Protocol Title	**The Role of Social Partners in Buffering Physiological Indicators of Stress in Adolescents: Dyadic Social Buffering**
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Student Investigator	NA
Scientific Assessment	Nationally-based, federal funding organizations
IND/IDE # (if applicable)	N/A.
IND/IDE Holder	N/A.
Investigational Drug Services # (if applicable)	N/A.
Version Number/Date:	V.3.3 3.31.23

PROTOCOL COVER PAGE

REVISION HISTORY

Revision #	Version Date	Summary of Changes	Consent Change?
V2	08.02.19	Added Youth self report measures: 3 RSQ scales and 1 PALS scale	no
V3	4.29.21	Study is Redesigned for ONLINE collection (no face-to-face sessions). Consent process will be online (REDCap), sessions will be conducted over HIPAA-Compliant Zoom, recorded to Box, parent & youth report will be collected via REDCap. There will be no cardiac measures taken, or nurse Tanner exams. Instead, we will measure salivary alpha amylase from saliva already obtained, and gather self/parent report of physical development. Payments were updated to suit current visits, and self-reports from parent & youth were modified for greater consistency. Specifically, all youth complete the same youth packets, and the parents in the 3 active conditions each complete the parent packet; the contents of the packets don't change. For clarity, we now have assent/consent versions for each of the 4 conditions.	YES
V3.1	9.23.21	Prior to enrollment, noticed and repaired inconsistency between assent and protocol. Assent forms now include text declaring ourselves to be mandated reporters, as the protocol states. Added personnel, as we are ready to launch collection.	
V3.2	11.12.21	Add Zach Miller, Kalina Chang to personnel, and replace recruitment flyer with version that matches the V3.1 protocol and has accurate contact information.	

		In addition, in an effort to expand the pool of potential participants, so that we have a full and inclusive sample, we would like to reach out to local private schools and request to include our flyers either in their newsletters, parent groups, social media pages, etc. The PI, Dr. Gunnar, would call and/or send the attached letter making this request. Essentially, the letter requests permission to post the approved flyer/info. Note that the letter, attached as a supporting document, references two studies from the same grant. We will need one common letter, but each study will seek approval of that letter within it's IRB protocol. The mod requesting that for the other study is: MOD00028164 Modification #27 for Study MISTiC Social Buffering We also want to offer each participant a fruit snack at the start of the session. Prev research indicates that people cannot mount a strong cortisol response when they are low in blood sugar, so a fruit snack will create a common baseline for all participants. The snack can be declined without consequences to the study, and no change will be made to the consent form.	
V3.3	3.31.23	Remove Naushil Sridhar from personnel. We wish to use social media (eg., Facebook, Twitter, Instagram) and list serves (eg., ICD or CEHD list	

		serve) to share contents of the approved 11.12.21 recruitment flyer. We would share that flyer, only modified for format. We wish to increase the pool of potential partcipants. For social media posts, commenting will be disabled, to prevent sharing of personal information or inappropriate comments.	

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ABBREVIATIONS/DEFINITIONS

- TSST-C: Trier Social Stress Test - Children
- CORT, AUC: salivary cortisol, area under the curve
- sAA: salivary alpha amylase
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-
- ICD: Institute of Child Development

1.0 Objectives

1.1 Purpose: The purpose of this experiment is to determine whether social buffering by friends of stress physiology remains effective later in puberty when friends share the load versus when they provide support but are not undergoing the stressor with the target child. There are four conditions: (1) Friend and Target both undergo the stressor, (2) Friend provides support but does not undergo the stressor, (3) Unfamiliar Peer and Target undergo the stressor, and (4) Alone (no partner).

2.0 Background

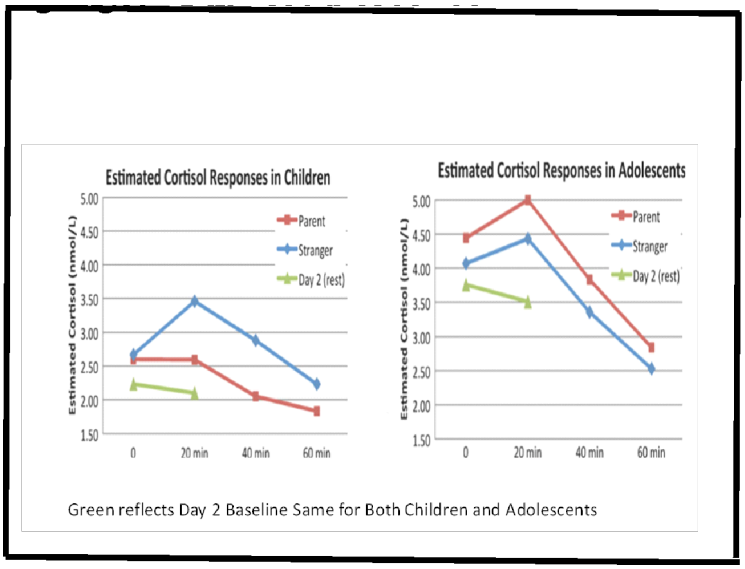
2.1 Significance of Research Question/Purpose: Adolescence is a dynamic period of change that both increases vulnerability to emotional and behavioral problems and creates opportunities for healthy development (Suleiman & Dahl, 20117). Becoming emotionally autonomous from parents is a critical developmental task of adolescence. While normative and essential, this autonomy also poses risks, including loneliness and social vulnerability (Laursen & Hartl, 2013). Traditionally, researchers have emphasized psychological processes secondary to hormonally induced physical changes to explain the increased risks of this period of development (Laursen & Hartl, 2013). Neuroscientists have begun to examine neurobiological changes that may also underlie these risks (Suleiman & Dahl, 20117; Wong, Yeung, & Lee, 2016). Recently, our group identified a normative pubertal change that could form a significant risk for stress-related problems. Specifically, puberty appears to disrupt the capacity to use one of the most powerful stress protective mechanisms in mammals: social stress buffering, or the ability to use the presence of relationship partners (parents, friends) to reduce physiological stress responses (Doom et al., 2015; Gunnar & Hostinar, 2015; Hostinar et al., 2015; Hostinar et al, 2014). This disruption of social buffering effectiveness is likely to be age-limited as, under the same conditions we used, adults (including college students) gain stress-buffering relief from the presence and availability of friends and partners (see review, Gunnar, 2017). *Because poorly regulated physiological stress-response systems can contribute to the development of affective disorders in vulnerable youth, it is critical that we understand the scope of this loss of buffering effectiveness.* If the loss is limited to the HPA axis where we have demonstrated it, that is important enough, but if it extends to other stress- and emotion-mediating systems, it could be highly significant for our understanding of the increase in affective disorders in young adolescents. *In our opinion, the proposed work is significant because normative changes in stress- and emotion-regulatory processes over the adolescent transition are currently under-appreciated as potential contributors to the increase in emotional and behavioral disorder observed during this period.*

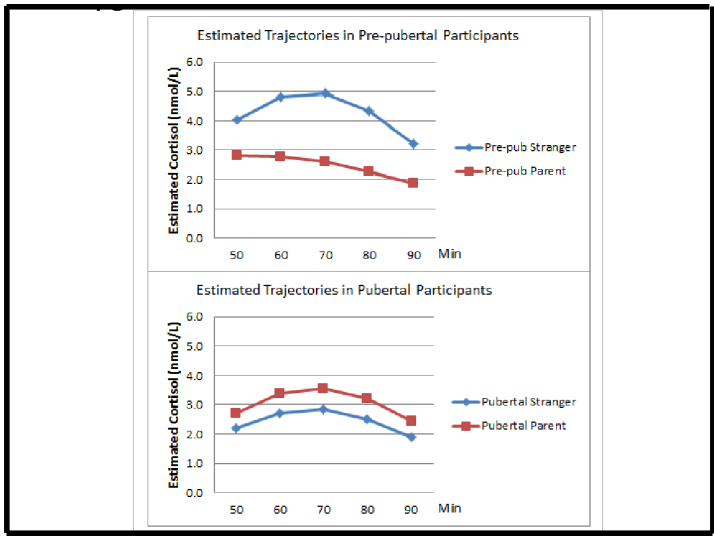
2.2 Preliminary Data: See 2.3, where Literature & Data are integrated. **Note that these lay the ground work for additional studies not proposed here, but associated with this NICHD grant.**

2.3 Existing Literature: Social buffering, a key concept in the psychobiology of stress, describes a phenomenon in which the presence and availability of social partners reduces activity of threat- and stress-mediating neurobiological and neuroendocrine systems (Gunnar, 2017). It is a key pathway through which social support reduces stress (Uchino t al., 2012) and enhances health (Kaplan et al., 19770. Impaired social buffering is a primary pathway through which adverse childhood experiences *get under the skin* to affect development (Hostinar et al, 2014). Social buffering is common to social mammals, although the most effective partners vary by species, sex and development (Hennessy et al., 2009). In the lexicon of social support, social buffering is *received* support as someone actually is present to provide help (Uchino, 2009). Received social support can be counter-productive if it heightens social evaluation (the support person becomes yet another evaluator) and/or reduces a sense of competence (I am here because you are not capable of helping yourself) (Thorsteinsson & James, 1999). Notably, social buffering has its roots in attachment security and is related to friendship quality (Allen et al., 2015). Drawing on evolutionary theory, attachment figures are described as "prepared safety signals" that throughout evolution have become associated with threat-reduction (Hornstein et al., 2016). *The pubertal shift away from parents and towards peers (who are not attachment figures), potentially creates conditions in which adolescents may be somewhat bereft of potent social buffering support figures.* Finally, Social Baseline Theory (Beckes & Coan, 2011) argues that being with others is our baseline state. Social partners reduce stress, in part, because the load is shared. In addition, when we are alone, we activate vigilance and threat-preparedness systems. Most of the work derived from theories of social support, prepared safety signals, and social baseline theory has focused on adults. Work on social buffering has involved both adults and infants of a variety of species (Hennessy, Kaiser, & Sachser, 2009). *But, despite its centrality to stress regulation, in humans we know relatively little beyond the infant years about the normative development of social buffering.*

Social Buffering of the HPA Axis and Development: The HPA axis is one of the two central arms of the mammalian stress system whose activity affects physical and emotional development (Gunnar, 2017). We have shown that social buffering becomes an increasingly powerful HPA axis regulatory mechanism over the first year of life, so that by one year the presence of the child's attachment figure in secure relationships completely blocks elevations in cortisol to events that are distressing to the infant (Ahnert et al., 2004; Gunnar & Donzella, 2002).. Parents remain effective social buffers throughout childhood. Among 9- and 10-year olds, preparing for the Trier Social Stress Test (TSST) with a parent completely blocked elevations in cortisol to this social evaluative stressor (Fig 1; Hostinar et al., 2015). Puberty results in a waning of parental HPA stress buffering effectiveness. Preparing for the TSST with the parent vs experimenter had no effect on 15- and 16-year olds or on 11-14 year olds who were at

more advanced pubertal stages (Fig 2; Doom et al., 2016). Supportive friendships are associated with many positive outcomes for children, while their lack is associated with negative affect (Laursen & Hartl, 2013). However, counter to our initial expectations, preparing for the TSST with a friend *increased*, rather than reduced the cortisol response for adolescents (Doom et al., 2015). Having the friend "help" may actually have amplified social evaluation. Because so many stressors of adolescence involve social evaluation, it was striking to find that being supported by a peer actually increased the HPA stress response. This led us to hypothesize that early adolescence may be a period of time when social buffering effectiveness decreases, both when provided by parents and by peers. This change in social buffering may enhance the young adolescent's risk for stress-related physical and mental health problems.





School-aged children do seem to be able to use a friend's presence to reduce cortisol to self-reported negative events that both the child and friend are experiencing (Adams, Santo, & Bukowski, 2011). Beyond our own work, we know of only two studies examining friends as social buffers of the HPA axis in adolescence. One (Bryd-Craven et al., 2008) experimentally examined co-rumination and found that when girlfriends were assigned to a co-rumination condition, their cortisol levels were higher than in the comparison condition. The other (Calhoun et al., 2014) examined youth 11-16 years who were accompanied by a friend who they were allowed to talk with AFTER the TSST. They found no evidence that positive qualities of the friendship influenced HPA recovery, but negative qualities slowed recovery.

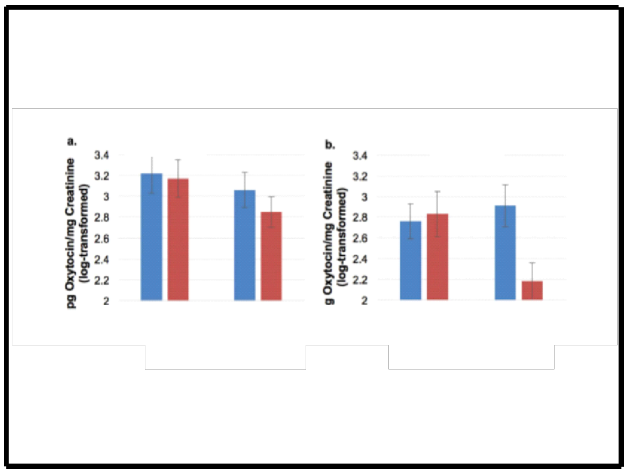
(Note: Background to Aim 1 removed, as only relevant to separate MRI study, submitted as separate IRB study.)

Background Aim 2: To determine the pervasiveness of shifts in the effectiveness of social buffers over the peripubertal period. *Hypothesis 2a. If the waning of parental buffering seen in cortisol responses reflects a general physiological change during puberty, this waning will also be observed in the sympathetic and parasympathetic systems and in oxytocin production, as well as in the neural systems*

examined in Aim 1. Stress-mediating systems do not respond uniformly and are frequently dissociated (McEwen, 2006). The sympathetic nervous system (SNS, norepinephrine) tends to track effort as well as threat, while the HPA axis and the sympathoadrenal (SAM, epinephrine) axis tends to be more narrowly responsive to physical and psychological threats (Goldstein & Kopin, 2008; Ursin, Baade, & Levine, 1978). We have measured salivary alpha amylase (sAA, an indirect index of norepinephrine) in response to the TSST with and without parental buffering and found no buffering effect on sAA despite strong effects on the HPA axis (Doom, Hostinar, VanZomeren-Dohn, & Gunnar, 2015). There are two reason that sAA may not have been responsive to social buffering in that study. First, only in the speech preparation period when the children sit silently and think can autonomic activity in response to emotions be clearly differentiated from autonomic activity in response to the physical demands of standing and talking. We were not able to isolate the speech period in Doom et al., 2015, but will be able to in the proposed studies. Second, sAA does not provide a measure of the SAM system which is needed in order to threat/stress more closely. Assessing pre-ejection period (PEP) which is a better measure of SAM might have yielded different results (see study 3). We know of one other study examining social buffering of the SNS in teenage youth (Lougheed et al., 2016). In this study, girls gave a short talk in front of an experimenter while galvanic skin responses (GSRs) were being measured. The girls were accompanied by their mothers who were assigned to either put their hand over their daughter's hand or not. GSR was lower in the touch than no-touch condition, but only for mother-daughter pairs who were low in quality. This study did not include a "no mother" condition, so it may be that the mother's mere presence reduced sympathetic reactivity in high quality relationships. However, it is critical to have a no buffer condition in order to map developmental changes in parental (and peer) social buffering.

Stress buffering can involve both reduction of threat perceptions and mobilization of protective processes that restore pre-stress functioning. Increased activity of the *parasympathetic system (PNS)* may be enhanced by the presence of supportive social buffers. *Oxytocin may mediate these responses as it has regulatory impacts on the autonomic nervous system and on the HPA axis (Heinrichs et al., 2003; Uvnas-Boberg, 1997).* Adults who show large cortisol responses in combination with large oxytocin responses to the TSST exhibit faster PNS recovery (Engert et al., 2016). Nasal oxytocin administered before the TSST reduces cortisol responses to the TSST and is most effective when combined with the presence of a social buffer (Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003). We (Doom, Doyle, & Gunnar, 2016) examined urinary oxytocin before and after the TSST when children and adolescents were provided with either the mother or a best friend (Fig 5). Adolescents showed lower levels of oxytocin than children; more importantly, while we did not see an increase in oxytocin in response to the TSST, we did find that in both children and adolescents, preparing with their mothers maintained oxytocin levels. Notably, boys preparing with a friend actually showed suppression of oxytocin, suggesting different mechanisms of social support for boys and girls that emerge with puberty. *In all of the proposed studies, we will not only*

measure activity of the HPA axis, but also activity of the autonomic nervous systems and of oxytocin.



Social buffering is an example of received support. Often there is one person who is being stressed and another who is providing support. Received support can backfire if the support person evaluates the support recipient or acts in a way that makes the recipient feel less competent (Uchino, 2009). Giving support also relieves stress and perhaps enhances one's sense of competence (Eisenberger, 2013). Teens are strongly motivated to become part of a group. Satisfying this need with high quality friendships positively predicts adult health (Allen, Uchino, & Hafen, 2015). From an evolutionary perspective, being with others has tremendous threat-protection value, and may be our baseline state (Beckes & Coan, 2011). While a teen might take a friend with her as received support when getting her ears pierced, it is also very common for youth to experience stressors in a group or with their friends who are going through the same stressful experience. During high-stakes exams or while walking school hallways where older kids are making fun of younger kids, it is intuitive that being with a friend helps make those situations less stressful. But, the friend is not only there as a support figure. Instead, both friends are giving AND receiving support from one another in these situations. We will examine a social evaluative stressor under four between group conditions: (1) two friends both experiencing the stressor, (2) two unfamiliar peers both experiencing the stressor, (3) one recipient and one giver of social support, and (4) one individual experiencing the stressor with no social support. *We predict that when two friends are in it together, being both recipients and givers of support, being with a friend will reduce physiological stress responding. However, when one friend gives support and the other receives it, the friend's presence will increase stress responding (as in our previous study, (Doom et al., 2016).*

Background Aim 3: **To examine the emergence of sex differences in social buffering effectiveness with puberty.** Basal cortisol levels increase from childhood to adolescence as does HPA reactivity to social evaluative stressors (van den Bos et al., 2016). These changes are related to pubertal stage (Adam, 2006). Sex differences in the cortisol response to social evaluative stressors are not consistently observed until later in puberty when, similar to adults, males elevate salivary cortisol more to these stressors (e.g., Bouma et al., 2009). Among adults, the patterns of sex differences in HPA axis functioning are complex, and are reflected in some but not all measures (Kudielka & Kirschbaum, 2005). Notably, the larger cortisol response for men is found in salivary (free/unbound) cortisol but not total cortisol. Women in the luteal phase of their cycle showed similar cortisol levels to men, but not women in follicular phase (Kudielka & Kirschbaum, 2005). Thus, it is likely that this finding relates to estrogen and progesterone influences on cortisol binding globulin, and not to reactivity of the axis, per se. Nonetheless, as girls begin to cycle more regularly and produce increased levels of

estrogen and progesterone across the cycle, we might expect to see similar variations in salivary (free) cortisol levels and reactivity. Therefore, we will have girls report on the date of their last period and whether their periods are regular. We can use these data if we find sex differences in cortisol reactivity.

With the exception of our oxytocin findings, we have not observed sex differences in our social buffering work. However, in adults, sex differences have sometimes been found. To the extent that changes in social buffering effectiveness track pubertal development, it would make sense that sex steroids may play a role in this phenomenon. As these steroids differ among girls and boys, we might expect them to influence sexually-dimorphic patterns of social buffering as puberty proceeds. We will collect a sufficient amount of saliva to be able to assay reactivity in sex steroids if we find sex differences in social buffering emerging with increased pubertal development. Among adults, generally speaking, there is more evidence of HPA axis buffering for men and SNS buffering for women (Gunnar, 2017). Women, it is argued, cope more by tending and befriending, which might suggest greater estrogen-oxytocin interactions, while men cope more by fight and flight, which might suggest more testosterone-sympathoadrenal interactions (Taylor et al., 2000). However, if coping strategies tend to differ between men and women, it is not clear when these differences emerge. Youth report increased reliance on friends vs. parents as they move from childhood through adolescence, and this reliance does differ by sex of parent and youth (Furman & Burhmester, 1992). Teens also report a decrease in emotional closeness to parents with pubertal development, although less so for girls and their mothers (Steinberg, 1987). These changes in gender differences in parent-child and friend-friend relationship from childhood to adolescence may have implications for social buffering. Indeed, as described above, there is one study suggesting that 15-year-old girls may still use their mothers to regulate sympathetic responding to a mild social evaluative stressor (Lougheed, Koval, & Hollenstein, 2016).

3.0 Study Endpoints/Events/Outcomes

3.1

3.2 Primary Endpoint/Event/Outcome: salivary cortisol, salivary alpha amylase
Secondary Endpoint(s)/Event(s)/Outcome(s): Exploration of possible sex differences in social buffering in different conditions later in puberty but not earlier in puberty.

4.0 Study Intervention(s)/Investigational Agent(s)

4.1 Description: Note that there are no investigational agent(s) (e.g., drug, device) being evaluated. The study qualifies as an intervention due to random assignment into social buffering condition, to determine the effect of social partner to stress response.

4.2 Drug/Device Handling: NA

4.3 Biosafety: NA

4.4 Stem Cells: NA

5.0 Procedures Involved

5.1 Study Design: **Rationale:** Adolescents experience social evaluation stress frequently. However, it is likely that often they are not alone, but with friends who are also going through the same experience. Thus, it is possible that under these conditions, social buffering by friends does not wane over the peripubertal period. Participants will be assigned to social conditions while engaging in an evaluative stressor task.

All sessions will occur ONLINE in a modification to accommodate COVID-19.

During Session 1, which will occur using the HIPAA-compliant version of Zoom the full consent & assent process will take place with parent & youth completing forms via REDCap. All youth who complete the TSST will have a full Session 1, while families whose youth merely attend as support will be given the study description by email or phone, and will be sent a link to REDCap for signing consent/assent. Parents will supply background information on family income, education and composition. Parents and the youth will complete the MacArthur Health and Behavior Questionnaire items for the Internalizing, Externalizing and ADHD scales, and reports of pubertal development. Youth will complete the Network of Relationships Questionnaire and other similar surveys in which they respond to questions about their relationships with their parents, their friend, and peers in general. Session 2 occurs approximately 2 weeks after Session 1. If the youth is in the one of the close friend conditions, their same sex close friend will also accompany them online. If they are in the unfamiliar peer condition, an unfamiliar peer of the same age and sex will be recruited and arrive online for session 2, likelywith their parent. The TSST involves developing and delivering a speech and doing mental arithmetic while being judged/evaluated by two judges while being filmed over HIPAA-compliant Zoom. We will collect saliva for cortisol and alpha amylase assessment. Breakout rooms will be used in Zoom to create the different environments described below. For example, the session will start with a friendly experimenter who guides people through the online session, and participants will be sent to breakout rooms to either wait their turn or engage in the TSST task, and return to the main room with the friendly experimenter. We have successfully used this procedure in a recent study nearly identical to this one, with the exception of the Peer conditions.

There are four conditions in this experiment: Close Friend Who Also Completes the TSST, Close Friend Provides Social Support, Unfamiliar Peer Who Also Completes the TSST, and No Partner. The Close Friends and Unfamiliar Peer will complete the Network of Relationships Questionnaire and similar surveys about their relationship with the Target youth (Close Friend Conditions) or their own same sex best friend (Unfamiliar Peer). Both Close Friends and Unfamiliar Peer who complete the TSST will undergo the same procedures as the Target Child during session 2,

including salivary measures. Thus, we will have 50 boys and 50 girls who undergo the same procedures in parallel with the Target youth. We will have an additional 25 boys and 25 girls who provide social support but do not go through the same procedures.

5.2 Study Procedures:

200 target children, 11- 14 years of age will be tested in one of four conditions: 1) Close Friends Both Tested, 2) Unfamiliar Peers Both Tested, 3) Close Friend Gives Support, and 4) No Social Support.

Social Buffering Manipulations: Will include 4 between-subjects conditions and both sexes. Participants will be randomly assigned to Close Friend-Both Tested, Unfamiliar Peer-Both Tested, Received Support from Close Friend, and No Social Partner conditions. Definition of Close Friend. We will ask the parent to have the child select the close same-sex friend they want to have accompany them online. The parent then has to contact that child's family and get their permission for us to contact them. We have successfully conducted studies in which the child brings their best friend.

Stressor Paradigm: TSST paradigm with the child facing a camera and two judges (one male, one female). After consent, parents are invited to exit the call (or leave the room); in our recent experience with a Zoom'd TSST, youth and parents readily agreed to this separation. A period of 'rest' begins where youth watch approximately 20-25 minutes of a mildly amusing child appropriate animated movie. The experimenter then sends them to a breakout room to begin the TSST sequence. They are greeted by 2 "judges", given instructions for the speech and told that they have 5 min to prepare. During that time, the judges turn off their audio/video but clearly remain in the breakout room. When 5 minutes elapse, the judges reappear. If the condition is one of support, the Peer or Friend is sent back to the main room and either released from the call or told to continue preparation for another 10 minutes until it is their turn. The judges give final speech instructions, and tell the youth to begin their speech, and then transition to the math. That participant is then excused back to the main room. If the condition calls for a Peer or Friend to perform the TSST, they are then sent to the judging breakout room and deliver their speech/math. After each participant completes the speech/math, they return to the main room and are greeted by the friendly experimenter, who guides them through REDCap links to the various surveys. At several points in session 2, saliva samples are obtained.

Note: In all conditions, one participant will be the target, even though in two of the conditions (Close Friends and Unfamiliar Peer Both Tested), both youth will go through the TSST and all physiological measures will be obtained. The reason for this is two-fold: (1) when we recruit we will know

which youth is the target and this is the youth we select to balance pubertal stage and age, while in the close friend conditions, the close friend only needs to be within two years the age of the target youth; (2) in the "Close Friend Gives Support" condition, the friend will not be assessed and will only be there for moral support and to provide information on his/her friendship with the target youth. The collected data from the non-target youth will be analyzed using non-sponsored funds available to the PIs and co-regulation analyses may be conducted.

Note: These sessions will be recorded, as part of the challenge, to document the session, and to make behavioral response to the task available for later coding.

Pubertal Development: All youth will also complete the Morris and Udry (1980) pubertal development questionnaire, which allows placement in Tanner stages for hair, testicles, and breast development.**Questionnaires.**

Demographic Questionnaire: Parents will complete information on pre-tax family income, education level of parent(s), composition of the household, and medications that the child regularly takes.

Daily Diary: The parent and the youth both will report the following for the child that day: the time of wake up, estimated duration of sleep, medication usage, illness/fever, physical activity, and caffeine consumption.

Relationship Quality: Participants will complete the Network of Relationships Inventory - Behavioral Systems Version (NRI-BSV, Furman & Buhrmester, 2009) for the parent who accompanies them and their close friend. This questionnaire is based on an integration of attachment and Sullivanian theory and assesses three systems that are expected to be key in close relationships: attachment, caregiving and affiliation. The scales are: companionship, seek safe haven, seek secure base, provide safe haven, provide secure base, conflict, antagonism and criticism. We will also add 3 scales from the NRI-Relationship Qualities Version: Emotional Support, Approval and Satisfaction. Each scale consists of 3 items scored on a 5-point Likert scale. Psychometric qualities are high. Condition differences in quality of relationships will be examined and included as covariates if groups differ significantly. Otherwise, in follow-up analyses we will examine whether social buffering effects are stronger in relationships the child views as more supportive.

MacArthur Health and Behavior Questionnaire (HBQ) for 9-18 Year-Olds (Parent and Child, 2.1): This questionnaire was developed by Marilyn Essex based on the original HBQ for 4-8 year olds. We have used it many times. The scales we proposed to use here have high reliabilities (Cronbach

alpha's> .8). We will use the peer scales (acceptance/rejection, bullied, relational victimization, asocial, behavioral inhibition) and broad-band symptoms scales (Internalizing, Externalizing). These measures will be examined for balance across groups and if differences by condition, pubertal stage, or sex are found, will be entered as covariates.

Self-Report of Stress: As a manipulation check, participants will rate their arousal and emotional state at various points during each study using Likert-type scales

Version 2.0 addition: Added Child-self-report RSQ for Peer, Family, & Academic Stress, and PALS Student report of adaptation to challenge in environment.

Physiological Measures.

Stress Hormones: We will assay saliva samples for stress sensitive hormones: **cortisol** and **alpha-amylase**. Saliva will be collected using SalivaBio Oral Swabs (SOS) or passive drool and stored frozen at −30°C until shipped for assay. All testing will be conducted in the late afternoon to control for circadian rhythm. Medications will be recorded from parent report and classes of drug codes will be used to group meds by presumed mechanisms of action and will be entered as covariates (Granger et al., 2009). Systemic glucocorticoid medications are an exclusion criterion. For each analyte, we will calculate area under the curve from intercept (AUCi) to provide a single measure for the main analysis.

5.3 Study Duration: Participants will attend two sessions. Session 1 will be 1-1.5hr; Session 2 will be 2-2.5 hrs. It is expected that it will take approximately 2 years to enroll all study participants, and that the study will end, including data analyses, approximately 5 years after enrollment begins.

5.4 Individually Identifiable Health Information: NA. We do not intend to use individually identifiable health information in this study, with the exception of date of birth. Birth date will be maintained in order to ensure the correct age of the participant at the time of participation. However, this will be deleted once age at participation is obtained. This data will be collected within REDCap, using appropriate limits on data-sharing to de-identify.

5.5 Use of radiation*: NA*

5.6 Use of Center for Magnetic Resonance Research*: NA*

6.0 Data and Specimen Banking NA

7.0 Sharing of Results with Participants

7.1

7.2

7.3 Results will only be shared with participants in aggregate.Regarding other biologic measures we take, abnormal values are commonly due to an interfering substance present in the sample (ex, milk for salivary cortisol). Without a body of evidence, it seems unethical to alarm families given an out-of-range assay value and therefore we do not plan to share individual results with participant.

8.0 Study Population

8.1 Inclusion Criteria: Healthy 11-14 year olds. Additional inclusion criteria are imposed in ensure that youth will be able to follow the study procedures, sufficient vision, hearing, and language skills to provide verbal and written assent, see and read stimuli presented on the computer screen, and hear verbal instructions provided by the experimenter and judges.

8.2 Exclusion Criteria: Premature birth (less than 37 weeks), congenital and/or chromosomal disorders (e.g. cerebral palsy, FAS, mental retardation, Turner Syndrome, Down Syndrome, Fragile X), Autism Spectrum Disorders, history of serious medical illness (e.g., cancer, organ transplant),serious psychiatric illness, or youth taking systemic glucocorticoids or beta-adrenergic medications.

8.3 Screening: Screening will occur by asking parents questions at the time of recruitment. To reduce confounding age and pubertal stage, we will use a stratified recruitment method. During recruitment, parents will be asked 3 questions about their child's physical development, and children will be initially categorized into Pre/Early and Mid/Late Puberty. As cells fill, participants will no longer be enrolled into those cells. Also, to increase minority representation we will also sample across zip codes with higher racial and ethnic minority representation.

9.0 Vulnerable Populations

9.1 Vulnerable Populations:

Population / Group	Identify whether any of the following populations will be targeted, included (not necessarily targeted) or excluded from participation in the study.
Children	Targeted Population
Pregnant women/fetuses/neonates	Excluded from Participation

Prisoners	Excluded from Participation
Adults lacking capacity to consent and/or adults with diminished capacity to consent, including, but not limited to, those with acute medical conditions, psychiatric disorders, neurologic disorders, developmental disorders, and behavioral disorders	Excluded from Participation
Non-English speakers	Excluded from Participation
Those unable to read (illiterate)	Excluded from Participation
Employees of the researcher	Excluded from Participation
Students of the researcher	Excluded from Participation
Undervalued or disenfranchised social group	Included/Allowed to Participate
Active members of the military (service members), DoD personnel (including civilian employees)	Included/Allowed to Participate
Individual or group that is approached for participation in research during a stressful situation such as emergency room setting, childbirth (labor), etc.	Excluded from Participation
Individual or group that is disadvantaged in the distribution of social goods and services such as income, housing, or healthcare.	Included/Allowed to Participate
Individual or group with a serious health condition for which there are no satisfactory standard treatments.	Excluded from Participation
Individual or group with a fear of negative consequences for not participating in the research (e.g.	Included/Allowed to Participate

institutionalization, deportation, disclosure of stigmatizing behavior).	
Any other circumstance/dynamic that could increase vulnerability to coercion or exploitation that might influence consent to research or decision to continue in research.	Excluded from Participation

9.2 Additional Safeguards:

Children are the targeted population for this study. Others that have been checked as "allowed" above are not being intentionally included or excluded. For example, we will not inquire about someone's membership in a disenfranchised group. Our careful consent/assent procedures designed to handle research with children should be sufficient to cover other vulnerable participants who may inadvertently enter our study.

All of the subjects will be children, ranging 11-14 years old. This study focuses on adolescents because its goal is an analysis of the impact of social buffering and puberty on stress-mediating systems. Children have been the focus of my research for the past 30+ years. My staff is highly trained in working with children and families. The graduate students working on the project will be Child Psychology and/or Child Clinical Psychology Ph.D. students. The undergraduate research assistants will, for the most part, be majoring in child development.

Our recent experience conducting a study using an online version of the TSST (Zoom/REDCap) with youth aged 14-16) was very positive and well received.

The risks of this study are generally minimal. For the youth, they largely include embarrassment while completing the puberty questionnaires and distress/anxiety during the public speaking task. Youth may experience concern when completing questionnaires dealing with emotional problem symptoms. Parents may experience concern when completing interviews about youth psychiatric symptoms. Any time you work with youth there is the possibility that they will reveal information about abuse and/or indicate that they are thinking of hurting themselves or others. We are mandated reporters, a fact that is revealed in both consent and assent forms. Parents provide consent, youth provide assent, and see below where that process is fully described to ensure that youth have full understanding and are free to decline.

10.0 Local Number of Participants

10.1 Local Number of Participants to be Consented: We plan to analyze at least 200 target participants. Of those 50 will participate alone, while 150 will participate with a social peer. Social peers will be enrolled as they will at least complete a questionnaire. Thus, we will enroll at least 350 children. To account for attrition between sessions/replacement for incomplete data, we anticipate up to 400 children may be enrolled.

11.0 Local Recruitment Methods

11.1 Recruitment Process: Families will be contacted by phone or email and the procedures will be described. If the family is interested, the first online visit will be scheduled. Copies of the consent and assent forms will be sent to families prior to the visit, along with directions to the University, and a reminder call will be a chance for families to confirm their interest in participation after having seen the consent forms. The formal consent process will occur at the first visit .

Regarding recruitment of the social peer to the target, when that peer is a friend: The family of the participant will contact the family of the friend make sure that they have permission to give us their contact information. Parents of friends who participate will then be emailed a REDCap link to a consent formthat they can complete and sign online before the session.If they do not respond to email in 3 days, our recruiter will call them for a reminder. There will be phone and email contact information so that parents can ask any questions they have before their child comes to theonline. In the event that we are unable to obtain consent for the friend prior to the online session, that session will be converted to the “alone” condition. If we have parental consent for the friend, but not assent by the time of the online session, the assent form will be provided to the friend at session 2, and will also have a line that the parent of the primary participant can sign as a witness to the consent process. We have successfully used this method in the past.

11.2 Identification of Potential Participants: Potential participants will be identified using the ICD registries of families interested in being contacted about research. Those families have agreed to be contacted, and once ICD Registry procedures have been followed, including proof of IRB approval, an encrypted list is provided to study staff. No protected records are involved. Study staff will make initial contact. In our 3/31/23 revision, we use social media (eg., Facebook, Twitter, Instagram) and list serves (eg., ICD or CEHD list serve) to share contents of the approved 11.12.21 recruitment flyer. We would share that flyer, only modified for format. We wish to increase the pool of potential partcipants. For social media posts, commenting will be disabled, to prevent sharing of personal information or inappropriate comments.

11.3 Recruitment Materials: We will recruit via telephone and email (as parents have given us both means of contacting them). We will also post flyers and put the flyer information on our website so that families can contact us. Materials are uploaded in ETHOS.

11.4 Payment: In the four conditions, as follows:

1. No Social Support: We would give the child up to $50 and parent up to $20 in debit cards for participating. 2. Unfamiliar Peers Both Tested: For each of the two participants: We would give the child up to $50 and parent up to $20 in debit cards for participating. 3. Close Friend Gives Support: We would give the child up to $50 and parent up to $20 in debit cards for participating. We would give the friend $20 debit card for attending, as well.

4. Close Friends Both Tested: For each of the two participants: We would give the child up to $50 and parent up to $20 in debit cards for participating.

We intend to use the prepaid debit cards: Greenphire ClinCard for compensation, and have included appropriate language in the consent document. (We will provide a Target gift card instead of the ClinCard if parents object to the terms and conditions of the ClinCard, tho in our experience, this is very rare.) Families will be mailed empty cards, which will be loaded following receipt of the card and completion of the sessionsWithdrawal of Participants

11.5 Withdrawal Circumstances: If participants are enrolled (consent signed) and subsequently determined to meet exclusion criteria, their data will be excluded from analyses.

11.6 Withdrawal Procedures: If they have a remaining session scheduled, we will cancel the session. If, however, cancelling will bring undue embarrassment to the child, in consultation with the parent, we will allow the second session to occur. Participants will not be contacted if their participation is complete.

11.7 Termination Procedures: Data will not be used after termination.

12.0 Risks to Participants

12.1 Foreseeable Risks:

Questionnaires: Youth or parents may experience concerns when completing questionnaires dealing with symptoms of behavior problems. Any time one works with youth there is the possibility that they will reveal information about abuse and/or indicate that they are thinking of hurting themselves or others. We are mandated reporters, a fact that is revealed in

both consent and assent forms. However, in over a decade of working with children and youth drawn from our participant files, we have never encountered a reportable incident, so we consider this risk to be very low.

Trier Social Stress Test for Children. This is a social evaluative stressor. Public speaking evokes anxiety in many individuals. Also, the task involves deception, in that the judges do not render judgements as part of the task

The TSST activates stress-mediating systems because it serves as a threat to the social self. Public speaking produces anxiety in most people. However, answering questions in class, speaking in front of the class or school, and solving problems in front of teachers and classmates is a common occurrence in children's lives. Thus, the TSST standardizes a common everyday stressor. To reduce risk, youth are told that they can stop without prejudice if they choose.

Regarding deception: Because they are told they are being evaluated (and this is a necessary part of the procedure), after each TSST session, the youth will be debriefed to make it clear that there were no judgements made about the speech or math performed. It will be explained that performance in a social evaluation task was used in order to elicit a physical stress response. The youth will never receive any negative evaluations from adults as part of being in this study. Our previous experience with this deception and debrief is that youth tolerate the experience very well.

Saliva Sampling. We will collect saliva using passive drool, so this risk is eliminated.

12.2 Reproduction Risks: NA

12.3 Risks to Others: NA

13.0 Potential Benefits to Participants

13.1 Potential Benefits: The only direct benefit to participants is the small financial remuneration and the knowledge that they have contributed to a better understanding of human development among youth.

14.0 Statistical Considerations

14.1 Data Analysis Plan: 200 primary participants, including 100 females and 100 males. Within each group there will be 50 early and 50 late puberty participants.

Study Design. This randomized control study will assign study participants into one of four conditions: Close friend both tested, Unfamiliar Peer both tested, Received support from close friend who is not tested, and no partner. To perform the randomization, participants will first be placed into one of four groups based on sex and pubertal stage: early puberty, female;

late puberty, female; early puberty, male; and late puberty, male. Next, participants within a group will be randomly assigned to one of four conditions equally in order to have a balanced design. Pubertal stage will then be treated as a continuous variable.

Statistical Methods

Preliminary Analyses to Determine Covariates Need for Primary Analyses. The purpose of collecting data on potential covariates is to identify variables that are correlated with both the independent variable (social buffering condition, sex, pubertal stage) and the primary outcome measures. Those covariates that are associated with the independent and outcome variables will be controlled for in the primary analyses. Those that are not will be used to describe the sample, but will not need to be included in the analyses to obtain unbiased estimates. For each potential covariate (listed as "other type" in section 4.3, Outcome Variables), we will conduct correlations or t-tests with pubertal stage, and t-tests, ANOVAs, or chi-square tests (as appropriate) with sex and condition. If data do not confirm to modeling assumptions, we will use non-parametric analogs instead.

14.2 Power Analysis: For the power analysis for the primary analyses, we calculated the minimum detectable effect size (MDES) assuming n = 200, α = .05, and power = .80 with a conservative estimate of 12 covariates in our model (see section 4.3). Power was calculated in R (R Core Team, 2018) using the pwr library (Champely, 2018). A power analysis was performed to quantify the MDES (measured as f^2) for a three-way interaction between the social buffering condition, sex, and pubertal stage within a multiple regression model controlling for all potential covariates, two-way interactions, and main effects (the most complex model considered in our primary analysis, see equation 1 below). Assuming these conditions, we should be able to detect an f^2 of 0.056. In other words, adding the three-way interaction to a model containing the covariates and all lower order interactions and main effects, the three-way interaction would need to explain an additional 5.6% of the variability to be detected. This falls between Cohen's criteria for a small (f^2 = .02) and medium-sized effect (f^2 = .15) (Cohen, 1988). With a sample size of 200, even if we lost 40 of the participants, assuming all the previous conditions except a sample size of 160, we would still be power enough to detect an f^2 of .070, or still within a small-medium effect size. As the number of covariates decreases, the size of the MDES will decrease. Therefore, this represents a conservative estimate of power for our primary analyses as we are likely to have fewer covariates in our models and will use multiple imputation to correct for missing data. For the two-way interaction and main effects models, described below, the power will be larger than these reported values as the

models will be simpler. In addition, we ran a power analysis that corrected for multiple comparisons using the conservative Bonferroni's adjustment. Because we expect to run 9 independent multiple regression models or fewer, we ran our models assuming an alpha of 0.05 / 9. Based on this reduced alpha, we expect to be able to detect an effect of .07.

14.3 Statistical Analysis: The primary analyses will consist of a series of multiple regression models. For each dependent variable, Cortisol AUCi, Alpha-Amylase AUCi,, we will begin by testing the three-way interaction between social buffering condition, sex, and pubertal stage for all outcomes. If the three-way interaction is not significant, we will then examine all two-way interactions. If the two-way interactions are not significant, we will then examine main effects only models. If the three-way interaction is significant, we will not examine any other model.

14.4 Data Integrity: *Multiplicity of testing*. We will correct for multiple comparisons using the Benjamini-Hochberg (BH) correction. The BH method works by controlling the false discovery rate, and relative to the Bonferroni's correction is more powerful, while still adequately protecting against Type I errors (Williams et al., 1999).

Missing data. If there is missingness in any of the dependent or independent variables, multiple imputation will be used (Little & Rubin, 2014; Peng et al., 2006; Schafer & Graham, 2002). Variables related to the missingness and the variables missing data will be included in the missing data model. In the event that missingness is not at random (MNAR), we will investigate the use of models for non-ignorable missing data.

15.0 Confidentiality

15.1 Data Security:

Only authorized research staff will have access to the data, and all data will be stored on encrypted servers or locked locations. Birth date will be maintained in order to ensure the correct age of the participant at the time of participation. However, this will be deleted once age at participation is obtained. In addition, recordings of the TSST session must be kept in order to code for participant, friend behaviors. Recordings and birth date will only be linked with participant ID number.

A password-protected document linking name and ID number will be kept until the conclusion of the study in case we must contact a family for any reason. We will use REDCap for this.This document will be destroyed at the conclusion of the study.

Password protection is used to protect data files. Because this session will be conducted online, we anticipate that all experimenter, parent, and youth reports will be completed on REDCap, and stored there or on

University Box. No consent/assent will be placed in participants' medical, employment, or educational records.

16.0 Provisions to Monitor the Data to Ensure the Safety of Participants

16.1 Data Integrity Monitoring.

All of the proposed procedures meet the NIH definition of minimal risk. The PI and study coordinator will meet regularly to ensure that study protocols are followed and best practices are applied. Study coordinator will bring any identified protocol violations to the PI in a timely fashion, and members will be re-trained if needed.

16.2 Data Safety Monitoring.

Overall Framework for DSMP

This project is considered a clinical trial because it involves a) random assignment to condition, and b) physiology is being measured.

There is no risk associated with the assignment to conditions (Close Friend-Both Tested, Unfamiliar Peer-Both Tested, Close Friend-Social Support Only, No Partner). All conditions resemble naturally occurring social situations that children might experience, and there is no inherent danger to one condition over another as there would be for, say, a drug vs placebo trial.

Further, in three of the conditions the presence of another person is expected to provide a social buffer whose stress reduction potency by condition is what is being tested. All target youth experience the same stressor, the TSST. The TSST mimics a naturally occurring stressor, as when the youth is asked to speak in front of the class and his/her performance is being evaluated. This can provoke anxiety.

The other potential risks of this study to which all of the participants are exposed regardless of condition have been eliminated in this online-only revision.

The steps taken to reduce or prevent these risks include allowing participants to refuse the nurse exam and use of proper saliva collection materials. These risks are all managed by the routine processes monitored by our IRB, including Serious Adverse Events (SAEs) such as deaths, hospitalizations, and life threatening events and Unanticipated Problems (UPs), will be managed and reported, as required, to the IRB and relevant monitoring authorities. All members of the project will have received human subjects training and certification in FDA Good Clinical Practice through the Collaborative Institutional Training Initiative (CITI) curriculum.

The individuals responsible for trial monitoring and advising the appointing entity. Because of the low level of risk in this study, the PI, Dr. Megan Gunnar will be responsible for reporting.

17.0 Provisions to Protect the Privacy Interests of Participants

17.1 Protecting Privacy: Once the participant has been tested, identifying information will be removed, and all materials will be identified with a participant number only. A master list with participant names, addresses and contact information will be maintained on REDCapseparately from the data and study materials. Only researchers involved in the study will have access to the data and the file that merges participant number and personal information.

Considerable efforts are made to make participants comfortable, despite the nature of the stressor in the second session. Consent and all study procedures will be conducted in private, tho online, rooms. During consent/assent multiple efforts are made to make it clear that participation is optional and the youth is in control of refusal. (Because cards will be mailed empty to prevent possible theft, cards will be loaded after the session is complete. However, we would not withhold payment to a participant who began a session and ended it due to discomfort. !) Collection of biological measures, administration of sensitive questionnaires are all done with a "matter of fact" attitude to minimize any feeling of embarrassment. Should a participant need to skip a portion or end a session early, experimenters are trained to accept this as a standard procedure when working with children rather than a problematic ending to the session. We have successfully guided hundreds of youth through these very procedures without undue problem, including longitudinal studies where the participants continue to return.

17.2 Access to Participants*:* We do not access medical records or other sources of private information.

18.0 Compensation for Research-Related Injury

18.1 Compensation for Research-Related Injury: All of the proposed procedures meet the NIH definition of minimal risk.

18.2 Contract Language: NA

19.0 Consent Process

19.1 Consent Process (when consent will be obtained):

-
- The consent process begins at recruitment. A full consent will occur online over Zoom, where we have been able to interact with them

privately in our experience. We control the environment at our end, and all staff take care to maintain a private environment. We send the consent to parents following recruitment, so that participants have typically 1-3 weeks to consider, before the online session and the full consent process.

- Researchers are trained and experienced consenters follow scripted protocols to determine that a potential participant understands the information. Open ended questions are gently used to assess understanding, for example, "can you tell me what we will do today?"
- At consent/assent, we make it clear that participation can end without negative consequences. Children are told that they can skip any task or stop the session as needed.
- Consent & Assent will be documented over REDCap.

19.2 Waiver or Alteration of Consent Process (when consent will not be obtained): N/A

19.3 Waiver of Written/Signed Documentation of Consent (when written/signed consent will not be obtained): N/A

19.4 Non-English Speaking Participants: N/A

19.5 Participants Who Are Not Yet Adults (infants, children, teenagers under 18 years of age):

- All participants will be children, recruited by contacting parents. Age will be verified with parent.
- Parental permission will be obtained from:
  - One parent, even if the other parent is alive, known, competent, reasonably available, and shares legal responsibility for the care and custody of the child.
-
- In the case of the "friend" to the target participant, we will obtain consent/assent by phone/email or Zoom. If we lack parental consent, that session converts to "alone", but if we merely lack assent, we will obtain that at the online TSST session, and include a line for target parent's signature as witness.
- All youth will be part of the consent process, will be asked questions to verify understanding, including the question of "when will you be allowed to skip a task or end the session?", and will sign an assent form.

19.6 Cognitively Impaired Adults, or adults with fluctuating or diminished capacity to consent:

- NA

19.7 Adults Unable to Consent:

- NA

20.0 Setting

20.1 Research Sites:

- Participants will be recruited from the ICD registry of potential participants, or self-identified thru flyer/website.
- Research will be conducted online, using HIPAA-Compliant Zoom and REDCap.

20.2 International Research: NA

21.0 Multi-Site Research

NA

22.0 Resources Available

22.1 Resources Available:

- There are sufficient potential participants on the registry to meet the needs of the study design/cell count. In the age range we would need over the years of this proposal there are 14,529 girls and 15,284 boys from which to draw.
- We anticipate it will take approximately 2 years to complete recruitment and testing.
- We do not anticipate the need for either medical or psychological resources that participants might need as a result of anticipated or unanticipated consequences of the research, as risk is minimal. However, Drs Gunnar & Thomas are widely connected to such resources at the University.
- All staff & lab personnel, including undergraduate students working with de-identified data, undergo required and available ethics training, and PI Gunnar's lab manager has over 25 years of experience training staff and conducting research with children. Weekly project meetings keep us adequately informed about the protocol, the research procedures, and their duties and functions.

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