR-9 between Time Points B and A (B/A) (Pearson's R=0.640 and p=0.025). Sum scores on the Childhood Trauma Questionnaire (CTQ) and the State-Trait Anxiety Inventory (STAI) assessing state anxiety before beginning the TSST were positively correlated with the change in miR-9 expression between Time Points B and A. There was no significant correlation between baseline mir-9 expression, nor changes in miR-9 expression and the Beck Anxiety Inventory (BAI), nor the Trier Inventory for the Assessment of Chronic Stress (TiCS). Finally, changes in gene expression between Time Point C and Time Point B (C/B) and Time Point C and Time Point A (C/A) were not correlated with any of the psychological measures tested.

Table 2.3. Summary of Repeated Measures ANOVA and Stepwise Linear Regression Analysis: miR-9

Repeated Measures ANOVA	• Significant change in miR-9 levels over time (F=4.91p=0.018) • Not modified by BDI, BAI, TiCS, CTQ, or STAI State Anxiety Before TSST
Stepwise Linear Regression	• Fold change (B/A) is dependent on STAI state anxiety before the TSST (p=0.011 model R^2=0.490) • Not dependent on BDI, BAI, TiCS, or CTQ
	• Baseline miR-9 expression is dependent on CTQ sum score (p=0.023 R^2=0.420) • Not dependent on BDI, BAI, TiCS, or STAI State Anxiety Before TSST

Table 2.3. Summary of Repeated Measures ANOVA and Stepwise Linear Regression Analysis.

Repeated measures ANOVA showed a significant change in miR-9 levels over time (F=4.91p=0.018) that was not modified by BDI, BAI, TiCS, CTQ, or STAI state anxiety before the TSST. Stepwise linear regression analysis favored a model wherein fold change (B/A) is dependent on STAI state anxiety before the TSST (p=0.011 model R^2=0.490) and is not dependent on BDI, BAI, TiCS, or CTQ sum scores.

Table 2.4. Baseline NFKB1 Levels and Psychological Measures

		Baseline NFKB1 Expression
BDI	Pearson's R	.030
	Significance (2-tail)	.930
BAI	Pearson's R	-.272
	Significance (2-tail)	.418
TiCS	Pearson's R	.066
	Significance (2-tail)	.848
CTQ	Pearson's R	-.027
	Significance (2-tail)	.938
STAI State Anxiety Before TSST	Pearson's R	-.071
	Significance (2-tail)	.836

Table 2.4. Baseline NFKB1 Levels and Psychological Measures

There was no significant correlation between levels of NFKB1 and sum scores for the psychological variables measured in this study, namely BDI, BAI, TiCS, CTQ, and STAI State Anxiety Before TSST.

Table 2.5. EVA Outlier Characteristics in Mean Values

Sample	Participant #2	Participant #11	Cortisol Responder Mean (+/- Stdev)
Age	35	58	43.17 (19.33)
Cortisol Response (in nmol/l)	20.35	9.34	13.60 (5.77)
BDI	3	1	6.43 (6.61)
BAI	10	3	3.75 (3.05)
TiCS	10	20	10.33 (7.24)
CTQ	59	57	39.33 (13.86)
SLE	60	71	43.22 (14.18)

Table 2.5. EVA Outlier Characteristics in Mean Values.

Legend: BAI: Beck Anxiety Questionnaire. BDI: Beck Depression Questionnaire. TiCS: Trier Inventory for the Assessment of Chronic Stress, Screening Scale. CTQ: Childhood Trauma Questionnaire. SLE: Stressful Life Events (based on a structured interview). Underlined values differ from the Cortisol Responder mean by more than 1 standard deviation.

CHAPTER 3: BIBLIOGRAPHY

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