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Neural Correlates and Peripheral miRNAs Associated with Stress-Related Telomere Shortening

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Stephanie Izzi

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Stephanie A. Izzi

We, the dissertation committee for the above candidate for the
Doctor of Philosophy degree, hereby recommend
acceptance of this dissertation.

Turhan Canli, Ph.D.
Associate Professor, Department of Psychology

Martha Furie, Ph.D.
Professor, Department of Pathology

Mary Kritzer, Ph.D.
Professor, Department of Neurobiology and Behavior

Gerald Thomsen, Ph.D.
Professor, Department of Biochemistry and Cell Biology

John Robinson, Ph.D.
Professor, Department of Psychology

This dissertation is accepted by
the Graduate School

Charles Taber
Dean of the Graduate School

Abstract of the Dissertation

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Telomeres are repetitive nucleoprotein complexes at the ends of chromosomes that provide a protective cap for genomic DNA. Telomere shortening leads to cellular senescence or apoptosis. Accordingly, telomere length is considered both a mechanism and marker for aging. Increased exposure to chronic stress is associated with shorter telomere length. In addition, differences in stress reactivity have been associated with telomere length. Here I will describe several studies identifying neural circuits associated with such telomere shortening, an evaluation of saliva as an appropriate biomaterial to measure telomere length, and an exploration of the role of microRNAs as mediators of stress-related telomere shortening. Abnormalities in the function of stress related neural circuitry might be one mechanism of stress-related telomere shortening. I examined the relationship between activation in stress-related neural circuitry at rest or during an acute psychosocial stress task, as well as telomere length in a healthy

population. I observed that subjects with shorter telomere length showed increased activation in the anterior cingulate cortex (ACC) and the amygdala at rest. I also found that telomere length negatively correlated with activity in the ACC, hippocampus and medial prefrontal cortex during an acute stress task. These findings suggest that increased neural reactivity in stress- or emotion-related brain regions may be one mechanism explaining individual differences in telomere length. The majority of studies of telomere shortening use leukocyte telomere length as a biomarker, which requires a blood draw. While telomere length varies across tissues, the rate of telomere shortening appears to be similar across tissue types. Based on within-subject comparisons, I determined that relative telomere length in saliva is correlated with telomere length in leukocytes, indicating that saliva is an appropriate proxy for blood to measure telomere length. To date, limited research has been done to identify the mechanism by which stress affects telomere length. Psychological stress has been associated with increased levels of specific miRNAs, and our laboratory has compiled a list of miRNAs that undergo expression changes in response to an acute stressor. These miRNAs are predicted to target several proteins that elongate and protect telomeres. However, our investigations do not support a role for miRNAs in stress-related telomere shortening.

To my wonderful mother and sister, for their constant support and inspiration

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List of Abbreviations

(5-HTT) - 5-HT transporter gene

(ACC) - Anterior cingulate cortex

(ACTH) - adrenocorticotropic hormone

(ANS) - autonomic nervous system

(ASL) - arterial spin labeling

(BOLD) - blood oxygenation level dependent

(CBF) - Cerebral blood flow

(CTQ) - Childhood Trauma Questionnaire

(CRH) - corticotrophin-releasing hormone

(FWHM) - full width at half maximum

(HPA) - hypothalamic pituitary adrenal

(LTL) - leukocyte telomere length

(miRISC) - miRNA-induced silencing complex

(MIST) - Montreal Imaging Stress Task

(MRI) - magnetic resonance imaging

(NEO) - Neuroticism Extraversion and Openness Personality Inventory

(PACE) - prospective acquisition correction

(PBMC) - peripheral blood mononuclear

(PET) - positron emission tomography

(PFC) - prefrontal cortex

(PTSD) - posttraumatic stress disorder

(qPCR) - quantitative polymerase chain reaction

(QUIPSS) - quantitative imaging of perfusion using a single subtraction

r(ROI) - region of interest

(SAM) - sympathetic adrenal medullary

(TSST)- Trier Social Stress Test

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Chapter 1: Neural Correlates of Short Telomeres

Introduction

Telomeres

Telomeres are repetitive non-coding nucleoprotein complexes that are located at the ends of linear chromosomes. The DNA sequence of telomeres consists of tandem repeats of a short sequence (in vertebrates, this sequence is TTAGGG) that serve to protect genomic DNA (McElligott and Wellinger, 1997; Meyne, 1989). At the very end of the telomeric repeat sequence, there is a 3' single-stranded overhang that can fold back and invade the end of the double-stranded telomere sequence, creating a structure known as a T-loop (Stansel et al., 2001). The T-loop interacts with specific proteins responsible for protecting the telomere (Figure 1), as well as a special polymerase called telomerase that can elongate these structures (Chan and Blackburn, 2004). Telomeres are essential for normal cellular function as they maintain the integrity of the genome and prevent the loss of genetic material during cell division (Aubert and Lansdorp, 2008).

Telomeres provide a solution to the so-called end-replication problem. In eukaryotic cells, DNA polymerase performs semiconservative DNA replication on both strands during cell division, but can only synthesize DNA in the 5' to 3' direction and requires a short RNA primer, which is later removed. The leading strand is copied continuously to the end of the chromosome, while the discontinuous lagging strand is copied in fragments. The RNA primers are then

removed, and the internal gaps are filled in by extension of the discontinuous DNA and ligation. However, removal of the most distal RNA primer leaves a 5' terminal gap. Following subsequent rounds of DNA replication, if only the semi-conservative DNA replication machinery operates this gap would result in progressively shorter daughter strands (Olovnikov, 1973). If telomeres reach a critically short length, problems arise. First, genomic DNA can become damaged and impact cellular function. Second, short telomeres can cause chromosomal fusions and missegregation in subsequent cell divisions (Vajen et al., 2013). Finally, the 3’ single-stranded overhang of critically short telomeres is recognized by the cellular DNA damage response as a double-stranded break, leading to cellular senescence or apoptosis (Henderson and Blackburn, 1989).

Telomere maintenance is essential for the continued viability of proliferating cells. Although telomere attrition rate varies among individuals and tissues, the end-replication problem results in an average shortening of 50-100 base pairs of terminal telomeric repeat sequence with each doubling of cultured human somatic cells (Harley, 1995; Huffman et al., 2000). This erosion eventually results in a 'critically short' telomere on one or more chromosomes. It is unclear what constitutes a dangerously short telomere at the molecular level; the number of double-stranded repeats and the length of the single-stranded 3' overhang may both play a role. Whatever this length may be, the presence of critically short telomere(s) induces one of three fates: proliferative senescence, apoptosis or continued proliferation accompanied by genomic instability (Stewart and Weinberg, 2000).

Telomeres, aging and disease

In germ, stem and certain highly proliferative cells, telomere shortening is prevented by telomerase, an enzyme that adds nucleotides specifically to telomeric repeats (Blackburn, 1992). In most adult somatic cells, however, telomerase activity is absent or extremely low. Telomeres therefore shorten in most tissues as a function of cellular proliferation. Interestingly, telomere attrition is correlated with increasing age in all human tissues in which it has been tested, which is presumed to reflect the accumulated cell divisions associated with tissue renewal (Collins and Mitchell, 2002). Telomere shortening is one of the best-understood mechanisms known to impose a limit on the growth of normal cells in culture, as critically short telomeres lead to replicative senescence in culture (Collado et al., 2007). Telomere length in vivo has been shown to predict age in humans (Canela et al., 2007), although as discussed later length is influenced by epidemiological factors other than age, so the accuracy of using telomere length to predict chronological age has been variable.

Although the exact role of telomeres in aging is complex, telomere length has been viewed as both a marker and a mechanism of biological aging and increased disease risk (Epel, 2009; Frenck, Blackburn, and Shannon, 1998). Some of the most compelling evidence for the role of telomere shortening in aging and disease comes from premature aging syndromes. Several human premature aging syndromes are characterized by a faster rate of telomere attrition with age, and these have provided important insights into the consequences of accelerated telomere shortening.

These syndromes include dyskeratosis congenita, Werner syndrome and Bloom syndrome, among others. Each of these involves mutations in proteins involved in telomere maintenance and elongation. All of these syndromes result in decreased telomerase stability, poor telomere maintenance and shorter telomeres during development, at birth and throughout life. Patients show increased chromosomal instability with age, consistent with a faster rate of telomere loss (Mochizuki et al., 2004). These patients develop many of the pathologies shown for the telomerase-deficient mouse model, such as short stature, hypogonadism and infertility, defects of the skin and the hematopoietic system, bone marrow failure, and premature death (Marrone et al., 2005). These studies provide clear evidence for a crucial role of telomeres in aging and age-related diseases, although the exact role of telomere shortening remains poorly understood.

Relatively short leukocyte telomere length (LTL), as measured by age-corrected average telomere length in leukocytes, is a predictor of several disease states. These include increased risk of several cancers including breast (Shen et al., 2007) and renal cancer (Shao et al., 2007). The association between short telomeres and heart disease has also been well studied; short LTL is correlated with increased occurrence and severity of heart disease (Brouilette et al., 2007), as well as increased mortality from cardiovascular disease and infection (Cawthon et al., 2003) and increased risk of hypertension (Demissie et al., 2006). Insulin resistance has also been associated with short LTL in several studies (Demissie et al., 2006, Aviv et al., 2006, Gardner et al., 2005).

Stress and telomeres

Telomere length is influenced not just by age, but also by environmental factors. The increased long-term exposure to air pollution faced by residents in Hong Kong (Hou et al., 2012) and US toll booth workers (Hoxha et al., 2009) has been correlated with relatively short telomere length, although short-term exposure has been associated with a rapid increase in telomere length (Hou et al 2012). Lifetime exposure to any of several pesticides is also linked to significantly shorter telomeres (Lhou et al., 2012). Women who lived near sites polluted by illegal waste dumping in Italy had shorter telomeres than controls; furthermore, telomere length was inversely proportional to the distance from the dump-site (De Felice et al., 2012).

Of particular relevance in the context of this dissertation and the work conducted in the Canli laboratory, several measures of psychological stress have been consistently associated with altered telomerase activity and shorter telomere length. In the first such study, the telomere length of mothers who were caregivers to chronically ill children was compared to that of controls (mothers of healthy children) by Epel and colleagues (2004). These investigators observed a positive correlation between the number of years caregivers had provided care and LTL, even after controlling for age. Furthermore, those who scored higher on a questionnaire assessing perceived stress had significantly shorter telomeres, regardless of caregiver status. A study examining caregivers of spouses with Alzheimer's disease found similar results (Damjanovic et al., 2007). Causal evidence linking telomere length and stress exposure has been provided by

Kotrschal and colleagues (2007), who found that mice exposed to chronic stress over a period of six months have significant reductions in telomere length compared to controls. In humans, shorter leukocyte telomere length has been linked to a variety of chronic stress events. Women who work unusually long hours, or who report particularly stressful work conditions, have significantly shorter telomeres relative to part-time or nonworking women; these differences are as great as those between smokers and nonsmokers (Parks et al., 2011). Relative telomere length in white blood cells was significantly shorter in those with relatively low household income and those with poor diet, an association that is not explained by other measures of health or lifestyle factors (Sheilds et al., 2011). A study of twins with different socioeconomic statuses revealed that those with low socioeconomic status reported higher stress levels and had significantly shorter telomere length than their twins (Cherkas et al., 2006). In a study of patients with and without chronic osteoarthritis pain, Sibille et al. (2012) found reduced telomere length in those with chronic pain and high stress versus those with no pain and low stress. In another study, Drury et al. (2011) found that Romanian orphans (living under unquestionably difficult conditions) also have shorter telomeres when compared to age-matched children in foster care.

In addition to exposure to chronic stress, individual differences in stress reactivity have been associated with short telomere length (Tomiyama et al., 2012) and altered telomerase activity (Epel et al., 2010). Short LTL is associated with high cortisol in response to an acute stress task in the Trier Social Stress Test (TSST), which exposes participants to social stressors including public

speaking and negative social feedback (Epel et al., 2010). Additionally, shorter telomeres are associated with increased heart rate and cortisol reactivity in children (Kroenke et al., 2011). Shorter telomeres are found in rape victims who develop posttraumatic stress disorder (PTSD) compared to those who do not (Malan et al., 2011). Hostility, which correlates with heightened stress reactivity, is inversely correlated with telomere length and positively correlated with telomerase activity, in men but not women (Brydon et al., 2012).

Several studies have linked the experience of childhood trauma to short telomeres and to dysregulation of the stress response. For example, adults reporting a history of maltreatment had significantly shorter telomeres than those who did not report maltreatment across several similar studies (Kananen et al., 2010; O'Donovan et al., 2011 and Tyrka et al., 2010). In addition, perceived stress, rather than objective measures of stress, can be associated strongly with telomere length. For example, in two studies comparing caregivers to controls, it was individual differences in the anticipatory appraisal or perceptions of a given stressful event that predicted telomere length (Epel et al., 2010; O'Donovan et al., 2012).

It has been hypothesized that individuals with certain personality characteristics or predispositions that give rise to greater or more frequent cortisol output in response to stressors may also have shorter telomeres (Tomiyama et al., 2012). For example, animals with anxiety phenotypes are more likely to show exaggerated response to stressors as well as to experience premature aging (Viveros et al., 2007). In humans, shorter LTL has been linked

to both symptoms of depression (Hoen et al., 2011; Simon et al., 2006) as well as the number of years experiencing depression (Wolkowitz et al., 2011). Simon et al. (2006) demonstrated shorter telomeres in patients with mood disorders or bipolar disorder compared with healthy control subjects. This was replicated by Hartmann et al. (2010) and Elvsashagen et al. (2011). Wolkowitz et al. (2011) also reported shorter telomeres in those with mood disorders. Those diagnosed with schizophrenia, major depressive disorder and bipolar disorder were all found to have significantly shorter leukocyte telomeres compared with healthy controls (Zhang et al 2010). Even among healthy participants, shorter telomere length has been associated with individual differences in personality, such as neurotic traits and pessimism (O'Donovan et al., 2012). Positive stress-coping mechanisms, such as physical exercise (Puterman et al., 2010) and meditation (Epel et al., 2009), have been associated with longer telomeres.

The stress response

Stress is a term that is widely used in psychobiology literature, but it is rarely well defined. Generally, stress describes the set of physiological changes that occur when an organism is faced with physical or psychological threat. While it is easy to imagine extrinsic physical threats eliciting such a response, the fact that psychological conditions can have the same effect is more difficult to comprehend. Rumination about past events or anticipation of future threats can cause similar physiological responses. Psychologically stressful conditions can include an element of novelty, something that one cannot control (uncontrollability), or social evaluative threats. These events elicit responses

similar to those of a physical threat (Dickerson & Kemeny, 2004).

The ability of physiological systems to dynamically adjust to changing environmental demands is known as allostasis (McEwen and Stellar, 1993). The stress response is considered an adaptation that allows an organism to return to homeostasis after being confronted with a threat. In order to accomplish this, the organism must increase arousal, alertness, vigilance, focused attention and cognitive processing of the threat. These processes are performed by limbic brain structures, which include the hippocampus and amygdala, as well as the prefrontal cortex. Stressors also trigger physiological and behavioral responses that are aimed at reinstating homeostasis. This 'stress response' is reflected in the rapid activation of the sympathetic nervous system, which leads to the release of noradrenaline from widely distributed synapses and adrenaline from the adrenal medulla. A slower and prolonged cortisol and vasopressin release by the hypothalamus then follows. These physiological responses are referred to as the sympathetic adrenal medullary (SAM) and hypothalamic pituitary adrenal (HPA) axes (Figure 2; Munck, Guyre, and Holbrook, 1984).

The immediate activation of the SAM axis in response to a stressor is known as the "fight or flight" response (Selye, 1936). It is thought to have evolved to prepare a body for action through the increase of heart rate and blood pressure and a reduction in activities related to rest, such as digestion. Therefore, cardiovascular reactivity (indexed by heart rate and blood pressure) and direct measure of sympathetic nervous system activity (norepinephrine and epinephrine levels in the blood) are biomarkers of the stress response.

Activation of the HPA axis is a slower and longer-lasting response to a stressor. The HPA axis is a neuroendocrine system that comprises a complex set of interactions and feedback loops among the hypothalamus, the pituitary gland, and the adrenal glands. When the HPA axis is activated by a stressful situation, the hypothalamus secretes vasopressin and corticotrophin-releasing hormone (CRH) into blood vessels that connect the hypothalamus to the anterior portion of the pituitary gland. CRH then travels to the anterior portion of the pituitary though the portal blood vessel system, and vasopressin travels via axonal transport to the posterior pituitary. CRH and vasopressin then trigger the secretion of adrenocorticotropic hormone (ACTH). ACTH then travels to the adrenal cortex of the adrenal gland through the blood. Upon reaching the adrenal cortex, ACTH stimulates the synthesis and release of glucocorticoids into the blood. In humans, ACTH causes the release of the glucocorticoid cortisol, which is able to cross the blood-brain barrier and has widespread effects on much of the body and the brain. Levels of cortisol in blood or saliva, or ACTH in saliva, are considered measures of HPA activation and a major biomarker of the stress response (de Kloet et al., 2005; Chrousos and Gold, 1992).

Mechanisms of stress-related telomere shortening

Chronic activation or dysregulation of the physiological stress response is the presumed mechanism by which psychological stress affects chromosomes. Although the exact mechanism by which psychological stress relates to telomere length remains unclear, there is evidence for the involvement of several

interrelated pathways: inflammation, oxidative stress and exposure of tissues to stress hormones (Lin et al., 2012). Psychological stress can increase markers of inflammation (Kiecolt-Glaser et al., 2003) as well as oxidative stress (Epel et al., 2004), and both inflammation and oxidative stress are associated with short telomeres (Aviv, 2004). The molecular mechanisms by which these pathways lead to telomere shortening remain unknown, but it has been observed that inflammation triggers T cell proliferation and enhances the leukocyte turnover rate, one known cause of telomere shortening. The GC-rich telomere repeats have been shown to be particularly sensitive targets of oxidative damage (Kawanishi and Olkawa 2004). Oxidative stress is thought to contribute to telomere attrition by promoting telomere erosion during cellular replication (Aviv, 2004).

Increased exposure of cells to stress hormones has long been suspected as a mechanism of stress-related telomere shortening. Epel et al (2006) found that low leukocyte telomerase activity is associated with high autonomic reactivity to a laboratory stressor and with elevated nocturnal epinephrine. Conversely, short LTL is associated with elevated epinephrine and cortisol levels. In older women, short telomere length is associated with high urinary catecholamine (Parks et al., 2009). Chronic exposure to stress can cause lasting changes in the function of both the SAM and HPA axes, including modifications of the negative feedback loops that regulate cortisol output and help the body achieve homeostasis (McEwen, 2007). These changes can lead to abnormal cortisol levels and increased recovery periods in response to acute stressors (McEwen,

1998). Additionally, psychopathology status and certain individual differences in personality have been linked to differences in both the perception of stressful events and the cortisol response to these events (Oswald et al., 2006). Choi and colleagues (Choi, Fauce, and Effros, 2008) demonstrated that human T lymphocytes exposed to cortisol have significantly reduced telomerase activity. Tomityama and colleagues (2012) demonstrated that individuals with shorter telomeres show heightened cortisol response to a standardized acute psychological stressor compared to individuals with longer telomeres.

In addition to increased hormone exposure, increased activation of the SAM axis leads to increased inflammation and oxidative stress (Padgett and Glaser 2003; Glaser and Kiecolt-Glaser, 2005), both of which have been found to erode telomeres (Willerson and Ridker 2004, and von Zglinicki 2002). In summary, both previous exposure to chronic stress and individual differences in response to stress can influence SAM and HPA activation, suggesting both may be mechanisms for increased circulating levels of stress hormones, inflammation and oxidative stress and therefore potentially for altered telomerase activity or telomere length over time.

Neural correlates of the stress response

Appraisal of stressors and the subsequent stress response are regulated by key structures in the brain. It is therefore worthwhile to explore differences in these regions as they relate to telomere length, as stress-related telomere shortening may ultimately be traced to differences in neural function during stress or at

baseline. While extensive research has been conducted using acute psychological stress paradigms to measure hormone and physiological outputs in response to stress, the brain mechanisms related to psychological stress in humans have only been addressed relatively recently. The brain is a key target for understanding the mechanisms underlying both adaptive and dysregulated stress responses, and therefore stress-related telomere shortening. The hypothalamus, a small structure just above the brainstem, integrates information from both endocrine and sympathetic systems and initiates a cascade of hormone release that trigger the psychological and behavioral response to stress. The appropriate regulation of that response then depends upon dynamic relationships between limbic and prefrontal regions that can serve to up- or down-regulate the stress response as needed.

The brain regions involved in the stress response were first examined in animal models. Common animal models of stress include cohousing of territorial animals, exposure to predator pheromones, cage relocation, confinement to open areas, restraint or exposure to unpleasant stimuli such as shock. Observations of animals under these conditions or with psychopharmacological manipulations or lesions in various areas of the brain, have provided evidence for several key brain structures involved in the stress response. Specifically, the anterior cingulate cortex (ACC), the prefrontal cortex (PFC), the insula, the amygdala and the hippocampus are the regions most commonly implicated in the stress response from animal studies (discussed in more detail below).

It is only relatively recently that advances in neuroimaging technology allowed researchers to investigate the neural correlates of stress in humans directly. Several imaging technologies have been developed. Among the most popular are positron emission tomography (PET) and magnetic resonance imaging (MRI). PET scans utilize changes in a tracer that can label blood flow, glucose utilization, or other metabolic markers identified by a radioactive tracer to areas of cellular activity in the brain (Raichle, 1998). Functional MRI techniques are more sensitive and do not require exogenous tracers, as they rely on magnetization to create endogenous tracers. These include blood oxygenation level dependent (BOLD) contrast and arterial spin labeling (ASL) perfusion contrast methods. The BOLD signal relies on the reaction of oxygenated blood to magnetization and is typically measured by the difference in oxygenated blood flow (which is higher during neural activity) under distinct conditions (Rosen, Buckner and Dale, 1998). ASL utilizes magnetized water in the blood, and the fMRI scanner measures the exchange of this water with that of brain tissue to give a signal of absolute blood flow in the brain (Petcharunpaisan, Ramalho and Castillo, 2010).

In order to use these techniques to study stress, several paradigms have been developed to elicit a stress response in the scanner. One such paradigm is the Montreal Imaging Stress Task (MIST), which is a math-based cognitive stress paradigm developed by Dedovic and colleagues (2005). It is effective because it combines the cognitive challenge of the serial subtraction task with social evaluation and an element of uncontrollability, as the mathematics change and

the tasks are timed. The math problems dynamically adjust in difficulty and speed based on the performance of the participant, such that the task is completed with 50% accuracy for all participants. In the experimental condition, in addition to displaying the math problems, the interface also displays a countdown bar showing the remaining time, as well as a bar providing feedback (sometimes false feedback exaggerating the low performance) about the current participant's performance in relation to the average user (Figure 3). The experimental condition is either contrasted with a rest condition, during which the participant sees the interface but does not have to solve math problems, or the control condition, during which the participant has to solve problems but is told their performance is not evaluated and they do not see the countdown bar or the false feedback bar. In this way, the mental arithmetic is held constant across both conditions, yet the importance of completing the task and the social evaluative aspects such as the false feedback bar are unique to the experimental condition. These block types are intermixed and displayed in three runs. To further introduce social evaluative threat, participants are told between runs of the experiment that they need to do better during the experimental condition of the next run. The cognitive challenge of the math is held constant, but the stress of the social evaluation and time constraints are unique to the experimental condition. Therefore, brain regions with changes in activity during the experimental condition relative to control conditions can be linked to stress. Brain regions associated with stress in the MIST paradigm include the cingulate, the prefrontal cortex, the occipital cortex, the insula and in some cases the

amygdala and anterior cingulate cortex (Pruessner et al., 2008; Dedovic et al., 2009). These are discussed in detail below.

Activity in the ACC is increased during the stressful condition in the vast majority of studies of major acute-stress imaging paradigms. Neuroanatomical evidence suggests that the ACC may play a critical role in the sympathetic stress response (Craig, 2003). Numerous fMRI and PET studies have demonstrated enhanced activation of the ACC in response to increases in cognitive challenge such as attentional demand and/or task difficulty (Gusnard and Raichle, 2001; Paus et al., 1998), which are known to elicit a sympathetic stress response (Callister, Suwarno, and Seals, 1992; Light and Obrist, 1983). ACC activation has been shown to correlate with a variety of sympathetic stress response measures, including increase in blood pressure (Critchley et al., 2000; Gianaros et al., 2005) and changes in heart rate and mean arterial blood pressure (Critchley et al., 2003). Increased activity in the ACC correlates with cortisol output, and it has therefore been suggested that it may play a role in the appraisal process and error monitoring, detecting social evaluative threat and inducing stress perception (Pruessner et al., 2008).

Activation of the PFC was thought to play a purely inhibitory role in regulating the HPA axis in the stress response; however, current research suggests that there are differences in the role of the large PFC depending on the task, when certain PFC sub-regions can be activated and act in very different ways. The prefrontal cortex has been implicated across nearly all stress paradigms studied, although the specific sub-region, and whether or not the area

is activated or deactivated, varies greatly. Ventral and dorsal regions of the PFC have been related to reduced cortisol reactivity, suggesting they may play a role in the regulation of stress (Eisenberger, et al. , 2007; Kern, et al., 2008). In other studies, general increases of activity in the medial PFC were reported, and a meta analysis of PFC activation in response to stress suggests its role in the regulation of cortisol secretion may be stressor-specific and depend on the sub-region activated (Herman et al., 2003 and Herman et al., 2005). In the majority of the studies, a decrease was reported in ventral regions of the medial PFC during stress tasks. The results for the orbital frontal cortex were also consistent, although less widely reported, with decreases in activity in this PFC sub-region observed during serial subtraction, anticipation of public speaking in healthy controls, and the MIST. The role of the PFC in dysregulation of the stress response has been studied in those with stress disorders. The PFC is smaller in those with disorders such as PTSD, and PFC activation has shown an inverse functional relationship with emotional tasks (Shin et al., 2006). In animal studies, the PFC is activated during mild stressors and stimulates dopamine release (Lindvall and Bjorklund, 1984). While the prefrontal cortex as a whole likely plays a variety of important regulatory roles during stress, it is difficult to make clear judgments about the role of this large and diverse region of the brain.

The insula has also consistently been implicated in stress response regulation in different acute stress paradigms; however, whether it is activated or deactivated in response to the stress condition appears to depend on the task. A study of a response-conflict task (word and color matching task; Gianaros, et al.,

2005; Gianaros, et al., 2008) and one MIST study (Dedovic et al., 2009) report increases in insula activation. However, several public-speaking studies (Tillfors, et al., 2001; Wager, et al., 2008) and MIST studies (Dedovic et al., 2009; Pruessner, et al., 2008) report deactivation of the insula during the stress condition. In studies of those with PTSD, the insula seems to be more active than in non-PTSD subjects at rest (Rosso et al., 2014).

Although the hippocampus is clearly important for stress reactivity, its exact role in the stress response has been difficult to discern. Animal studies have found the hippocampus to exert an inhibitory effect on the HPA axis (Herman and Cullinan, 1997). Stimulation of the hippocampus results in a decrease in glucocorticoids (Dunn and Orr, 1984), while lesions in the hippocampus result in an increase in corticosterone or ACTH and a prolonged stress response (Herman et al., 1995; Sapolsky, Krey, and McEwen, 1984). Hippocampectomy studies in rats also suggest an inhibitory role of the hippocampus, as hippocampectomized rats show an exaggerated response to mild stressors such as cage relocation and open-field tests (Herman, Dolgas, and Carlson, 1998; Kant, Meyerhoff, and Jarrard, 1984). There is also evidence that the hippocampus can play in excitatory role in the regulation of stress, particularly dorsal regions (Dunn and Orr, 1984), indicating that the inhibitory effects of the hippocampus may be region-specific. In human imaging studies, increases in hippocampal activity occur during the recall of stressful events (Sinha et al., 2004) and during a version of the MIST (Dedovic et al., 2009). Decreases in hippocampal activity were reported in a serial subtraction paradigm using PET and fMRI (Critchley et

al., 2000), and in PET and fMRI versions of the MIST (Pruessner et al., 2008). In the majority of human neuroimaging studies, increased activation in prefrontal regions, particularly the ACC, has been reported during stressful task conditions (Critchley et al., 2000; Gianaros et al., 2005; Kern et al., 2008; Pruessner et al., 2008; Sinha et al., 2004; Wager et al., 2009; Wang et al., 2005). The ACC has been hypothesized to reflect a general role in the regulation of autonomic arousal (Craig, 2003), and its activation correlates with a variety of autonomic and endocrine responses, including increases in blood pressure (Critchley et al., 2000; Gianaros et al., 2005), changes in heart rate (Critchley et al., 2003), changes in electrodermal activity (Critchley et al., 2002), higher perceived stress (Wang et al., 2007), and increased cortisol output in response to stress (Wang et al., 2007).

Present study

Individual differences in the experience of, perception of, and physiological reactivity to stress relate to telomere length. Differences in the function of stress-related neural circuitry may shed light on stress-related telomere shortening in both chronically stressed populations and among individuals who perceive experiences as particularly stressful. However, to date, the relationship between neural processes associated with stress and telomere length has not yet been investigated. The present study implemented a version of the MIST (in which deception is minimized) to investigate the relationship between neural activation to an acute stressor in key stress-related regions and telomere length. Additionally, arterial spin labeling data were collected with subjects at rest to

evaluate differences in resting brain activation related to differences in telomere length. In both cases, biological measures of stress (cortisol activity) and psychological evaluations (anxiety and traumatic life events) were collected to assess the impact of mediating factors on telomere length.

I found that heightened activation in the ACC, hippocampus and midbrain correlated with shortened peripheral blood mononuclear (PBMC) telomere length. Additionally, resting brain activation was obtained for healthy individuals with relatively long or short telomeres in either PBMCs or whole saliva. I found that those with short telomeres had significantly higher resting activation in the amygdala, PFC and ACC. I believe that this research represents an exciting first step in evaluating the roles that individual differences in brain activity play in stress-related telomere shortening.

Materials and Methods

MIST Experiment:

Participants

Thirty-two healthy right-handed Caucasian males with no history of psychiatric or neurological illness (mean age of 24.12, with age ranging from 18-52) were included in the final analyses for this study. Participants were recruited using flyers posted at Stony Brook University and in the community as well as local newspaper advertisements. Participants were pre-screened for these exclusion criteria: history of psychiatric or neurological illness, substance abuse in the last six months, psychoactive drug use, use of drugs that impact cortisol production (such as anti-depressants, anti-anxiety medication, progesterone, Adderal, beta blockers, etc.), metal in the body, left-handed, non-native English speaker, astigmatism, BMI above 30 or below 18, thyroid disease, or smoking. The Committee on Research Involving Human Subjects at Stony Brook University approved this study. All participants provided informed consent and received payment for their participation.

fMRI procedure

Experimental sessions began at the Social Cognitive and Affective Neuroscience (*SCAN*) Center between the hours of 12 pm and 2 pm to control diurnal cortisol variations. Participants were asked to refrain from eating, drinking, or exercising for at least 1 hour prior to their arrival. Following consent, the experimenter explained the task to the participants and asked them to complete a short training

session to familiarize them with the task. Participants then entered the scan room and completed the task and several structural and resting scans. Following the scan session, the participants completed questionnaires, were debriefed, and then were escorted to the Stony Brook Hospital to complete a blood draw.

fMRI task

The Montreal Imaging Stress Task (MIST) was used to induce stress in the scanner (Dedovic et al., 2005). The MIST consists of series of computer-based arithmetic challenges with customized difficulty levels and time limits to ensure a standardized failure rate, as well as a social evaluative component provided by the experimenter.

The task included rest, control and experimental conditions presented in a block design. During the rest condition, subjects viewed the arithmetic interface, but no arithmetic problems were presented. During the control condition, subjects solved mental arithmetic problems using a difficulty based on subject performance, but because there was no time limit, subject performance was approximately 90 percent. The experimenter encouraged the participants to respond quickly and accurately during the control condition, but emphasized that performance did not count during this condition. Subjects used a response box to navigate to the correct answer, which is always a value between 0 and 10. During the experimental condition, difficulty level was again based on subject performance, and in addition a time limit was imposed that is 10% less than the subject's average response time. This time limit adjusted over the course of the

experiment based on subject performance such that the participant was correct approximately 50% of the time. During the experimental condition a countdown bar and a depiction of the subject's performance in relation to optimal performance were displayed on the screen. Rest, control and experimental blocks were randomly intermixed and then divided into three separate runs. After runs one and two, the experimenter provided negative evaluative feedback to the participant. Participants were informed that their performance was being monitored during the experimental condition in the control room and that their performance was considerably less than what could optimally be achieved. Participants were then told that they needed to improve their performance and try harder so that their data will be usable. After the final run, participants were told that their performance during the last run was satisfactory, and they were thanked for participating.

Saliva sampling

To assess cortisol levels, ten saliva samples were obtained from each participant over the course of the experiment using Salivettes® (Sarstedt; Rommelsdorf, Germany). Three baseline cortisol samples were acquired in the scanner directly before the first MIST run. Saliva samples were then taken after each of the three MIST runs and 10, 20, 30 and 45 minutes following the end of the fMRI session, respectively. Participants were instructed to gently chew on the cotton swab for approximately 1-2 minutes. Following the experiment, salivettes were stored at -20ºC until they were shipped to the analysis facility (Dr. Nicholas Rohleder, Laboratory for Biological Health Psychology, Brandeis University, Boston). Each

sample was assayed in duplicate using a CLIA cortisol chemiluminescence immunoassay (CLIA catalog number RE62019) with a sensitivity of 0.16 ng/ml (IBL International, Toronto, Canada). Inter-assay and intra-assay coefficients of variation (CV; equals 100*Standard Deviation/Sample Mean) were less than 4%.

Blood drawing

Following the scan session, 30 ml of blood was drawn by registered nurses at the Stony Brook University Hospital General Clinical Research Center (GCRC). PBMCs were immediately extracted using Leucosep® tubes (Greiner bio-one, Frickenhausen, Germany) and frozen at −80°C until analysis. DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit.

Telomere analysis

Relative mean telomere length (RTL) in PBMCs was determined using a quantitative polymerase chain reaction (qPCR) method developed by Cawthon (2002). Using this method, the telomeric repeat copy number and single-copy gene (β-globin) number are measured by qPCR. The relative amount of telomeric repeat (T) and β-globin (S) product give an average telomere measurement per cell (the T/S ratio). The relative telomere length is calculated for each sample by dividing the individual T/S ratio by the experimental average.

PCR was done using the BioRad DNA Engine Opticon 2 Real-Time PCR Detector (Bio-Rad Ltd, Hercules, CA, USA) and Sso Fast EvaGreen Supermix (Bio-Rad). Primers used were adapted from Zhang et al. (2009) and are as follows:

telomere forward: 5'-

CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'

telomere reverse: 5'-

GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'

β-globin forward: 5'-GCTTCTGACACAACTGTGTTCACTAGC-3'

β-globin reverse: 5'-CACCAACTTCATCCACGTTCACC-3'

Primers were included at a concentration of 200 nM, and 3 ng/μL of genomic DNA was used. The thermal cycling conditions included an initial 95^{o}C incubation for 5 minutes, followed by 33 cycles of 95^{o}C for 15 sec, 54.3^{o}C for 1 minute and 72^{o}C for 15 sec for telomere qPCR, and 37 cycles of 95^{o}C for 15 sec, 56^{o}C for 30 sec and 72^{o}C for 30 sec for *β-globin* qPCR. Each run included melting curve analysis to verify PCR products. Samples were run in duplicate in 96-well plates. Any duplicates that varied by more than 7% were re-run. Control samples (a sample of pooled DNA from several subjects) were included in each plate, and intra-plate variability was adjusted by using a normalizing factor obtained by dividing the T/S ratio of each control DNA by the average T/S for the same DNA from each run (Farzaneh-Far et al., 2010). Three-fold dilution (from 0.74 to 60 ng) of HeLa cell genomic DNA was used to create a standard curve. Analysis was performed using Opticon Monitor software (Bio-Rad). Telomere length measurements were made in a blinded fashion, with no knowledge of subject identity or associated data.

fMRI data acquisition and analysis

A 3 Tesla Siemens TrioTim whole body scanner (Siemens Medical, Erlangen, Germany) with a 12 channel head coil was used to acquire 468 T2*-weighted whole-brain volumes with an EPI sequence for analysis of BOLD signal. The following parameters were used: TR = 2500 ms, TE = 30 ms, flip angle = 90°, matrix dimensions = 64 x 64, FOV = 256, 256 mm, slices = 34, aligned to the AC-PC, slice thickness = 4mm, slice acquisition = interleaved, gap = 0.

Standard preprocessing procedures were performed in Statistical Parametric Mapping software (SPM8), starting with slice time correction for acquisition order, followed by realignment for motion correction, normalization to standard Montreal Neurological Institute space, and spatial smoothing using a Gaussian kernel with 8 mm full width at half maximum (FWHM). First-level single subject SPMs were created from a model that specified the onset of each condition. Motion for all participants was below 3 mm. Scan to scan motion over 0.5 mm in any participant was detected and repaired using the interpolation method in ArtRepair. No more than 10% of scans for any participant were interpolated. Following motion correction, preprocessing procedures were repeated on corrected data, and first-level analyses were de-weighted.

At the second level, random effects analyses were conducted to test for statistical differences between the control and experimental conditions using contrasts created for each individual at the first level. To assess activation of the anterior cingulate cortex during the experimental-greater-than-control contrast, region of interest (ROI) analyses were performed using the AAL atlas (Tzourio-

Mazoyer et al., 2002) and WFU Pickatlas (Maldjian et al., 2003). A height threshold set to FWE $p<0.05$ and an extent threshold of 10 voxels was used to correct for multiple comparisons for the whole brain and ROI analysis for the experimental greater than control contrast. Only peak activations that are significant at $p<0.05$ after FWE correction are reported.

To assess correlations between telomere length and brain activation, normalized telomere length values for each subject were entered as a covariate using a one-sample t-test for the experimental- greater- than- control contrast using a height threshold set to $p<0.001$, uncorrected, with an extent threshold of 10 voxels. Pearson's correlations were conducted using SPSS 18 (2010, Stony Brook University) to assess potential relationships with age.

Resting Activity Experiment:

Participants

Fifty-three healthy male adults with a mean age of 23.3 (with an age range from 18-52) participated in this study. All participants were right-handed native English speakers without a prior history of neurological or psychological illness. The study was approved by The Committee on Research Involving Human Subjects at Stony Brook University. Participants provided informed consent and received payment for their participation.

DNA collection

Telomere length was measured in either blood (33subjects) or saliva (20 subjects). Following the informed consent procedure, 10 mL of blood was collected and immediately processed to extract PBMCs according to the Leucosep® instruction manual. Cell pellets were frozen and stored at -80^{o}C. For saliva collection, Oragene® Discover saliva collection kits were used. Subjects were instructed to avoid eating or drinking 30 minutes before collection. Saliva samples were stored at -20^{o}C prior to processing. DNA was extracted from PBMCs using the Qiagen DNEasy kit. DNA was extracted from saliva using the Oragene DNA Genotek kit.

Telomere length measurement

Relative mean telomere length was measured as described for the MIST study.

Arterial Spin Labeling data acquisition

Perfusion images were collected using a 3 Tesla Siemens Trio Trim whole body scanner (Siemens Medical, Erlangen, Germany) with a 12-channel head coil. Resting-state arterial spin labeling data was collected with a pulsed arterial spin labeling (PASL) sequence using the Q2TIPS method – a quantitative imaging of perfusion using a single subtraction (QUIPSS) II method with a thin-slice TI1 period saturation. A proximal inversion with control for off-resonance effects (PICORE) Q2T tagging scheme was used. A total of 91 interleaved label and control volumes were acquired during a 3-minute 57-second scan (12 slices, TR = 2500 ms, TE = 11 ms, inversion time 1 = 700 ms, inversion time 2 = 1800 ms,

saturation stop time = 1600 ms, FOV = 256 mm, flip angle = 90 degrees, voxel size = 4x4x8 mm, slice thickness = 8 mm). The first acquired image was used as the M_0 image. Siemens prospective acquisition correction (PACE) was used during the PASL scan to generate motion-corrected, perfusion-weighted and relCBF maps. Resting-state perfusion data was collected following structural and functional scans related to a passive picture-viewing task. Prior to the start of the resting-state scan, participants were asked to close their eyes for the duration of the scan.

Arterial Spin Labeling image processing

Cerebral blood flow (CBF) maps were created using an in-house script developed using Interactive Data Language (IDL; Exelis Visual Information Solutions, Inc.). PASL motion corrected maps were first normalized using the EPI template in SPM 8 (http://www. fil. ion. ucl. ac. uk/spm/) to generate images with a 91 x 109 x 91 resolution in MNI space. Images were then smoothed using a 4 mm FWHM Gaussian kernel. Following control-tag calculations, perfusion maps were converted to quantitative CBF value (qCBF) maps in physiological units (ml/ 100 g/min) using the General Kinetic Model (Buxton, et al., 1998) for PASL. Arterial blood magnetization was computed based on local tissue magnetization, using typical T_2 and water partition coefficients as described in prior literature (Cavusoglu et al., 2009; Wong, Buxton and Frank, 1998).

Region of interest analysis

ROIs for the amygdala, median prefrontal cortex, anterior cingulate cortex, hippocampus and visual regions were defined on anatomical grounds using the AAL atlas in WFUPickatlas (Maldjian, 2003). Eigenvariates for these ROIs were then extracted using SPM 8. The relationship between activity in these regions and behavioral measures was examined using IBM SPSS Statistics 19.

Arterial Spin Labeling statistical analysis

As RTL was measured from either blood (PBMC) or whole saliva samples, the Z-score from the T/S ratio from each subject (either from saliva or PBMC extraction) was obtained, and these scores were used to calculate relatively long or short telomere lengths. Based on relative telomere length, subjects were split into two groups (Long RTL and Short RTL). A t-test was performed to determine significant differences in activation in each region of interest between Long RTL and Short RTL groups.

Psychological assessments

For both studies, subjects were evaluated for anxiety, childhood trauma and stressful life events, which are known to influence telomere length. Anxiety was assessed using the Neuroticism Extraversion and Openness Personality Inventory (NEO-short form; Costa and McCrae 1992). Subjects were asked to complete the Childhood Trauma Questionnaire (CTQ; Bernstein and Fink 1998) and were given a structured life stress interview to assess overall life events (Appendix A).

Results

MIST Study:

In this study we assessed correlations between brain activity during the MIST stress task and telomere length in a healthy population.

Telomere length and age

The average age of participants was 24.1, and participant age ranged from 18-45. Telomere length was not significantly correlated with age (r = -0.24, p = 0.20); therefore, age was not used as a covariate in any subsequent analyses between telomere length and brain activation.

Cortisol response to the stress task

We compared peak cortisol and cortisol at each time point among participants and found no overall significant group differences in cortisol response to the task using a repeated measures ANOVA (F (7, 217) = 1.71, p = 0.11). Consistent with previous studies using the MIST (Pruessner et al., 2008), 41% of our participants demonstrated a significant inra-subject increase in cortisol response following the task. Thus, we split participants into two groups: those who showed a cortisol response to the task (n =13), and those who did not (n = 19). We then conducted a two-factor (group x time) ANOVA, which revealed a significant group x time interaction (F (2.04, 72.22) = 15.49, p > 0.001), Greenhouse-Geisser corrected for sphericity; the cortisol response in 'responders' significantly differed over time from the 'non-responder' group. A follow-up t-test comparing the cortisol sample

taken immediately prior to the stress task (Baseline) and 10 minutes following the stress task (Peak) revealed that responders only showed a significant increase in cortisol in response to the stress task ($t(12) = 4.59$, $p = .001$), not the control condition.

Neural activation during the stress task

Whole-brain analyses of the experimental-greater-than-control condition (that is, a comparison of those brain regions that showed increased activity during the experimental condition relative to the control condition) revealed significantly greater peak activation in the occipital and medial prefrontal regions and parietal regions. A region-of-interest analysis revealed significant activation of the anterior cingulate. These activations are presented in Figure 4. All peak values exceeded a threshold of $p< 0.05$, -corrected. In a comparison of experimental-less-than-control condition, no deactivations were observed in any brain region at these thresholds.

Telomere length and stress-related neural activation

Whole-brain analyses of the experimental-greater-than-control condition using telomere length as a covariate revealed that telomere length was negatively correlated with activation in the anterior cingulate cortex, the hippocampus, and the midbrain. No regions of the brain were positively correlated with telomere length. Again, all peak values exceeded a threshold of 0.05 FWE corrected. These results are presented in Figure 5.

Neural activation during the stress task and cortisol

During the experimental- greater-than-control contrast, no regions of the brain significantly correlated with cortisol response to the task (Peak – Baseline) after correcting for multiple comparisons using family-wise error correction in the whole group or responders.

Telomere length and cortisol

There were no significant relationships between cortisol increase in response to the task (Peak – Baseline) and telomere length in the entire sample (t = 0.176, p = 0.334, n = 32) or among responders only (p = 0.680, n = 13).

Psychological factors

No correlation between telomere length and number of stressful life events as measured by the CTQ or structured interview or with anxiety score was observed.

Arterial Spin Labeling Experiments

In these experiments, resting brain activation was examined to observe differences in ASL activation in those with short or long relative telomere length.

Telomere length and age

The groups did not differ in age (and age did not significantly correlate with telomere length) in these studies.

Resting state activation and telomere length

Activity in three stress-related brain regions was significantly higher in those subjects with short relative telomere length (Figure 6); the amygdala, medial prefrontal cortex, and anterior cingulate cortex. Specifically, I found the peak activity in an 8 mm sphere in the left amygdala to have an average activity of 30.1 mL/100g /min for long RTL and 35.3 mL/100g /min for short RTL ($p= 0.038$), an 8mm sphere in the right medial prefrontal cortex (BA9) region to have an average activity of 41.5 mL/100g /min for long RTL and 47.5 mL/100g /min for short RTL ($p= 0.033$) and an 8mm sphere in the anterior cingulate cortex to have an average activity of 39.1 mL/100g /min for long RTL and 45.1 mL/100g/min for short RTL ($p= 0.028$). There was no significant difference in activity in the hippocampus or visual cortex regions (used as controls) between those with short and long RTL.

Psychological factors

There was no significant difference between long and short RTL groups in terms of anxiety score, or the number or severity of stressful events as measured by the CTQ or the structured life stress interview.

Discussion

Previous studies have demonstrated that shorter telomere length is associated with chronic stress, particularly stressful life events (Epel et al., 2004; Hou et al 2012; Damjanovic et al., 2007; Parks et al., 2011; Sheilds et al., 2011) and increased reactivity to acute stressors (Epel et al., 2010; Tomiyama et al., 2012; Kroenke et al., 2011). While psychological and physiological measures of stress are associated with stress-related telomere shortening, structures in the brain ultimately regulate these outputs. Here I present evidence that differences in activity in these structures in response to acute stress and at rest may play a role in stress-related telomere shortening.

First, we investigated the relationship between brain activation during a validated acute stressor and telomere length. Shorter telomere length was associated with increased activation in key regions associated with response to stress: the anterior cingulate cortex and the hippocampus.

Given the potential roles of SAM and HPA axes in stress-related telomere shortening, differences in brain activation in regions regulating these axes correlating with telomere shortening are not surprising. The SAM and HPA axes, and consequently hormone output, are regulated by key structures in the brain, including the hippocampus, the amygdala, the medial prefrontal cortex, and the anterior cingulate (Herman et al., 2005).These regions of the brain have higher densities of glucocorticoid receptors than other regions (Gold, Drevets, and Charney, 2002) and are able to influence HPA axis activity through direct or

indirect feedback on the hypothalamus. In the majority of human neuroimaging studies, increased activation in prefrontal regions, particularly the ACC, has been reported during stressful task conditions (Critchleys et al., 2000; Gianaros et al., 2005; Kern et al., 2008; Pruessner et al., 2008; Sinha et al., 2004; Wager et al., 2009; Wang et al., 2005). The ACC has been hypothesized to reflect a general role in the regulation of autonomic arousal (Craig, 2003), and its activation has been shown to correlate with a variety of autonomic and endocrine responses including increases in blood pressure (Critchley et al., 2000; Gianaros et al., 2005), changes in heart rate (Critchley et al., 2003), changes in electrodermal activity (Critchley et al., 2002), higher perceived stress (Wang et al., 2007), and increased cortisol output (Wang et al., 2007). Evidence for the role of the ACC in autonomic regulation comes from studies specifically attempting to disentangle autonomic arousal from cognitive tasks. Critchley and colleagues (2000) demonstrated that dorsal ACC activation is related to effort expended in both cognitive (difficult n-back task, where subjects must discern whether a stimulus matches one from n steps earlier) and physical (isometric hand grip) challenges. Furthermore, activity in the dorsal ACC is correlated with mean arterial blood pressure in these studies.

Additional support for the involvement of the ACC in the generation and regulation of autonomic arousal comes from studies of patients who are unable to generate autonomic arousal due to denervation of the autonomic nervous system (Mathias, 2000). When engaging in mental arithmetic tasks or isometric hand exercises, these patients do not show increases in heart rate or blood

pressure; however, they show hyper-activation of the dorsal ACC (Critchley et al., 2005) suggesting that in the absence of negative feedback, systems involved in the generation and regulation of autonomic arousal may work even harder. These findings appear to be in agreement with animal studies suggesting that the infralimbic region of the rat brain, which may correspond to the ACC in primates (Preuss,1995), plays a role in this regulation of the stress response, as lesions of this region result in blunted corticosterone secretion. In summary, animal models, human lesion studies, and human imaging studies all support the notion of a role of the ACC in regulating autonomic arousal generated by stressful cognitive and social evaluative situations.

Although the hippocampus has been shown to have an inhibitory effect on the HPA axis (Herman and Cullinan, 1997), the hippocampus could contribute to increases in the stress response. This function has been shown in studies where the memory of previous stressful experiences is being explicitly manipulated (Sinha, et al., 2004) or in anticipatory situations during which participants may be recalling previous anxiety-inducing situations (Wager et al., 2009). In a study of social phobia, the hippocampus was found to have increased activity when subjects were told to prepare a speech (Lorberbaum et al., 2004). It has been suggested that hippocampal activity increases immediately after an acute stressor, as it is required in combination with the amygdala for the formation of memories associated with strong emotions, such as fear and stress (Gray and Rawlins, 1986).

My findings from the MIST study lend support to the idea that individual

differences in stress reactivity relate to differences in telomere length, even among healthy populations. The regions of activation that associate with telomere shortening are not surprising. These findings are consistent with a model of stress-related telomere shortening in which accumulation of damage to cells either by frequent or heightened response to acute stressors induces telomere damage.

Although exposure of cells to cortisol has been proposed as a mechanism of stress-related telomere shortening, we found no correlation between telomere length and cortisol in this study. The only in vivo study that has demonstrated such a relationship subjected women to a social stress task (TSST; Epel et al 2010). This study focused on fourteen women who were caring for someone with dementia and nine healthy controls. Our participants are healthy young adult males with minimal life stress. Furthermore, the MIST elicits a much more subtle cortisol response in only 40% of participants (Dedovic, 2005), compared to 70-80% in the TSST (Kudlelka, Hellhammer and Kirschbaum, 2007). Therefore, our ability to investigate differences in telomere length or ACC and hippocampal activation in relation to cortisol reactivity was limited. Additionally, studies suggest that 5-10% of subjects with no previous exposure to MRI scanners will have a cortisol response to being in the scanner that is large enough to interfere with results (Melendez and McCrank, 1993).

By collecting brain activation data at rest, we also discovered that subjects with short telomeres had heightened activity in the amygdala, ACC and PFC when they were unconstrained by any specific task demands. These brain

regions are all associated with the stress response. As stated previously, increased ACC activity is thought to influence both SAM and HPA activation in response to stress. The amygdala has long been known to be involved in stress appraisal. An abundance of evidence suggests that the amygdala is a key region mediating the biological response to stress. Activation of the amygdala initiates changes that resemble the stress response (Gray, 1993). Damage to the amygdala will usually alter one or more of the neuroendocrine, autonomic or behavioral measures of the stress response (Shekhar et al., 2005).

Within the brain, the dopamine, norepinephrine, and serotonin biogenic amine systems that innervate the cortex, and in particular the prefrontal cortex, also are activated in response to threatening stimuli (Bliss et al.1968; Thierry et al.,1976; Weiss et al.,1981; Deutch and Roth,1990; Tanaka et al., 1990; Inoue et al.,1993). Metabolic activation of the dopamine system in the rat medial PFC is one of the most intensively studied central neurochemical correlates of the stress response (Deutch and Roth, 1990). Exposure to mild stress preferentially activates this PFC (Lindvall and Bjorklund,1984). Several animal studies have demonstrated evidence of amygdala control of stress-induced metabolic activation of the dopamine and adrenergic stress response systems in the PFC, as well as a role for the amygdala in integration of behavioral and neuroendocrine components of the stress response (Goldstein et al.1996). Activation of these regions at rest may reflect a chronic activation of a mild stress response.

These results may also reflect increased rumination associated with short

telomeres. The PFC and ACC (Denson et al., 2009 and Johnson et al., 2009 and amygdala (Canli et al., 2006, Ray et al., 2005 and Siegle et al., 2002) are brain regions that have been suggested to be associated with excessive self-referential thinking and rumination. In any case, my dissertation data suggest that differences in baseline activation in key regions of stress-response regulation may influence stress-related telomere shortening.

There were several limitations to these dissertation studies. First, both studies recruited healthy, mainly young, Caucasian male subjects from Stony Brook University. This, coupled with our exclusion criteria (which were convenient for several other studies using these subjects), provided little variation in psychological measures of stress such as anxiety and stressful life events. With a larger variation in these qualities, we may be able to assess the relative contributions of stressful life events, maladaptive personality characteristics and brain activation on telomere length. Other populations (females, older people, those with mental illness, the chronically stressed, etc.) may have exaggerated or different neural responses to stress or baseline activation at rest. The functional brain activity results only demonstrate a correlational relationship, which of course poses limitations in determining causal effects. Nonetheless, these results associate shortened telomeres with heightened activation in brain regions involved in emotion regulation and stress. They are therefore consistent with a model of stress-related telomere shortening in which accumulation of cell damage either by chronic activation of stress response or frequent or heightened response to acute stressors induces telomere damage.

Future Directions

Several future studies could expand upon the work presented in this dissertation and address some of its limitations. First, these studies were correlational in nature and may not reflect a causal relationship between these brain activations and stress-related telomere shortening. Replicating these studies with a larger number of subjects would be an excellent first step in strengthening these results.

Females, the elderly, those with mental illness, the chronically stressed, or other populations not may have exaggerated or different neural responses to stress or baseline activation at rest, so studies should be performed including such groups. Both telomere length and stress reactivity differ between males and females. Telomeres tend to be longer in females and may be regulated differently due to hormones (Gardner et al 2014). Sex is one of the largest individual differences impacting cortisol response, with men demonstrating 200-400% increases in free cortisol in response to a stressor, compared to women only showing a 50-100% increase, despite similar baseline levels (Kirschbaum, et al, 1999; Kudielka, Hellhammer, and Kirschbaum, 2007; Wolf et al., 2001). These sex-related differences in cortisol response to an acute stressor depend on the hormone levels; women in the luteal phase of their menstrual cycle show increases in cortisol comparable to those of men, while women in the follicular phase and women using oral contraceptives demonstrate reduced cortisol responses (Kirschbaum, et al.,1999). Although male-only studies are a problem of biological and psychological studies in general, the few studies that do

compare male and female neural activation to a stressor demonstrate differences. For example, Buchanan and colleagues (2010) demonstrated that damage to the PFC could have sex-specific consequences for stress reactivity. Both male and female patients with lesions to the PFC had increases in self-reported stress. Women with PFC damage showed an increase in cortisol response to the TSST, while PFC volume was correlated with a decreased cortisol response in men.

The stress response is affected by age, as older adults have higher baseline cortisol levels and heart rate but the response to acute stressors is lower in these populations (Kudielka et al., 2004). There are also significant differences in stress response as a function of psychological health (Takahashi et al., 2005), social position (Hellhammer et al.,1997), and social support (Heinrichs et al., 2003).

Different stress paradigms may elicit distinct stress responses and may elucidate varied relationships with telomere length. Eliciting activation in both the HPA axis and the SAM system in psychological stress paradigms requires the use of particular types of stressors as certain qualities of stressful events trigger activation of these systems more than others (Dickerson and Kemeny, 2004). Furthermore, what may trigger activation of one system might not trigger activation of the other. For example, SAM activity, while elevated during stress, is also elevated in response to a variety of cognitive challenges that may not be considered subjectively stressful and that may not elicit increases in HPA axis activity. This is consistent with the idea that the SAM system is increased in

instances in which an individual is confronted with a challenge he or she believes can be mastered with effort (Frankenhaeuser et al., 1980). HPA axis activity, on the contrary, tends to be greatest in subjectively stressful situations that involve uncontrollability, social evaluative threat, or novelty (Dickerson and Kemeny, 2004; Mason,1968).

Future studies could also work to further elucidate the molecular mechanisms of stress-related telomere shortening. In addition to associating stress reactivity with telomere length, we could measure the expression of telomere-related genes, such as telomerase. Telomerase activity could also be measured, along with cortisol, catecholamines and markers of oxidative stress. A more complete picture of the correlations among aspects of the stress response and telomere length would strengthen the evidence for mechanisms of stress-related telomere shortening that could be studied in vitro and with longitudinal studies.

Finally, a study of genetic variation that may moderate stress-related telomere shortening would be very interesting for future research. Several stress-related genes have variants that moderate the stress response in human stress paradigms, such as glucocorticoid receptor variants (Wust et al., 2004, van Rossum et al., 2003), opioid receptor genes (Chong et al., 2006), and brain-derived neurotrophic factor (Schule et al., 2006), among others (Ising and Holsboer, 2006). Interestingly, it has been shown that variants of the transcriptional control region of the 5-HT transporter gene (5-HTT-linked polymorphic region, 5-HTTLPR), which codes for the serotonin transporter, have

different effects on risk for depression (Kendler et al., 1995), amygdala reactivity to negative stimuli (Hariri et al., 2002), and functional connectivity between the amygdala and PFC (Heinz et al., 2005); specifically, the short variant increases stress reactivity. Furthermore, it has been demonstrated that life stress modulates these effects in gene-environment interactions, such that long-variant carriers with the greatest number of life-stress events exhibit higher increases in amygdala and hippocampal activation at rest than short-variant carriers with the fewest life-stress events (Canli et al., 2006). These interactions would be particularly interesting to study in regards to effects on stress-related telomere shortening, given the association between both stress response and life stress on accelerating telomere shortening.

Chapter 2: Saliva as a Biomaterial for Telomere Length Measurement

Introduction

At birth, telomere length is similar across somatic tissues in humans (Youngren et al., 1998, Okuda et al., 2002), most likely due to the developmental activity of telomerase, the enzyme that lengthens telomeres (Blackburn 2005). Telomerase activity is greatly reduced in early life in almost all somatic tissues (Yui et al 1998), and telomere length shortens with each cell division throughout the lifespan. As stated before, many environmental factors are thought to accelerate the rate of telomere shortening, and telomere length has become a popular measure of cellular health. Telomere length is typically measured in whole blood, buffy coat or isolated leukocytes (Mather et al., 2011). But as various cell types have different replicative histories, it is quite possible that their rate of shortening differs from blood cells and may be influenced by different environmental conditions. While blood is a useful biomaterial for telomere length measurement, it requires a phlebotomist to obtain. It has also been problematic for psychological studies because blood draws can be a source of stress. One study of fear of needles found that 17.3-25.6% of young adults surveyed had a fear of needles (Nir et al., 2003). Other biomaterials may be better suited and more easily collected for studies, if the rate of shortening is the same as blood telomere shortening. The current study aimed to address the feasibility of

measuring telomere length in saliva, a biological sample that can be collected in a less invasive manner than blood.

Telomere length in somatic tissues

Telomere length appears to vary across somatic tissues in adults (Gardner et al., 2006). Studies from mice and cultured human cells suggest that some of the shortest telomere lengths are found in kidney and brain cells, while the longest are in gonadal tissue (Prowse and Greider,1995; Aviv and Aviv, 1997). This variability was also reflected in cloned animals; when cattle are cloned from different somatic cell types from the same donor, they have different telomere lengths (Miyashita et al., 2002).

The rate of telomere shortening among different intra-individual tissues appears to be similar, however. Several studies demonstrate a correlation in telomere length among different tissues within the same individual. For example, studies found telomere length in PBMCs within individuals is correlated with adipose tissue, muscle (Daniali et al., 2013), synovium and skin telomere length (Friedrich et al., 2000). The relationship of chronological age to relative telomere length was also consistent for various tissues in these studies.

As stated, blood is the most common biomaterial for psychobiological study of telomere shortening. Telomere length in various types of blood cells (T and B lymphocytes, for example) also differs, but the rate of shortening remains consistent (Lin et al., 2009). A handful of studies have used saliva as a biomaterial to measure telomere length, such as when blood use was

inappropriate due to leukemia (Rollison et al., 2011) or they utilized an uncommon, unvalidated method for telomere length measurement (Lanhert, 2005). To our knowledge, however, no study has directly compared relative telomere length in PBMCs and saliva samples in healthy subjects.

Saliva

Saliva has been used as a biomaterial for a number of genetic investigations. These are almost exclusively genotyping or other sequencing or PCR-related studies, for example, polymorphism studies (French et al., 2002) and commercial genetic sequencing such as 23andMe (Williams-Jones, 2003). DNA yields from whole saliva are significantly (up to 70%) lower than yields from other cell types, such as buccal cell swabs and blood samples (van Schie and Wilson, 1997; Terasaki et al., 1998; Walsh et al., 1992). However, the quality of DNA collected from whole saliva has been shown to be comparable to that obtained by other methods if contamination from food or other particulate is carefully avoided (Rogers et al., 2007).

Saliva is typically a clear secretion from major and minor salivary glands and from gingival crevicular fluid, composed of 99% water. The other 1% of saliva is a mixture of various cell types. These include acinar (excretory) cells from the parotid gland, the minor salivary glands and sublingual and submandibular glands from which salivary secretions originate. Additionally, cells from the ducts of these glands, buccal cells from the mouth epithelia and leukocytes from mucosal secretions are present. Saliva can also include varying

amounts of blood from gums, food debris and mouth bacteria. The amount of saliva secreted and the relative contribution of various cell types to saliva can be variable among individuals and can be influenced by gender, age, health and environmental conditions (Humphrey and Williamson, 2001, del Vigna de Almeida et al., 2008).

Present study

The goal of the present study was to determine whether salivary telomere length can be used as a proxy for blood telomere length as a biomarker of disease and aging. Saliva can be collected through noninvasive measures and requires minimal training to obtain. I aimed to determine whether the rate of telomere attrition in blood and saliva are similar, that is, if intra-individual blood and salivary telomere length are correlated. Blood and saliva were collected from 30 healthy participants, and relative telomere length was determined by qPCR. We have determined that relative telomere length in saliva is correlated with telomere length in PBMCs, indicating that saliva is an appropriate proxy for blood to measure telomere length.

Methods

Blood and saliva collection

Blood and saliva samples were collected from 30 healthy, non-smoking subjects. Following the informed consent procedure, 10 mL of blood was collected and immediately processed to extract PBMCs according to the Leucosep® Instruction Manual. Cell pellets were frozen and stored at -80^{o}C.

Saliva was collected using Oragene® Discover saliva collection kits. Subjects were instructed to avoid eating or drinking 30 minutes before collection. Saliva samples were stored at -20^{o}C prior to processing.

DNA extraction

DNA was extracted from PBMCs using the Qiagen DNeasy kit. DNA was extracted from saliva using the Oragene DNA Genotek kit.

Telomere length measurement

Telomere length measurement was performed as in Chapter 1.

Analysis

PBMC and salivary relative telomere lengths were compared using Pearson correlation and Spearman's Rank-Sum Test. SPSS software (IBM) was used for these statistics.

Results

Demographics

Subjects ranged in age from 18 to 49. The range of relative telomere length in blood was 0.31 to 2.51, and in saliva was 0.08 to 2.9. The composition of subjects was 8 females and 22 males. Subjects expressed some discomfort with saliva collection, but reported preferring it to the blood draw.

Telomere length in blood and saliva

There was a strong positive correlation between salivary and PBMC telomere length in individuals. Those with shorter relative PBMC telomere length also had shorter salivary telomere length (Figure 7). The Pearson's correlation was 0.6463 with a p-value of 0.000115.

Discussion

These results demonstrate that saliva can be used as a proxy for blood to measure telomere length, as the relative telomere length in saliva correlates highly significantly with the RTL in PBMCs. Short leukocyte telomere length has been associated with health problems and environmental and psychological factors (Epel et al., 2004; Hou et al 2012; Damjanovic et al., 2007; Parks et al., 2011; Sheilds et al., 2011; Epel et al., 2010; Tomiyama et al., 2012; Kroenke et al., 2011). Blood collection for isolated PBMCs requires venipuncture. This procedure is invasive and potentially stressful and requires a trained phlebotomist, making it expensive and personnel-limited. Saliva is an excellent alternative to blood as a biomaterial for studying telomere length, as saliva collection requires little training and is non-invasive.

There are, however, some precautions that must be considered when using saliva as a biomaterial for measuring telomere length. First, saliva is at risk for far more contaminants than is blood, mainly from food, blood from oral lesions, and oral bacteria. Contamination from oral bacteria should be irrelevant for telomere study, as bacteria lack telomere sequences (similar sequences may exist, but certainly at low levels). PCR measurement of relative telomere length requires a single-copy gene, namely beta-globin, and although some bacteria do possess a beta-globin homolog, its sequence differs extensively from human beta-globin (Hardison, 1998). Exact sequences would depend on bacteria type, which was not evaluated in this study.

Contamination from food debris was limited because subjects were asked to avoid eating or drinking for 30 minutes prior to the study, although compliance with this directive was not evaluated. Blood from oral lesions may be difficult to test for but may be an important source of contamination, as absolute telomere length in blood and saliva may differ. Therefore, significant amounts of blood contamination may skew relative telomere length measurements. Any visual contamination from blood or food should disqualify a sample from study, and it may be useful to utilize blood-specific antigen markers if contamination is suspected.

This study was limited to healthy, mainly young, male subjects who were not under stress. Different subpopulations may have different relative amounts of cell types, as well as different flow rates of saliva. As 2 mL of saliva needs to be obtained per subject, critically slow flow rates may be a problem for sample collection. Flow rate and saliva composition can be affected by age, stress, infection, and hormone levels (del Vigna de Almieda et al., 2008), so it may be useful to assure that results hold true for different sub-populations. In order to use this method for telomere length measurement, the screening and collection method must be well standardized.

Given the relatively low yield of DNA from saliva, the method by which telomere length can be measured in this biomaterial is limited to protocols requiring small amounts of DNA. Several methods of telomere length measurement are currently used. Three of the most popular methods of telomere length measurement are Southern blotting using probes to telomere restriction

fragments, flow-FISH, which uses telomere-specific probes and chemiluminescence measures in flow cytometry, and qPCR as described previously (Aviv et al 2011). Given the low DNA yield from saliva, my study was limited to the Cawthon qPCR method (2002), which measures average relative telomere length using very little DNA.

Future Directions

. The results of this study need to be replicated with another cohort, to assure that these correlations are upheld in different subpopulations. Salivary composition in women is influenced by hormone level (Humphrey et al., 2001), and while women were included in this study they represent only 26% of the study sample. The elderly and those with diseases will also have different complications that affect salivary composition and flow rate (Humphrey et al., 2001). A survey of telomere length in saliva and blood in a variety of subpopulations, which may have different compositions and flow rates, would help determine whether the correlation between salivary and PBMC telomere length holds in all populations. Furthermore, we should replicate this under conditions of stress, in case the amount of saliva or the relative composition of cells differs significantly.

Several subjects complained of discomfort during saliva collection. The protocol requires subjects to fill a vial with saliva, keeping their mouth open and their head down for several minutes. A study comparing the amount of stress caused by venipuncture versus saliva collection would be helpful in assessing potential influences of stress on these studies.

Lastly, other biomaterials should be evaluated as potential proxies for telomere length measurement. Saliva was chosen as a convenient and inexpensive proxy for blood draws. Buccal cells would be equally noninvasive

and are a homogenous cell source, but to date they have not been evaluated for use in telomere length measurement.

Chapter 3: The Role of miRNA in Stress-Related Telomere Shortening

Introduction

Mechanisms of stress-related telomere shortening

Despite copious investigations associating psychological stress with short telomeres, studies of the mechanism of stress-related telomere shortening are less numerous. Those studies that do exist propose several non-mutually exclusive mechanisms to explain how psychological stress may cause shortened leukocyte telomeres. Inflammation, oxidative stress, and an endocrine reaction in response to psychological stress all contribute to shortened telomeres. Psychological stress is known to trigger an inflammatory response (Danese et al., 2007, Pace et al., 2006). Older adults who have experienced childhood adversity show both higher amounts of inflammatory markers, such as interleukin-6 and C-reactive protein, and shorter telomere length in blood cells (Kiecolt-Glaser et al., 2011). Inflammation is also associated with increased proliferation of immune cells and, as a consequence, with greater telomere shortening through cell proliferation (Goronzy et al., 2006). Oxidative stress has been associated with increased perceived stress and shorter telomere length in peripheral blood mononuclear cells (Epel et al., 2004). Telomeres are sensitive to damage by oxidative stress, as demonstrated by experiments showing increased erosion under conditions of high levels of reactive oxygen species (ROS) in vitro (von Zglinicki, 2002).

The endocrine system is another plausible route for mediating the effects of psychological stress and physiological response. The correlation between high levels of circulating cortisol (a primary stress hormone) and increased oxidative stress and telomere length has been established (Behl et al., 1997). Exposure to cortisol reduces telomerase activity and expression in human T lymphocytes in vitro (Choi *et al* 2008). Similarly, higher levels of circulating cortisol in response to a laboratory stressor have been associated with shorter telomere length in buccal cells of 5 to 6 year-old children (Kroenke et al., 2011). Overall, it appears that stress-induced secretion of cortisol may down-regulate the activity and expression of telomerase, leading to more rapid erosion of telomeres in leukocytes.

If exposure to stress hormones influences telomere shortening, it is possible that this occurs via the differential regulation of microRNAs (miRNAs), which are known to respond acutely to a broad range of cellular stressors (Leung and Sharp, 2010; Mendell and Olsen, 2012), including cortisol exposure (de Kloet et al., 2009) and psychological stressors (Morita et al., 2003; Gidron et al., 2010; Meerson, 2010; discussed below). To date, little is known about miRNA involvement in telomeric homeostasis. Epigenetic modifications play a key role in telomere length (Garcia-Cao et al., 2003; Gonzalo et al., 2005), *hTERT* (the enzymatic component of telomerase) regulation (Gigek et al., 2009); however, little is known about the role of miRNA-mediated regulation of telomeric genes. In recent experiments, my colleague Magdalena Jurkiewicz determined that a set of miRNAs change expression in PBMCs in those individuals who demonstrate a

cortisol response to a social stress task, the TSST (Jurkiewicz et al., submitted). Here we explore the possibility that these miRNAs are involved in stress-related telomere shortening by regulating telomere-related gene expression in response to stress.

MicroRNAs

MicroRNAs are short (20-23 nucleotides), single-stranded, endogenous RNAs that regulate post-transcriptional gene expression through translational repression or transcript degradation (Ma et al., 2011). miRNA genes are located in intronic regions of both protein-coding and -noncoding genes and in intergenic and exonic regions of the genome. They are transcribed by RNA polymerase II into long primary miRNA transcripts that can range from several hundred nucleotides to kilobases in length and contain single miRNAs or miRNA clusters. Primary miRNAs are subsequently cleaved in the nucleus into hairpin precursor miRNAs by a ribonuclease (RNase) III double-stranded RNA-specific endonuclease called Drosha. They are in turn exported into the cytoplasm to be further cleaved by the 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. The miRNA then serves as a guide to areas of complementarity found on mRNA transcripts and mediates gene silencing through translational repression and/or mRNA degradation (Figure 8; Caroll et al., 2013).

MicroRNAs with increased expression in PBMCs after an acute stressor provide an extremely promising avenue of investigation into a potential molecular mechanism linking psychological stress to short PBMC telomeres. Furthermore, testing whether microRNAs predicted by sequence to target telomeric proteins actually target such proteins would provide important insight into telomere regulation and diseases involving telomeric dysfunction, including many types of cancer (Blasco, 2001).

MicroRNAs and stress

Changes in expression of mRNAs in response to a stressor have been observed in several studies. Previous studies have demonstrated changes in PBMC gene expression in individuals undergoing chronic or acute stress. Widespread changes in the gene expression of circulating leukocytes were observed within 30-120 minutes following acute stress, namely the TSST. Over the course of the TSST, changes were observed in cell cycle, cell signaling, adhesion, and immune pathways, among others (Nater et al 2009). Changes in gene expression were also observed in the context of academic presentation stress (Morita et al., 2003). Changes in miRNA expression in response to stress, however, have not been studied as robustly. MicroRNA changes 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 (Gidron et al., 2010). Psychological stress has been associated with increased levels of specific miRNAs in blood in humans (Gidron et al., 2010) and in the mouse amygdala (Meerson, 2010). Recently, chronic stress associated with test-taking

anxiety has also been associated with increases in several miRNAs (Honda et al., 2013).

Experiments done by Magdalena Jurkiewicz (Dissertation 2013) were the first to our knowledge to examine global changes in miRNA expression in response to an acute laboratory stressor. The study measured miRNA changes in PBMCs from subjects classified as cortisol responders. Global miRNA expression was assessed before, during and after an acute laboratory stressor, the TSST. The TSST is a laboratory stress paradigm designed to induce acute psychosocial stress in subjects. Briefly, the standardized laboratory stressor consists of a 5-min preparation period, a 5-min free speech task during which participants undergo 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. This protocol generally causes a predictable cortisol response in about 50-70% of participants (Kudlelka, Hellhammer and Kirschbaum, 2007).

In Dr. Jurkiewicz's study, a cortisol response was seen in 11 participants, and from these subjects 33 miRNAs showed significant expression changes over the course of the TSST (Figure 9). We evaluated whether these miRNAs were predicted to target proteins involved in telomere elongation and maintenance.

Telomere elongation and maintenance proteins

Several proteins are important to the maintenance of telomere length and therefore may be implicated in stress-related telomere shortening. Shelterin is the main protein complex that protects telomeres from damage. The shelterin complex is composed of six telomere-specific proteins: TRF1, TRF2, POT1, TIN2, TPP1 and Rap1. Shelterin allows cells to distinguish the 3' single-stranded overhang of telomeres from DNA damage, by folding telomeric DNA into structures called t-loops where the 3' overhang invades the double-stranded DNA of the telomere, hiding it from cellular DNA repair machinery (Smogorzewska and de Lange, 2004). Shelterin is required for the protection of telomeres from cellular DNA damage responses, which would cause cell cycle arrest. Specifically, TRF1 and TRF2 bind to double-stranded telomere DNA, while POT1 binds to the single-stranded 3' overhang. TIN2 and TPP1 hold these three proteins together in the shelterin complex, which helps to establish telomeric specificity (de Lange 2005). These proteins hide the telomeres from cell repair signaling and prevent non-homologous end-joining and homology-directed repair mechanisms (Palm and de Lange, 2008).

Telomerase, the enzyme responsible for adding telomeric repeats to the end of telomeres, has been a protein of much interest in telomere shortening research as a possible method of reversing shortening. Although telomerase is expressed at very low levels in most adult somatic tissues, these levels are both quantifiable and dynamic in leukocytes (Counter et al., 1998, Epel et al., 2010). Increased telomerase activity has been proposed as a mechanism that could

protect telomeres from shortening, or possibly reverse stress-related telomere shortening. Telomerase contains two components, a reverse transcriptase encoded by the *hTERT* gene, and an associated RNA template, *TERC* (Cohen et al., 2007). Telomerase function is primarily regulated by the expression of *hTERT*, as *TERC* is ubiquitously expressed in most tissues (Feng et al., 1995). Additional proteins implicated in proper telomerase function include Est1, Est3, and dyskerin, which are thought to play roles in localization of telomerase to the nucleus and TERC processing (Smogorzewska and de Lange 2004).

The regulation of telomerase activity involves several proteins acting either as transcription factors or localization factors. Specifically, *hTERT* expression is regulated by myc-1 and Sp1, transcription factors that upregulate transcription of telomerase (Kyo et al., 2000). Akt-mediated phosphorylation is required for the nuclear localization of hTERT (Kimura et al., 2004, Kawagoe et al 2003) and appears to be a key mechanism for regulating telomerase activity (Chung et al., 2012). Yet another protein involved in the regulation of telomeres is Pinx1, which inhibits telomere elongation by binding to shelterin proteins and preventing telomerase from accessing telomeres (Soohoo et al., 2011).

Telomere proteins and stress

The only telomere-related protein that has been studied in the context of stress is telomerase. Telomerase levels in PBMCs are altered in response to acute stress (Epel et al., 2010) and in those individuals who report higher life stress (Epel et al., 2004). Furthermore, relaxation techniques increase telomerase transcription

and activity. For example, participants in a meditation retreat had higher levels of telomerase and telomerase activity than non-participants (Jacobs et al., 2010). Acute psychological stress can promote increases in cortisol, catecholamines, and oxidative stress (Benetos et al., 2001; Gardner et al., 2005; von Zglinicki 2002), factors that may regulate telomerase activity. For example, exposure to cortisol in vitro dampens telomerase activity several days later (Brouilette et al., 2003; Cawthon et al., 2003; Choi et al., 2008).

Present study

The goal of this study was to evaluate the potential role of miRNA as a mediator of stress-related telomere shortening. Telomere shortening is associated with stress, and cortisol is one of the potential mediators of this shortening. MicroRNAs also appear to be regulated by stress, as a subset of miRNAs changed expression in response to an acute stressor in those who demonstrated a cortisol response. I evaluated these miRNAs for predicted targets involved in the telomere elongation and maintenance pathway. I then evaluated this subset by transfecting miRNA mimics into HEK293 cells and measuring the effect on target levels through immunoblotting. I found that the chosen miRNAs do not appear to regulate telomeric proteins.

Methods

MicroRNAs and targets were selected from a dataset generated by Magdalena Jurkiewitz (detailed above). These targets were evaluated by Western blot to determine if miRNA transfection in cell culture caused a decrease in target protein expression.

miRNA target candidate selection

Functional profiling of miRNA expression data was performed by the computational gene network prediction tool Ingenuity Pathway Analysis version 9.0 113 (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 involved in telomere elongation and maintenance.

Cell culture and miRNA transfection

MicroRNA 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 miRNAs. Human embryonic kidney (HEK 293, Sigma-Aldrich) cells under passage 8-10 were seeded in 6-well plates in medium containing Eagle's Minimum Essential Medium (Sigma) + 2

mM glutamine + 1% non essential amino acids (Sigma) + 10% fetal calf serum (Sigma). Once confluent, cells were transfected with 0, 50,100 or 200 nM miRNA mimic: mir-515-5p (C-301063-01-0593), mir-375 (C-301063-03-00324), mir-129-3p (C-301063-01-0005) or mir-9-5p mimic (C-301063-653) and control mimic from Dharmacon using Lipofectamine 2000 (Life Technologies) transfection reagent.

Transfection conditions were optimized and efficiency was verified using siGLO Green transfection indicator (Thermo Scientific) read on the FLUOstar microplate reader (BMG Laboratories).

Immunoblotting

After transfection, HEK 293 cells were incubated for 48 hours in order to assess changes in 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 by Western blot was performed using 50 µg of protein from each condition, which was repeated in quadruplicate.

Protein was loaded onto a pre-cast 12% SDS-polyacrilamide gel (Bio-Rad). A semi-dry protocol was used to transfer proteins onto nitrocellulose membranes using the BioRad semidry electrode. Blocking was performed using 1% casein blocking buffer (BioRad) for 2 hours at room temperature, followed by overnight incubation with primary antibody in blocking buffer at 4^{o}C, another blocking step and incubation with secondary antibody in blocking buffer for 2 hours at 4^{o}C. The antibodies used were a polyclonal rabbit Sp1 (sc-59) or Pinx1

(sc-374113) monoclonal antibody or mouse TPP1 (sc-365838) or hTERT (sc 377511) antibody from Santa Cruz was used at a dilution of 1:100, along with a fluorescently tagged goat anti-rabbit secondary antibody (Rockland 611-130-122) or goat anti-mouse secondary antibody that was used at a dilution of 1:10,000 (Rockland 610-131-121). 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). Protein levels were normalized to β-actin to ensure equal loading, and control lysate (Jurkat, Santa Cruz) served as a positive control to ensure the ability of the antibody to bind target protein.

Signal intensity was assessed with the Odyssey Infrared Imaging System, and band quantification was performed with ImageJ Software (developed at the National Institutes of Health and available at http://rsb.info.nih.gov.libproxy.cc.stonybrook.edu/ij/). Results from two independent experiments were included in the analysis of Sp1, h-TERT, TPP1 and Pinx1 to confirm targeting results by miRNAs mir-515-5p, 375, 129-3p and mir-9-5p. Statistical tests were conducted using one-way ANOVA with the Tukey post-hoc test.

Results

miRNA and target candidates

Of the miRNAs that demonstrated expression changes in response to the TSST, 16 have human, mouse or rat orthologs (Table 1) and therefore could be used for pathway analysis in IPA. Ingenuity IPA predicted that 13 of these miRNAs have putative target sites in mRNAs involved in telomere elongation and maintenance (Table 2). These targets were cross-referenced with MirBase Target database and TargetScanHuman database, which use different algorithms to predict miRNA targets. Targets were evaluated for those with highly predicted target sites. MicroRNAs with multiple targets in telomere maintenance were selected, namely mir-515-5p, mir-375, and mir-129-3p. mir-9-5p was selected a priori as a miRNA of interest. mRNA targets for analysis were chosen based on the number of miRNAs targeting them and their specificity to the telomere elongation and maintenance pathways. Specifically, we chose four mRNAs to evaluate. SP1 and TPP1 were predicted to be targeted by mir-515-5p and mir-375, which were both downregulated over the course of the TSST. h-TERT, the protein in telomerase, was predicted to have three putative target sites for mir-129-3p. Finally, Pinx1, an inhibitor of telomerase, was chosen for evaluation of its putative regulation by mir-9-5p, an miRNA that was downregulated in response to the TSST.

Transfection efficiency

Conditions for HEK 293 cell transfections using lipofectamine were optimized such that 75% (plus or minus 8.6%) of cells displayed siGLO green uptake in three trials (consistent with published results using HEK293 cells for transfection of miRNA mimic, e.g. Decrock et al., 2008). In each of 6 wells, 15.0uL lipofectamine was used in a 48hr inubation.

Target evaluation

MicroRNAs mir-515-5p, mir-375, mir-129-3p, and mir-9-5p were evaluated for their ability to downregulate TPP1, SP1, Pinx1 and hTERT by Western blot. Transfection of mir-375 resulted in the reduction of SP1 protein (but not TPP1 protein) when compared with a negative control mimic and an untransfected control ($p<0.05$). MicroRNA mir-515-5p did not result in a decrease in these proteins ($p<0.05$). Additionally, mir-129-3p did not appear to target h-TERT, nor did mir-9-5p appear to target PINX1 ($p<0.05$). These results are displayed in Figure 10.

Discussion

The only association we found among miRNAs and telomere regulation was the putative targeting of SP1 by mir-375. No further work was done in this dissertation to validate this targeting, because it has been independently verified in cervical cancer and several cell lines (Wang et al., 2011). SP1 appears to play major roles in cancer tumorigenesis and progression (Lu and Archer, 2009; Waby et al., 2010). While the role of mir-375 regulation of SP1 was not evaluated in the context of the role of SP1 in telomere regulation, there is enough evidence to suggest that this mechanism is possible. We have demonstrated that mir-375 is upregulated during an acute stressor. Our Western blot evidence, along with the extensive research conducted by Wang et al. (2011), demonstrates that mir-375 downregulates SP1 by binding its 3' UTR. Several lines of evidence demonstrate that SP1 cooperates with c-Myc to activate transcription of *hTERT* (Kyo et al., 2000). Five SP1 binding sites are found in the *hTERT* promoter, and SP1 binding is essential for *hTERT* transcription (Kyo et al., 2000). This regulation is accomplished in a cell type-specific manner, and other transcription factors are thought to be involved in *hTERT* transcriptional regulation, although the exact mechanisms remain unknown (Cong et al., 2002). SP1 may indeed play a role in stress-related telomere maintenance; however, it is probable that other factors contribute as well.

Determining the molecular mechanism of miRNA regulation in response to the TSST would help elucidate whether and how SP1 contributes to stress-associated telomere shortening. Our original hypothesis considered whether the

cortisol response elicited by the TSST was responsible for the upregulation of miRNAs such as mir-375, which downregulate proteins that protect or elongate telomeres, such as SP1. While there is no direct evidence of cortisol-regulated miRNA expression, it has been suggested by a survey of changing miRNA expression in response to cortisol treatment in cancer cells (Rainer et al., 2009), which has been replicated in vitro in several studies (Cochrane et al., 2011).

Overall, the miRNAs evaluated in our study did not appear to target telomere-related mRNAs. It is certainly possible that miRNAs do not play a large role in stress-related telomere shortening. These experiments were not enough to determine the extent of the role even these miRNAs may play in this process. Although the transfection efficiency of miRNA mimic was evaluated with siGLO Green, this is a proxy measure of mimic uptake under given conditions in a given cell line. It is possible that miRNA mimics were not transfected, processed or localized efficiently. Additionally, these miRNAs were evaluated in the HEK 293 cell line because these cells are easily transfected with miRNA mimic. However, the cellular context of the HEK 293 cells may not be conducive to allowing miRNAs to regulate telomere proteins. There may be competing mechanisms for regulating these proteins that mask the targeting by these miRNAs. If this is the case, repeating the experiment in a different cell line or with larger doses of mimic may elucidate targeting relationships that were hidden in these experiments. It may be best to evaluate these targets by more closely mimicking in vivo conditions using a leukocyte cell line or primary PBMCs.

Future Directions

More mRNAs should be evaluated as potential miRNA targets (see Appendix B for remaining targets). We did not exhaust the list of potential miRNAs and target mRNAs generated by the TSST experiment due to time and resource constraints. These could be evaluated by Western blot as described above. For all targets, qPCR should be used to confirm transfection efficiency. Any miRNAs that were found to target telomere-related mRNAs could be further validated using dual luciferase assays with 3'UTR clones of target genes, obtained from Genecopoeia. The full 3'UTR of each target could be cloned downstream of the firefly luciferase reporter gene in a dual luciferase (firefly/renilla) vector. In this assay, cells are cotransfected with the 3'UTR vector of interest and miRNA mimic, inhibitor, or negative control (Dharmacon) using the DharmaFECT Duo Transfection Reagent (Dharmacon), and luciferase activity is measured using the Dual-Luciferase® Reporter Assay System (Promega) and a FLUOstarOptima microplate reader (BMG Laboratories).

To evaluate the role of cortisol in regulating miRNA expression, a leukocyte cell line or primary PBMCs could be exposed to varying levels of cortisol using the protocol detailed in Choi et al., 2008, in which primary PBMCs were exposed to different concentrations of cortisol (concentrations that reflect in vivo levels reached after exposure to a stressor). The level of miRNAs could then be measured under each condition by qPCR to evaluate the transcriptional control of these miRNAs by cortisol. Any effect of these miRNAs on telomerase activity could be directly evaluated using the TRAPeze assay kit (Millipore). This

assay utilizes a synthetic oligonucleotide containing telomeric repeats and a PCR protocol to measure the rate of addition of repeats to the synthetic oligo. The effect of any of these miRNAs on telomere length would be more challenging to evaluate in cell culture, as telomere shortening would occur over many passages and telomere regulation would be altered by this process. If enough successful candidates were evaluated, an animal model could be established for testing the effect of miRNA in blood on telomere length over time by utilizing in vivo purified miRNA mimics (eg mirVANA, mirScript or Exiquon mimics for in vivo use). These have been successfully injected into several animals including mice, rats and zebrafish (Trang et al., 2011; Selvamani et al., 2012; Bridge et al., 2012) to evaluate the effect of miRNA manipulation.

It may be worthwhile to evaluate a larger range of miRNA concentrations in these and future miRNA targeting experiments. Here, a range from 25-200 nM of miRNA mimic was evaluated for a given target. The range of expression of miRNAs in different cell types can differ 1000-fold in in vivo experiments evaluating differences in tissue samples, cancer and normal cell lines (Gaur et al 2007), and the targeting of the miRNAs listed here may have been evaluated below a functional threshold. We are limited by the toxicity of the miRNA mimic, but a wider range of concentrations might illuminate functions missed at lower levels. A dose-response curve should be employed up to the range of cell toxicity. Additionally, as many of the mRNAs evaluated had putative binding sites for several miRNAs, it is possible that their regulation requires the additive effect

of two or more miRNAs. These miRNAs should be transfected in combination to observe any additive effects.

Another approach to selecting candidate mRNAs that are the most likely to be targeted by miRNAs that are altered after a stressor would be to evaluate variation in mRNA amounts in participants of the TSST. Due to limitations and other uses for the blood samples, these could not be evaluated in this sample set, but could be evaluated in future TSST subjects.

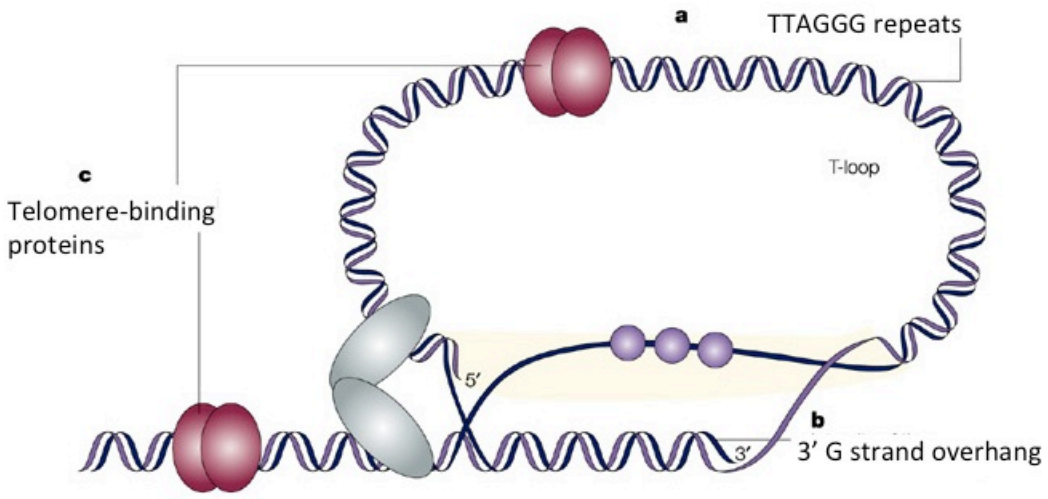


Figure 1. Structure of the telomeric T-loop. Telomeric capping requires at least three factors: a., minimal length of TTAGGG repeats; b., integrity of the 3' G-strand overhang; and c., telomere-binding proteins. Reproduced with permission from Blasco et al., 2002 (Figure 1).

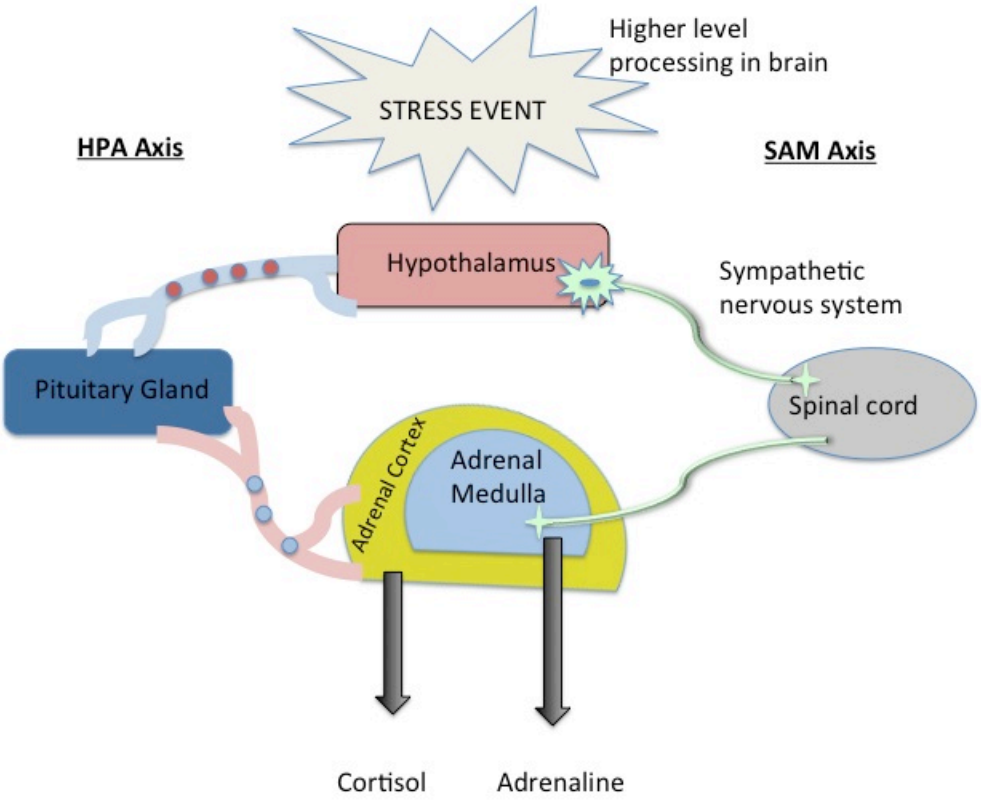


Figure 2. The SAM and HPA axes. In response to stress, the hypothalamus releases corticotropin releasing hormone (CRH). This stimulates the pituitary to release ACTH, which is then detected by the adrenal cortex which releases corticosteroids into the bloodstream. The hypothalamus arouses the ANS by direct innervation to stimulate the adrenal medulla, which releases adrenaline into the bloodstream. This results in the fight or flight response, which involves physiological changes such as faster breathing, increased heart rate and increased blood pressure.

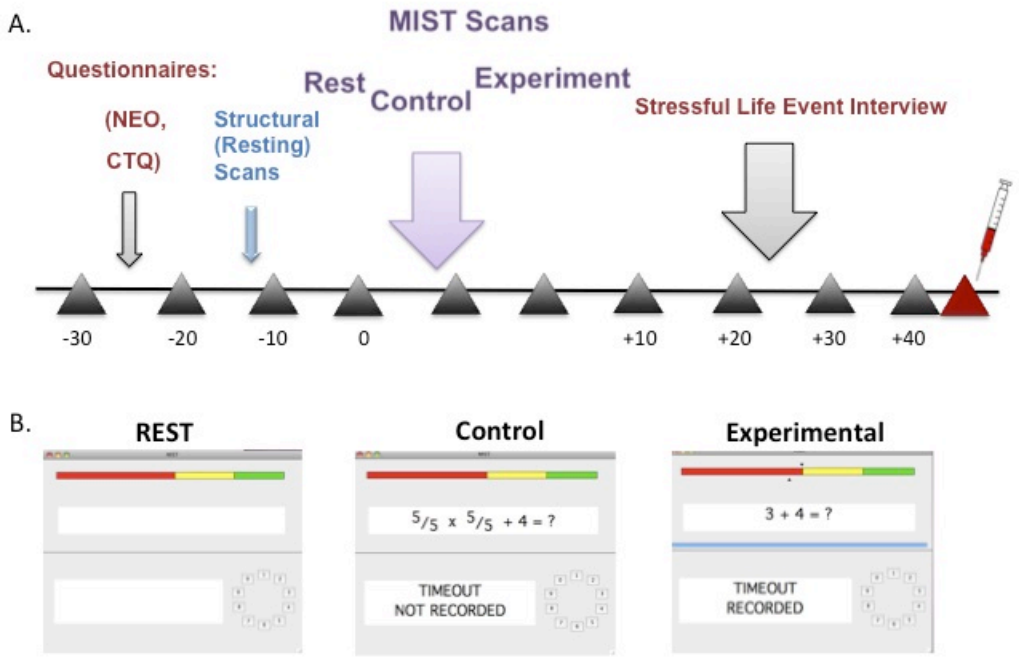
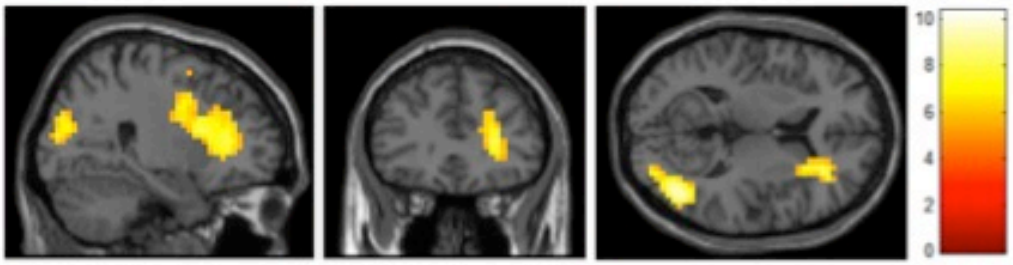


Figure 3. The Montreal Imaging Stress Task. A. The MIST is an fMRI-compatible version of the TSST that combines difficult arithmetic with explicit social evaluation to induce psychosocial stress in the scanner. Before the MIST scans, subjects are asked to complete psychological evaluations. Resting brain scans are then taken in the scanner. The MIST scans begin at time 0. After the MIST scans, subjects participate in a stressful life events interview and blood is taken. Saliva samples are taken at regular intervals during the scan session (black triangles) and time before and after MIST scans is labeled in minutes. B. Representations of what subjects see in Rest, Control and Experimental conditions in the scanner. The arithmetic task is divided into three conditions; a rest task where the participant simply sees the task interface, a control condition where the participant completes arithmetic problems, but is told their performance does not count, and finally an experimental task where the subject is told that the task will be assessed by the experimenter. In this condition, the arithmetic task is timed and the difficulty is gauged to be just beyond the participant's ability level. Between each run, the experimenter enters the room and provides negative feedback to the participant, informing them that their performance is being monitored during the experimental condition and that they are performing below an acceptable level.

A.

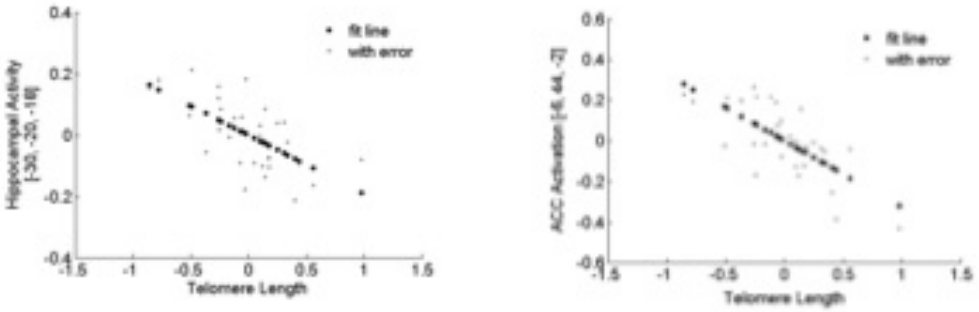


B.

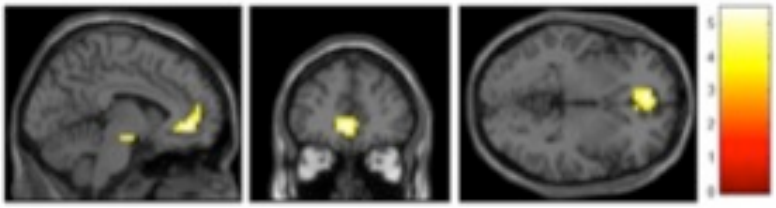
Cluster Size	T	Tal Coordinates (X, Y, Z)	Region
		Whole-Brain Analysis	
199	10.36	41, -65, 4	R Middle Occipital Gyrus
	7.93	26, -78, 13	R Middle Occipital Gyrus
428	8.30	22, 7, 21	R Putamen
	8.07	15, -3, 56	R Middle Frontal Gyrus
	8.05	23, 27, 16	R Anterior Cingulate
48	7.56	7, -48, 49	R Precuneus
	7.43	4, -55. 51	R Precuneus
14	6.21	4, -5, 2	R Thalamus
		ROI Analysis	
107	6.89	-7, 7, 28	L Anterior Cingulate
	5.65	4, 7, 28	R Anterior Cingulate
	5.42	8, 19, 26	R Anterior Cingulate

Figure 4: Whole brain analyses of the experimental-greater-than-control condition. A. Heat map of activity greater in experiment than control for whole group shown in sagittal, coronal and transverse views (generated by SPM8). **B.** Activity for whole-brain and region of interest (ROI) in experimental-greater-than-control condition. All peak values exceed a threshold of 0.05, FWE corrected. Cluster size refers to number of voxels included in peak analysis. T refers to the T-score, Tal coordinates are anatomical normalizations from the Talairach Atlas (Talairach and Tournoux, 1998).

A



B.



C.

Cluster Size	T	Tal Coordinates (X, Y, Z)	Region
		Whole-Brain Analysis	
66	5.47	-6, 44, -2	Left Anterior Cingulate
	4.32	-10, 52, 14	R Anterior Cingulate
12	6.62	-2, -12, -14	Midbrain
12	4.45	-30, -20, -18	L Hippocampus
	3.78	-14, -20, -25	L Parahippocampus

Figure 5: Brain activation during the MIST associated with telomere length. **A.** Relative telomere length is inversely correlated with activation in the ACC and the hippocampus during the experimental condition of the MIST. B. Heat map of activity greater in subjects with short telomeres during experimental condition, shown in sagittal, coronal and transverse views (generated by SPM8). C. Activity for whole-brain and region of interest (ROI) for activity greater in subjects with short telomeres during experimental condition,.All peak values exceed a threshold of 0.05, FWE corrected. Cluster size refers to number of voxels included in peak analysis. T refers to the T-score, Tal coordinates are anatomical normalizations from the Talairach Atlas (Talairach and Tournoux, 1998).

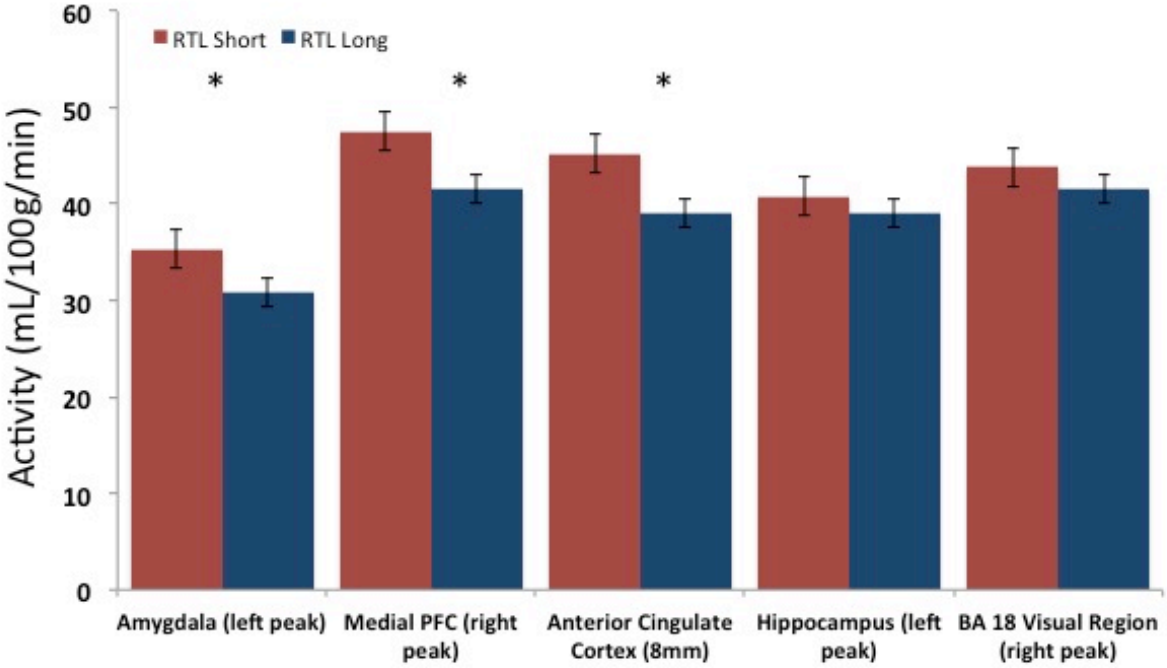


Figure 6. Amygdala, mPFC and ACC activity is higher in those with short telomeres. ASL data were collected for subjects with short or long RTL (median split). Significant differences (marked with *) were found for amygdala, medial prefrontal cortex and ACC.

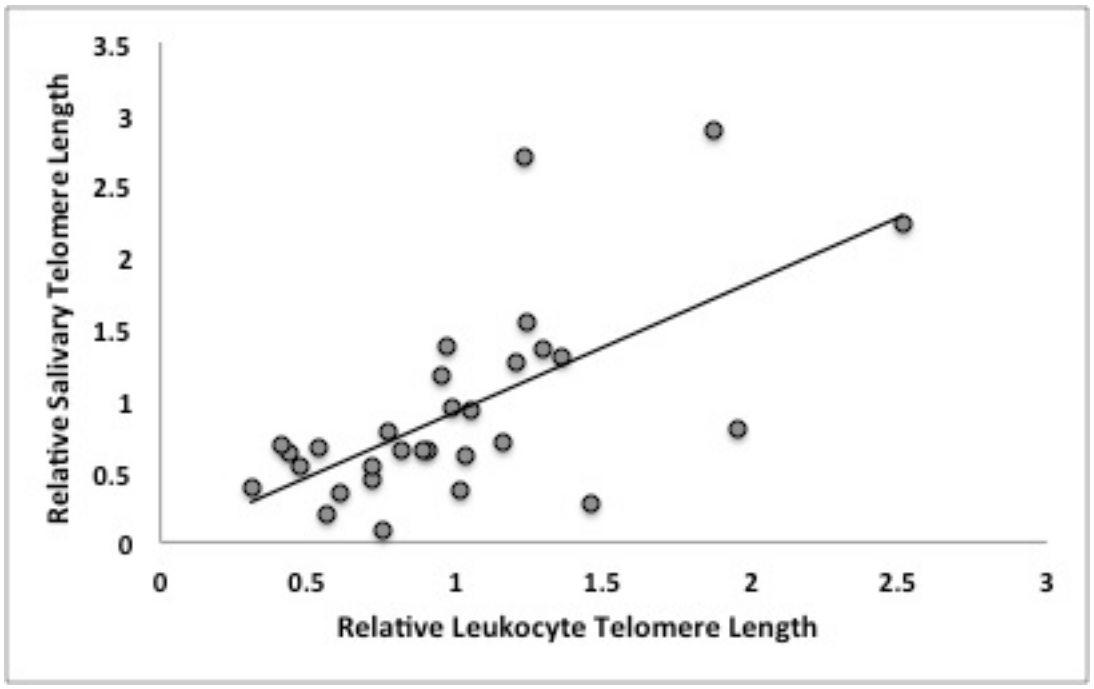


Figure 7. Blood and saliva RTL correlate highly. Relative telomere length was measured in blood and saliva from 30 individuals using qPCR method developed by Cawthon (2002).

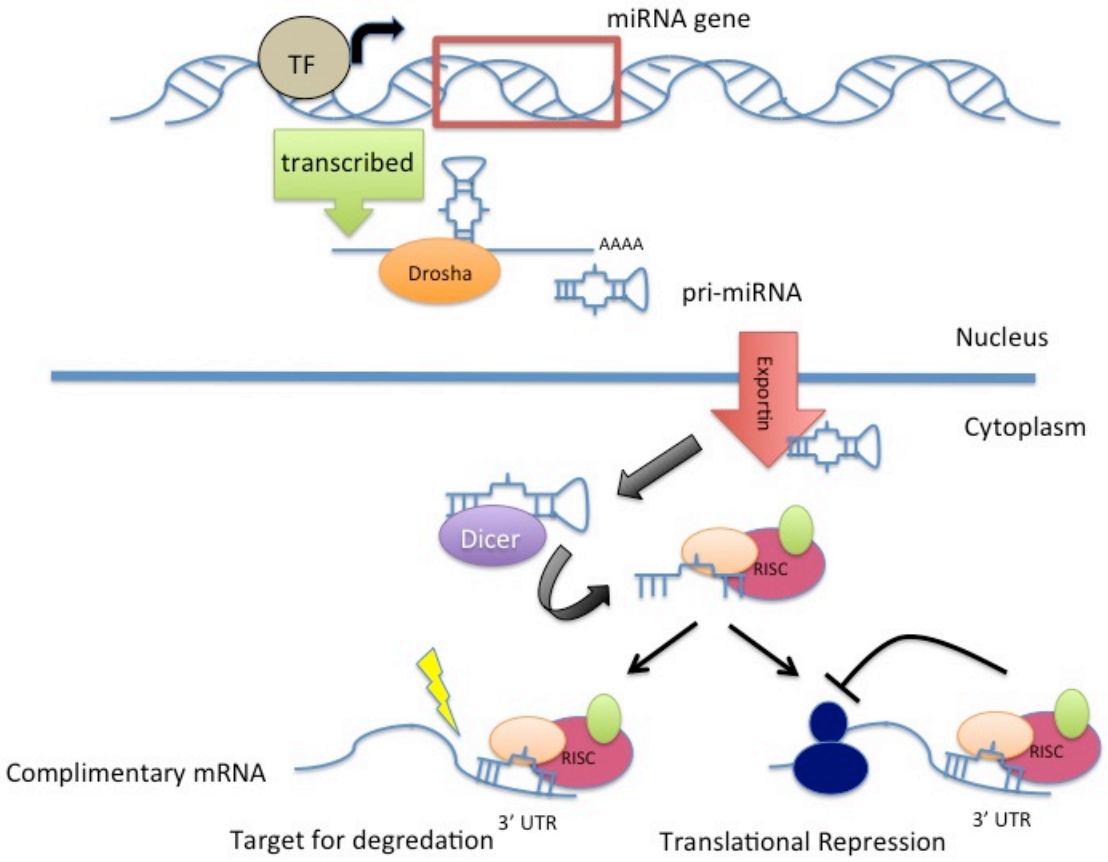


Figure 8. Common MicroRNA processing and mechanisms of action. The miRNA is transcribed from the genome and cleaved by Drosha, an miRNA processing complex. Exportin then transports the pri-miRNA from the nucleus to the cytoplasm. Dicer then further processes the molecule forming the mature miRNA. Mature miRNAs associate with the RISC complex to find a complimentary sequence in the 3'UTR of target mRNAs, where it can interfere with translation or mark the mRNA for degradation.

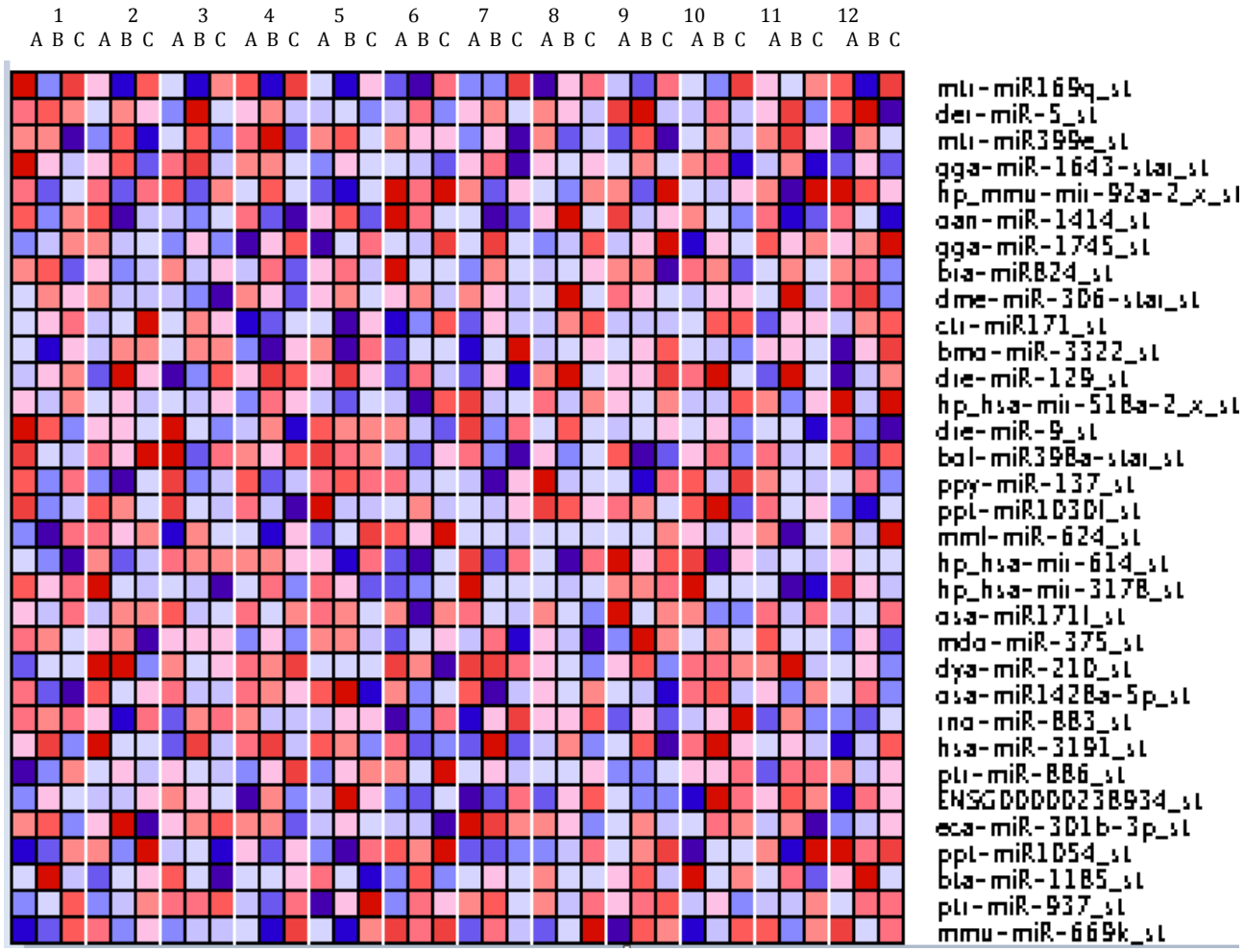
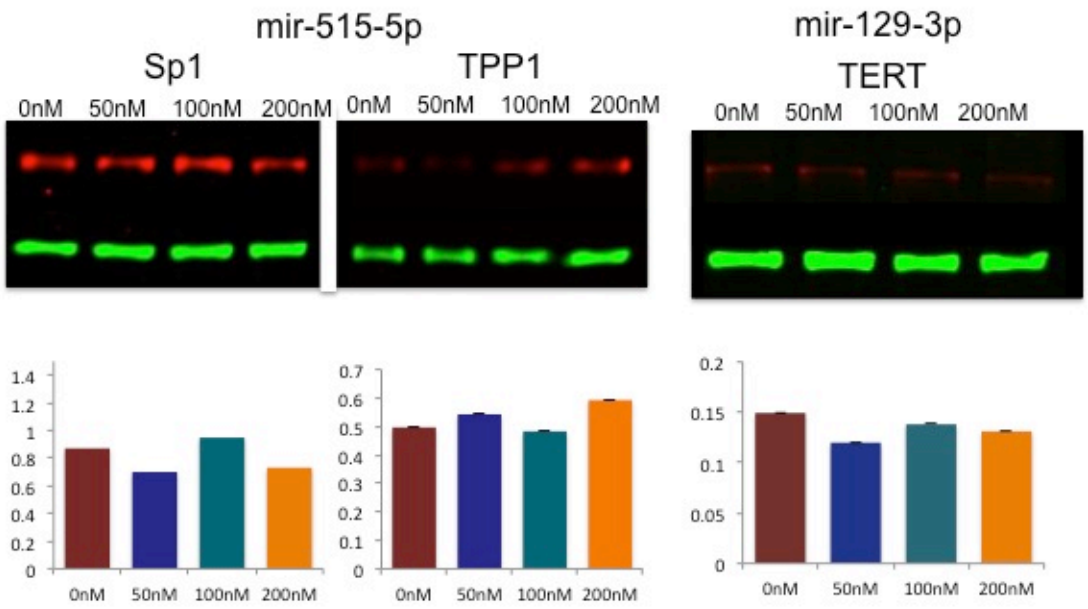


Figure 9. MiRNA expression inPBMCs before, during and after the TSST. Each row represents a miRNA probe-set and each set of three columns represents on individual (labeled with participant ID number) at Time Points A, B, and C. Each sample was run once on one microarray at each Time Point. The expression value represented by each square is $\log^2$ 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.


mir-515-5p
Sp1
TPP1
0nM 50nM 100nM 200nM
0nM 50nM 100nM 200nM
mir-129-3p
TERT
0nM 50nM 100nM 200nM
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.2
0.15
0.1
0.05
0
0nM 50nM 100nM 200nM

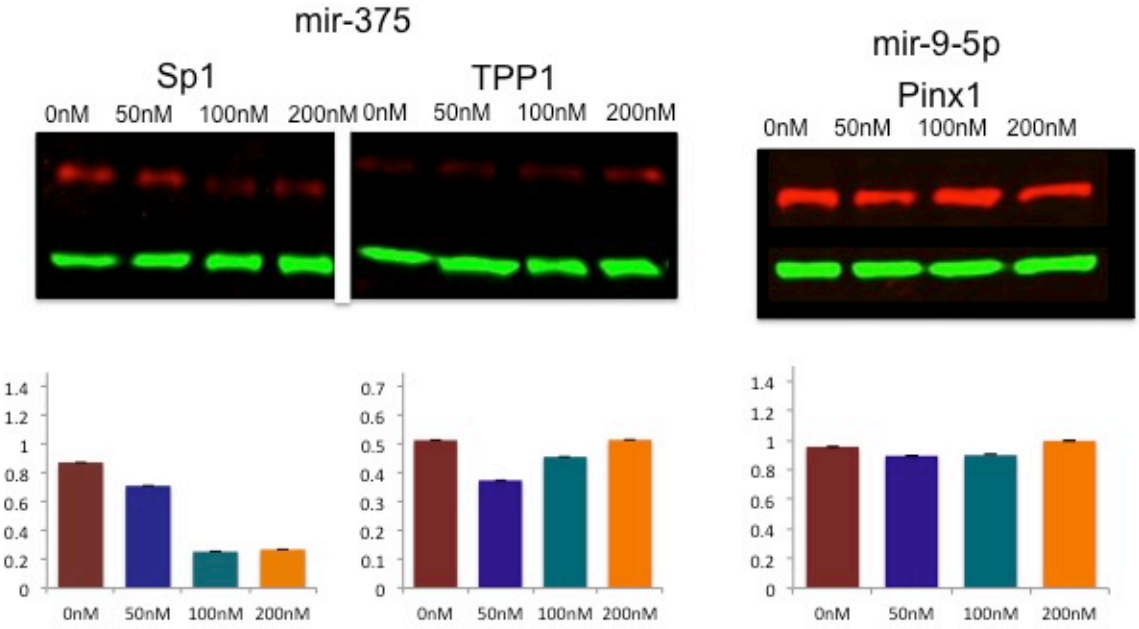

mir-375
Sp1
TPP1
0nM 50nM 100nM 200nM
0nM 50nM 100nM 200nM
mir-9-5p
Pinx1
0nM 50nM 100nM 200nM
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0nM 50nM 100nM 200nM

Figure 10. Western blot results of miRNA targeting proteins in telomere elongation and maintenance. Western blots were used to assess protein levels in HEK 293 cells after incubation with miRNA mimics. Western blot image from Odessy, and graphs depicting target protein level relative to actin (control) protein level are depicted for cells incubated with 0, 50, 100 or 200nM miRNA mimic. MicroRNA mir-375 incubation led to a significant decrease in SP1 (but not TPP1 when compared with untransfected control MicroRNA mir-515-5p incubation did not appear to change protein levels. Additionally, mir-129-3p did not appear to change h-TERT levels, nor did mir-9-5p appear to change PINX1 levels.

Affymetrix Probe Name	Human Ortholog	MirBase ID
mtr-miR169q_st	no human/mouse/rat homologue	
der-miR-5_st	no human/mouse/rat homologue	
mtr-miR399e_st	no human/mouse/rat homologue	
gga-miR-1643-star_st	no human/mouse/rat homologue	
hp_mmu-mir-92a-2_x_st	hsa-mir-92a-1	MI0000093
oan-miR-1414_st	no human/mouse/rat homologue	
gga-miR-1745_st	no human/mouse/rat homologue	
bra-miR824_st	no human/mouse/rat homologue	
dme-miR-306-star_st	no human/mouse/rat homologue	
ctr-miR171_st	no human/mouse/rat homologue	
bmo-miR-3322_st	no human/mouse/rat homologue	
dre-miR-129_st	hsa-miR-129	MIMAT0000242
hp_hsa-mir-518a-2_x_st	hsa-mir-518a-2	MI0003173
dre-miR-9_st	hsa-miR-9/ hsa-miR-9-5p	MIMAT0000441
bol-miR398a-star_st	no human/mouse/rat homologue	
ppy-miR-137_st	hsa-miR-137	MIMAT0000429
ppt-miR1030f_st	no human/mouse/rat homologue	
mml-miR-624_st	hsa-miR-624/hsa-miR-624-5p	MIMAT0003293
hp_hsa-mir-614_st	hsa-miR-614	MIMAT0003282
hp_hsa-mir-3178_st	hsa-mir-3178	MI0014212
osa-miR171f_st	no human/mouse/rat homologue	
mdo-miR-375_st	hsa-mir-375	MI0000783
dya-miR-210_st	hsa-mir-210	MI0000286
osa-miR1428a-5p_st	no human/mouse/rat homologue	MI0006126
rno-miR-883_st	rno-mir-883	MIMAT0015075
hsa-miR-3191_st	hsa-miR-3191	MI0005527
ptr-miR-886_st	hsa-mir-886	MIMAT0004980
ENSG00000238934_st	no human/mouse/rat homologue	MIMAT0004958
eca-miR-301b-3p_st	hsa-miR-301b	MIMAT0004980
ppt-miR1054_st	no human/mouse/rat homologue	MIMAT0005798
bta-miR-1185_st	hsa-miR-1185-5p	MIMAT0004980
ptr-miR-937_st	hsa-miR-937	MIMAT0004980

Table 1. MicroRNAs with expression changes over the course of the TSST and their human orthologs.

	Fold-Change					
MIR	**B vs A**	**A vs B**	**C vs A**	**A vs C**	**B vs C**	**C vs B**
mtr-miR169q_st	0.90	1.11	1.12	0.89	0.80	1.25
der-miR-5_st	1.10	0.91	0.90	1.11	1.22	0.82
mtr-miR399e_st	1.11	0.90	0.91	1.10	1.23	0.81
gga-miR-1643-star_st	1.06	0.95	0.85	1.17	1.24	0.81
hp_mmu-mir-92a-2_x_st	0.83	1.20	1.02	0.98	0.82	1.22
oan-miR-1414_st	0.83	1.21	0.80	1.26	1.04	0.96
gga-miR-1745_st	1.15	0.87	1.32	0.76	0.86	1.16
bra-miR824_st	1.02	0.98	0.83	1.20	1.23	0.82
dme-miR-306-star_st	1.07	0.94	0.89	1.13	1.20	0.83
ctr-miR171_st	1.09	0.92	1.21	0.83	0.90	1.11
bmo-miR-3322_st	1.03	0.97	1.23	0.81	0.84	1.20
dre-miR-129_st	1.23	0.81	1.13	0.88	1.09	0.92
hp_hsa-mir-518a-2_x_st	0.86	1.17	1.04	0.96	0.82	1.21
dre-miR-9_st	0.91	1.10	0.79	1.26	1.15	0.87
bol-miR398a-star_st	0.81	1.24	0.89	1.12	0.90	1.11
ppy-miR-137_st	0.82	1.22	0.93	1.08	0.88	1.13
ppt-miR1030f_st	0.89	1.12	0.83	1.21	1.08	0.92
mml-miR-624_st	0.91	1.10	1.17	0.86	0.78	1.28
hp_hsa-mir-614_st	0.82	1.21	0.95	1.05	0.87	1.15
hp_hsa-mir-3178_st	0.86	1.16	0.83	1.21	1.04	0.96
osa-miR171f_st	0.82	1.22	0.92	1.08	0.89	1.13
mdo-miR-375_st	1.11	0.90	0.90	1.11	1.23	0.81
dya-miR-210_st	1.04	0.96	0.80	1.25	1.30	0.77
osa-miR1428a-5p_st	0.89	1.13	0.83	1.21	1.07	0.93
rno-miR-883_st	1.07	0.93	1.20	0.83	0.89	1.12
hsa-miR-3191_st	1.09	0.92	0.90	1.11	1.21	0.83
ptr-miR-886_st	1.11	0.90	1.38	0.72	0.80	1.24
ENSG00000238934_st	1.22	0.82	1.18	0.85	1.04	0.96
eca-miR-301b-3p_st	1.09	0.92	0.87	1.14	1.25	0.80
ppt-miR1054_st	0.93	1.07	1.20	0.83	0.77	1.29
bta-miR-1185_st	1.07	0.94	0.89	1.13	1.20	0.83
ptr-miR-937_st	1.09	0.91	1.20	0.83	0.91	1.10
mmu-miR-669k_st	0.99	1.01	1.21	0.83	0.82	1.22

Table 2. Fold change differences in miRNA expression among three time points during the TSST.

Appendix A: Examples of Life Stress Interview Questions

Participant ID: _____ Date: _____

Age: _____

Negative and Positive Life Events Questionnaire (NPLEQ)

Listed below are a number of events, which may bring about changes in the lives of those who experience them.

- Circle the events that have occurred in participant's life and circle whether these were *Good* or *Bad*. If one event happened to be as well *Good* and *Bad*, please circle both.
- Show how much the event affected his/her life by circling the appropriate *number*, which corresponds with the statement (0 = no effect, 1 = some effect, 2 = moderate effect, 3 = great effect). If participant has not experienced a particular event in your life, please check N/A.
- Request age of event, take notes and ask if one or the other event happened more than once.
- You find backup pages for multiple events and note at the end.

Okay, a few demographic questions before we begin:

Where were you born? _____

Do you have any siblings? yes ☐ no ☐ If yes, how many? _____

Event		N/A	*Type of Effect*		*Effect of Event on your Life*			
					No effect	Some effect	Moderate effect	Great effect
A. RESIDENCE								
1. Bought or sold a house					No effect	Some effect	Moderate effect	Great effect
Youth/Adolescence (13-17)	*Age:* ____	N/A	Good	Bad	0	1	2	3
Adult (18 – 1year before interview)	*Age:* ____	N/A	Good	Bad	0	1	2	3
Last year	*Age:* ____	N/A	Good	Bad	0	1	2	3
Notes:								
Did this event happen more than once?		N/A						
	Age: ____		Good	Bad	0	1	2	3
	Age: ____		Good	Bad	0	1	2	3
	Age: ____		Good	Bad	0	1	2	3
2. Moved to another house					No effect	Some effect	Moderate effect	Great effect
Early 5 years (0-5)	*Age:* ____	N/A	Good	Bad	0	1	2	3
Childhood (6-12)	*Age:* ____	N/A	Good	Bad	0	1	2	3
Youth/Adolescence (13-17)	*Age:* ____	N/A	Good	Bad	0	1	2	3
Adulthood (18 – 1yr before interview)	*Age:* ____	N/A	Good	Bad	0	1	2	3
Last year	*Age:* ____	N/A	Good	Bad	0	1	2	3
Notes:								
Did this event happen more than once?		N/A						
	Age: ____		Good	Bad	0	1	2	3
	Age: ____		Good	Bad	0	1	2	3
	Age: ____		Good	Bad	0	1	2	3
	Age: ____		Good	Bad	0	1	2	3

1

Participant ID: _____ Date: _____

Age: _____

3. Frequent or long-distance moving (e.g. to another country)					No effect	Some effect	Moderate effect	Great effect
Early 5 years (0-5)	*Age:*____	N/A	Good	Bad	0	1	2	3
Childhood (6-12)	*Age:*____	N/A	Good	Bad	0	1	2	3
Youth/Adolescence (13-17)	*Age:*____	N/A	Good	Bad	0	1	2	3
Adulthood (18 – 1yr before interview)	*Age:*____	N/A	Good	Bad	0	1	2	3
Last year	*Age:*____	N/A	Good	Bad	0	1	2	3
Notes:								
Did this event happen more than once?		N/A						
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
4. Major house renovation					No effect	Some effect	Moderate effect	Great effect
Childhood (6-12)	*Age:*____	N/A	Good	Bad	0	1	2	3
Youth/Adolescence (13-17)	*Age:*____	N/A	Good	Bad	0	1	2	3
Adulthood (18 – 1yr before interview)	*Age:*____	N/A	Good	Bad	0	1	2	3
Last year	*Age:*____	N/A	Good	Bad	0	1	2	3
Notes:								
Did this event happen more than once?		N/A						
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
5. Homelessness (If yes, how long exactly?)					No effect	Some effect	Moderate effect	Great effect
Early 5 years (0-5)	*Age:*____	N/A	Good	Bad	0	1	2	3
Childhood (6-12)	*Age:*____	N/A	Good	Bad	0	1	2	3
Youth/Adolescence (13-17)	*Age:*____	N/A	Good	Bad	0	1	2	3
Adulthood (18 – 1yr before interview)	*Age:*____	N/A	Good	Bad	0	1	2	3
Last year	*Age:*____	N/A	Good	Bad	0	1	2	3
*Notes (**note length of homelessness**):*								
Did this event happen more than once?		N/A						
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3

Participant ID: _____ Date: _____

Age: _____

55. Conflicts with partner/spouse about parenting					No effect	Some effect	Moderate effect	Great effect
Youth/Adolescence (13-17)	*Age:*____	N/A	Good	Bad	0	1	2	3
Adulthood (18 – 1yr before interview)	*Age:*____	N/A	Good	Bad	0	1	2	3
Last year	*Age:*____	N/A	Good	Bad	0	1	2	3
Notes:								
Did this event happen more than once?		N/A						
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
56. Child (stepchild) started school					No effect	Some effect	Moderate effect	Great effect
Adulthood (18 – 1yr before interview)	*Age:*____	N/A	Good	Bad	0	1	2	3
Last year	*Age:*____	N/A	Good	Bad	0	1	2	3
Notes:								
Did this event happen more than once?		N/A						
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
57. Serious illness, accident or diagnosis of partner					No effect	Some effect	Moderate effect	Great effect
Youth/Adolescence (13-17)	*Age:*____	N/A	Good	Bad	0	1	2	3
Adulthood (18 – 1yr before interview)	*Age:*____	N/A	Good	Bad	0	1	2	3
Last year	*Age:*____	N/A	Good	Bad	0	1	2	3
Notes:								
Did this event happen more than once?		N/A						
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3
	*Age:*____		Good	Bad	0	1	2	3

Participant ID: _____ Date: _____

Age: _____

M. SPECIAL LIFE EVENTS IN THE LIFE OF ELDERLY PEOPLE								
113. (Female) started menopause					No effect	Some effect	Moderate effect	Great effect
Adulthood (16 – 1yr before interview)	*Age:* _____	N/A	Good	Bad	0	1	2	3
Last year	*Age:* _____	N/A	Good	Bad	0	1	2	3
Notes:								
114. Retirement from work					No effect	Some effect	Moderate effect	Great effect
Adulthood (16 – 1yr before interview)	*Age:* _____	N/A	Good	Bad	0	1	2	3
Last year	*Age:* _____	N/A	Good	Bad	0	1	2	3
Notes:								
115. Other negative event in later adulthood (specify):					No effect	Some effect	Moderate effect	Great effect
Adulthood (16 – 1yr before interview)	*Age:* _____	N/A	Good	Bad	0	1	2	3
Last year	*Age:* _____	N/A	Good	Bad	0	1	2	3
Notes (note event):								
Did this event happen more than once?		N/A						
	Age: _____		Good	Bad	0	1	2	3
	Age: _____		Good	Bad	0	1	2	3
	Age: _____		Good	Bad	0	1	2	3
	Age: _____		Good	Bad	0	1	2	3
116. Other negative event in later adulthood (specify):					No effect	Some effect	Moderate effect	Great effect
Adulthood (16 – 1yr before interview)	*Age:* _____	N/A	Good	Bad	0	1	2	3
Last year	*Age:* _____	N/A	Good	Bad	0	1	2	3
Notes (note event):								
Did this event happen more than once?		N/A						
	Age: _____		Good	Bad	0	1	2	3
	Age: _____		Good	Bad	0	1	2	3
	Age: _____		Good	Bad	0	1	2	3
	Age: _____		Good	Bad	0	1	2	3

Appendix B: List of miRNAs differentially regulated over the course of the TSST and their IPA predicted targets in telomere elongation and maintenance pathways.

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ID	Symbol	Observation 1 (Log Ratio)	Confidence	Symbol
MIMAT0000242	miR-129-5p	0.296	High (predicted)	PDPK1
MIMAT0000242	miR-129-5p	0.296	High (predicted)	MAPK1
MIMAT0000242	miR-129-5p	0.296	High (predicted)	HNRNPA1
MIMAT0000242	miR-129-5p	0.296	High (predicted)	HDAC2
MIMAT0000242	miR-129-5p	0.296	High (predicted)	ETS1
MIMAT0000728	miR-375-3p	0.147	High (predicted)	SP1
MIMAT0000728	miR-375-3p	0.147	Experimentally Observed,High (predicted)	PDPK1
MIMAT0004980	miR-937-3p	0.129	Moderate (predicted)	HDAC4
MIMAT0015075	miR-3191-3p	0.123	Moderate (predicted)	TPP1
MIMAT0015075	miR-3191-3p	0.123	Moderate (predicted)	PPP2R5D
MIMAT0015075	miR-3191-3p	0.123	Moderate (predicted)	HNRNPA2B1
MIMAT0015075	miR-3191-3p	0.123	Moderate (predicted)	ELK3
MIMAT0015075	miR-3191-3p	0.123	High (predicted)	ELF3
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	TPP1
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	SP1
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	SOS2
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	PTGES3
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	PPP2R1B
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	PIK3C2A
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	MAPK1
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	ELK3
MIMAT0004958	miR-130a-3p	0.123	High (predicted)	CDKN1A
MIMAT0005798	miR-1185-5p	0.095	Moderate (predicted)	TINF2
MIMAT0005798	miR-1185-5p	0.095	High (predicted)	SOS2
MIMAT0005798	miR-1185-	0.095	Moderate (predicted)	PPP2R2A

	5p			
MIMAT0005798	miR-1185-5p	0.095	Experimentally Observed	MAPK1
MIMAT0005798	miR-1185-5p	0.095	Moderate (predicted)	HDAC4
MIMAT0000267	miR-210-3p	0.058	Moderate (predicted)	PIK3R5
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	TNKS
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	SOS1
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	SHC1
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	PPP2R5D
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	PPP2R4
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	PPP2R2A
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	PIK3R3
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	PIK3C2A
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	MRE11A
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	HSP90AA1
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	HDAC5
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	HDAC4
MIMAT0000441	miR-9-5p	-0.135	High (predicted)	ETS1
MIMAT0004807	miR-624-3p	-0.138	Moderate (predicted)	RRAS2
MIMAT0004807	miR-624-3p	-0.138	Moderate (predicted)	PIK3C2B
MIMAT0004807	miR-624-3p	-0.138	Moderate (predicted)	MRE11A
MIMAT0004807	miR-624-3p	-0.138	Moderate (predicted)	EGF
MIMAT0015055	miR-3178	-0.216	Moderate (predicted)	MRAS
MIMAT0015055	miR-3179	-0.216	Moderate (predicted)	HDAC4
MIMAT0015055	miR-3180	-0.216	High (predicted)	HDAC10
MIMAT0015055	miR-3181	-0.216	Moderate (predicted)	ABL1
MIMAT0002826	miR-515-5p	-0.222	Moderate (predicted)	TEP1
MIMAT0002826	miR-515-5p	-0.222	Moderate (predicted)	PPP2R3A
MIMAT0002826	miR-515-5p	-0.222	Moderate (predicted)	PPP2R2A
MIMAT0002826	miR-515-5p	-0.222	High (predicted)	PIK3CG
MIMAT0002826	miR-515-5p	-0.222	Moderate (predicted)	PIK3C3
MIMAT0002826	miR-515-5p	-0.222	Moderate (predicted)	NRAS
MIMAT0002826	miR-515-5p	-0.222	High (predicted)	ATM
MIMAT0002827	miR-515-5p	-0.222	High (predicted)	HSP90AA1
MIMAT0002827	miR-515-5p	-0.222	Moderate (predicted)	HDAC9
MIMAT0002827	miR-515-5p	-0.222	Moderate (predicted)	ELF5
MIMAT0002827	miR-515-5p	-0.222	Moderate (predicted)	CDKN1A
MIMAT0002827	miR-515-5p	-0.222	Moderate (predicted)	ABL1
MIMAT0002827	miR-515-5p	-0.222	High (predicted)	SP1
MIMAT0000081	miR-92a-3p	-0.262	High (predicted)	SP1
MIMAT0000081	miR-92a-3p	-0.262	Experimentally Observed	RAF1
MIMAT0000081	miR-92a-3p	-0.262	High (predicted)	PIK3R3
MIMAT0000081	miR-92a-3p	-0.262	Moderate (predicted)	NRAS
MIMAT0000081	miR-92a-3p	-0.262	Experimentally Observed	MAPK1
MIMAT0000081	miR-92a-3p	-0.262	Experimentally Observed	MAP2K1
MIMAT0000081	miR-92a-3p	-0.262	Moderate (predicted)	HDAC9
MIMAT0000081	miR-92a-3p	-0.262	Moderate (predicted)	HDAC2
MIMAT0000081	miR-92a-3p	-0.262	Experimentally Observed	CDKN1A

MIMAT0000429	miR-137-3p	-0.290	High (predicted)	SP1
MIMAT0000429	miR-137-3p	-0.290	High (predicted)	PPP2R5C
MIMAT0000429	miR-137-3p	-0.290	High (predicted)	PIK3R3
MIMAT0000429	miR-137-3p	-0.290	High (predicted)	AKT2

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