Dr. Ned Kalin, chair of the Department of Psychiatry at the University of Wisconsin–Madison, has dedicated his career to decreasing the suffering of patients with psychiatric diseases. Alongside treating people, he has led research aimed at understanding how and why they develop problems like anxiety and depressive disorders. Those psychiatric illnesses cause millions of people in the United States immeasurable disability and suffering, sometimes leading to suicide.

One part of Dr. Kalin’s recent research examines the difference in brain function and structure in monkeys that serve as a model for the way human children develop anxiety disorders. Understanding those causes could help develop new treatments for people. The study drew attention for its original plan to separate some infant monkeys from their mothers and raise them together with other young monkeys in the attentive care of expert human staff. This method was expected to make the monkeys more likely to develop mild anxiety issues.

But a pilot study of young monkeys who were previously separated from their mothers for veterinary care reasons — some young monkey mothers do reject or neglect their infants — did not find those monkeys to be more vulnerable to anxiety. So Dr. Kalin and his colleagues decided not to separate infants from mothers, and to move ahead with a study of the natural range of individual differences in the development of anxiety.

Below is the plan — called a protocol — for this particular study approved by the UW–Madison animal care and use committee charged with reviewing this study. Animal care and use committees are required by federal law to ensure the ethical and humane treatment of research animals, and studies subject to their oversight cannot go forward without committee approval.

Dr. Kalin’s protocol was originally approved in 2014. He resubmitted the protocol in November of 2015 with amendments reflecting the changes prompted by the pilot study. In the version below, which was approved by an animal care and use committee in January of 2016, those changes are represented by passages crossed out and added in bold type. Black boxes in the document cover personal and security information redacted in order to protect the privacy and safety of staff and students, and the security of individuals and university research and property.

For more information on this study and other animal research at UW–Madison, visit animalresearch.wisc.edu.
UNIVERSITY OF WISCONSIN - MADISON ANIMAL CARE AND USE PROTOCOL: REVIEW FORM

Forms should be typed or in computer-printed format. PLEASE MINIMIZE formatting changes when preparing on computer.

PC & Macintosh word processing forms can be downloaded via the RARC homepage: http://rarc.wisc.edu/

Return completed forms to RARC (396 Enzyme Institute, 1710 University Ave., Madison, WI 53726).

Preferred method of delivery: attachment to e-mail (call 5-2696 or 2-7109 for e-mail address).

Form not required except for the page with PI signature, which must be sent or faxed (265-9040).

INVESTIGATORS: Animal protocols are assigned for review to the Animal Care and Use Committee(s) that provides oversight of the facility or facilities where the animals assigned to this protocol will be housed.

Questions? Call Debbie (262-7109), Helen (265-2696), or Holly (265-9241) at RARC, or consult the “Guide to Completing the Animal Use Protocol” on the RARC website.

Submission Deadlines by College or School:

• School of Medicine & Public Health: 4:00 pm the 15th of the month
• College of Agricultural and Life Sciences: 4:00 pm 1st of the month
• School of Veterinary Medicine: rolling deadline
• College of Letters and Science: 4:00 pm on the 1st of the month
• Graduate School: rolling deadline

NOTE: ALL PROTOCOLS ARE VALID FOR THREE (3) YEARS FROM DATE OF APPROVAL.

1. Principal Investigator/Project Director: Ned H. Kalin M.D.
   Telephone Numbers: Office: _______ Lab: _______ Animal Emergency: _______
   Home: Unlisted  Fax: _______ E-mail Address: ______@wisc.edu

   Alternate for animal emergency or study-related action/communication with Authority to act in the
   Investigator's absence:
   Name of Alternate for animal emergency/study-related action: _______
   Alternate Office Phone: _______ Alternate Phone: _______
   Alternate Email: ______@wisc.edu

   Alternate contact for clerical purposes only for this protocol:
   Name of Clerical Alternate: _______ Ph.D.
   Clerical Alternate Office Phone: _______ Clerical Alternate Phone/Email: ______@wisc.edu

2. University Department (of PI): Wisconsin Psychiatric Institute & Clinics (WISPIC)
   Office Address: 6001 Research Park Blvd., Madison, WI. 53719
   Unit & Division Number (UDDS): A-55-1000

3. Type of submission (underline appropriate category): NEW RENEWAL AMENDMENT
   If Renewal or Amendment, please give current protocol code (e.g. G00180): Code: G00738

4. This protocol is for: TEACHING or RESEARCH (Underline all that apply) BIOMEDICAL; BEHAVIORAL;
   OBSERVATIONAL; AGRICULTURAL; FIELD; OTHER (SPECIFY)

5. Title of this animal protocol: Effects of early experience on the development of anxiety and its neural substrate
6. **Classification of animal use** (will be completed by RARC administrative staff):  1  2  3  4  5

7. **Underline** the appropriate response to each question below:
   a) Will ANY surgery be performed on any animals?  **YES**  **NO**  If yes, fill out questions 24-30.  
   b) Will you be working with wild-caught animals?  **YES**  **NO**  If yes, fill out questions 31-34.  
   c) Will you be using nonhuman primates?  **YES**  **NO**  If yes, fill out question 35.

8. **Procedure locations:** Will any procedures on live animals (e.g., blood collection, injections, euthanasia, scans, etc.) be conducted in labs or other facilities outside of housing area? **Underline** one: **YES**  **NO**  If YES, enter information on the table below, using additional lines as necessary. "Precautions" refers to steps taken to prevent potential disease transfer upon return to normal housing.

   **NOTE:** Any location where animals are kept for more than 12 hours is considered HOUSING and should be included in Question 10.

   (hit "tab" in bottom right cell to add additional row)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Building/Room #</th>
<th>Length of stay (hrs)</th>
<th>Method of transport &amp; precautions, if any</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positron Emission Tomography (PET) scans</td>
<td>Rm. [unclear]</td>
<td>Typically less than 3, but can be up to 8</td>
<td>Animals will be transported within designated animal areas in the primary transport cage.  When transporting between buildings or in public corridors, a primary transport cage will be placed inside a rigid, opaque secondary leak-proof container with or without a small window for animal observation. Exposure kits will be kept in the transport vehicle, and both exposure kits and personal protective equipment (PPE) will be available. PPE will not be stored at Rm. [unclear] but will be brought along with the animals transported there.</td>
</tr>
<tr>
<td>Anesthesia administration and recovery for PET scans</td>
<td>Rm. [unclear]</td>
<td>Typically less than 3, but can be up to 8</td>
<td>Animals will be transported within designated animal areas in the primary transport cage.  When transporting between buildings or in public corridors, the primary transport cage will be placed inside a rigid, opaque secondary leak-proof container with or without a small window for animal observation. Exposure kits will be kept in the transport vehicle, and both exposure kits and PPE will be available. PPE will not be stored at Rm. [unclear] but will be brought along with the animals transported there.  Rm. [unclear] are used for recovery following anesthesia for PET scanning. Please see Positron Emission Tomography (PET) Imaging section of 17a for details.</td>
</tr>
<tr>
<td>Magnetic Resonance Imaging (MRI) scans</td>
<td>Rm. [unclear]</td>
<td>Typically less than 3, but can be up to 8</td>
<td>Animals will be transported within designated animal areas in the primary transport cage.  When transporting between buildings or in public corridors, the primary transport cage will be placed inside a rigid, opaque secondary leak-proof container with or</td>
</tr>
<tr>
<td>Anesthesia administration and recovery for MRI scans</td>
<td>Typically 10 minutes from time of arrival until anesthesia takes effect for performing MRI. Typically 30 minutes after MRI until the animal is safe to transport.</td>
<td>At Rm. is used for IM administration of Ketamine and recovery following anesthesia, which is given to prepare the subject for MR imaging. Please see the Magnetic Resonance Imaging section of question 17a for details.</td>
<td>At Rm.'s used for IM administration of Ketamine prior to MR scanning, using a transport box equipped with a restraint device, and recovery following anesthesia for scanning. When transporting between buildings or in public corridors, the primary transport cage will be placed inside a rigid, opaque secondary leak proof container with or without a small window for animal observation. Exposure kits will be kept in the transport vehicle, and both exposure kits and PPE will be available at and PPE will not be stored at but will be brought along with the animals transported there.</td>
</tr>
</tbody>
</table>

9. Species, Numbers, and Sources of Animals

NOTE: TOTAL NUMBERS ARE FOR THE ENTIRE THREE-YEAR LIFE OF THIS PROTOCOL.

a. Numbers of animals needed for experiments for 3 years:  

<table>
<thead>
<tr>
<th>Species of animal</th>
<th>Total for 3 years</th>
<th>Source of animals (e.g. commercial vendor, another UW-Madison protocol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhesus Monkeys (M. mulatta)</td>
<td>120 400</td>
<td>UW Madison primate colonies</td>
</tr>
<tr>
<td>Kingsnake (Lampropeltis getula)</td>
<td>1</td>
<td>Commercial vendor</td>
</tr>
</tbody>
</table>

b. Will any dogs or cats be obtained from Class B dealers? (Underline one)  

NOTE: Use of animals from Class B dealers requires permission from the Animal Care and Use Committee.

YES   NO

If YES, briefly explain how you have determined that the previous use of these animals will not compromise the animal's health and the research proposed in this protocol.
Colony Records Unit keeps complete colony records of all manipulations and drug treatments, as well as major and minor surgeries, performed on the monkeys throughout their lifetimes. This data is reviewed by the principal investigator (PI) and/or research staff, project director, and veterinary staff before assigning monkeys to this protocol. This facilitates multiple usage of all monkeys when possible, and ensures that previous use will not compromise an animal's health and the proposed research. With PI and veterinarian consent, the animals may receive blood draws for other protocols while assigned to this protocol.

10. Housing: Building(s)/facilities—including procedure room(s)—where the animals will be housed for more than 12 hours.

Nonhuman Primates:

(All surrogate/rear rearing will occur in this building)

Animals will be transported between these housing locations in a primary transport cage within a rigid, opaque secondary leak-proof container with or without a small window for animal observation. Animals will be transported in accordance with the SOP 1.02 (Animal Transport in an Enclosed Container), the primary transport cage will be placed inside a rigid secondary leak-proof container and, if necessary, moved by a vehicle that is dedicated to animal transport, is sanitized, and is temperature controlled, when required by research staff.

Snake:
Will be housed in a terrarium kept in room of . Prior to housing the snake, the ACUC will inspect room of and a SOP for the care and husbandry of the snake will be provided.

11. Explanation of Goals, Animal Use, and Choice of Species

a.

In straightforward, nonmedical, nontechnical language that would be understandable to a layperson (aim for a high school-senior reading level), outline the specific scientific goal(s) and significance of this research. Be convincing as to why this work is important for advancement of knowledge, improving human or animal health, or for the good of society. Spell out all acronyms at first occurrence. If this is a Renewal submission please provide a brief (2-3 sentences) description of your progress and productivity in the past three years to help the Committee evaluate animal usage. This description can be a citation(s) to a publication generated from this research or new directions that will be pursued in the next three years. If a published manuscript is not yet available, a brief description of any other progress can be provided, such as abstracts, oral presentations, or presentations at meetings.

Anxiety disorders and depression are very common, with marked disability to individuals and large social and economic costs to society. These are illnesses that start in early childhood, but frequently go untreated until much later. There is a significant need for the development of new treatments aimed at intervening in early childhood so that the long-term chronic effects of these illnesses on mental and physical health can be reduced or even prevented. We have developed an animal model to explore the factors that lead to the early risk of developing anxiety and depression and want to use this model to, ultimately, develop new ideas and paradigms for early interventions.

The purpose of these studies is to use the nonhuman primate model of anxiety to develop new strategies to decrease the vulnerability to develop anxiety and depressive disorders over early development. The studies proposed here could not be performed in humans because of their prospective and controlled nature, as well as the need to collect postmortem samples. The analysis of molecular mechanisms in the brain has the potential to lead to new treatments. These efforts will allow us to identify the exact brain regions affected, the changes in gene function in these regions, and the specific genes that are involved in increasing the early risk to develop anxiety and depression. Such information has the potential to identify new targets in specific brain regions that can lead to new ideas about treatment and even prevention of the long-term suffering associated with early adversity. For example, understanding the involvement of brain chemicals that have never before been implicated in anxiety, will allow the field to begin to search for medications that affect these newly identified systems. In addition, the molecular information, combined with the imaging data, may allow for interventions that target novel brain regions that are critically involved in anxiety and depression. We have found that there is a wide range of anxiety that occurs naturally in developing monkeys that does not appear to be affected by rearing experience. For example, we studied monkeys that had an adverse experience early in life (they were separated from their abusive mothers and peer reared) and somewhat surprisingly we found that these animals were not more anxiety prone than those reared with their mothers. Therefore, the strategy that we will take will be to study the molecular...
underpinnings of the wide range of individual differences in the development of anxiety which will provide important information about molecular mechanisms underlying vulnerability as well as resilience.

The two most important components that lead to the risk of developing anxiety and depression are genetic predisposition and early environmental adversity. Our work to date has studied anxiety and its underlying components in maternal reared animals. However, we believe it is important to move beyond the normal situation of studying maternal reared animals and begin to understand the effects of early adversity, as this is the most important environmental component that contributes to risk of developing anxiety and depression. The value of animal models lie in their capacity to manipulate and examine mechanisms that cannot otherwise be accomplished in human studies. While severe adversity (early physical trauma, sexual abuse, abandonment, and psychological abuse) is relatively common in humans, it is impossible to perform prospective, controlled studies in young children examining how these early stressors influence the brain and its molecular function. These stressors are the most important environmental risk factors for the development of anxiety and depressive disorders. The manipulation that we propose in monkeys is surrogate/peer rearing. In our research, surrogate/peer reared animals will be raised with a surrogate from birth until they are ready to be paired with a peer. All animals will be housed in pairs when mature enough. It is important to point out that this manipulation is not associated with the degree of trauma that occurs in human children exposed to abuse, isolation and/or abandonment. We have selected surrogate/peer rearing because it is currently an accepted early manipulation with a large database demonstrating that surrogate/peer reared monkeys have increased emotionality, which is characterized by enhanced environmental reactivity and increased anxiety. We believe that surrogate/peer rearing is the best model for our studies of anxious temperament because this paradigm begins at birth and occurs during the critical period of brain development that we must study to understand the earliest determinants of pathological anxiety.

The studies proposed here could not be performed in humans because of their prospective and controlled nature, as well as the need to collect postmortem samples. The analysis of molecular mechanisms in the brain has the potential to lead to new treatments. It is important to emphasize that currently there are no evidence-based treatments available for young children exposed to early adversity who face the risk of developing debilitating psychiatric disorders. While numerous studies have been performed examining the effects of surrogate/peer rearing in nonhuman primates, no studies have been reported examining the effects of this rearing modification on brain development using state of the art imaging and molecular methods. These efforts will allow us to identify the exact brain regions affected, the changes in gene function in these regions, and the specific genes that are involved in increasing the early risk to develop anxiety and depression. Such information has the potential to identify new targets in specific brain regions that can lead to new ideas about treatment and even prevention of the long-term suffering associated with early adversity. For example, understanding the involvement of brain chemicals that have never before been implicated in anxiety, will allow the field to begin to search for medications that affect these newly identified systems. In addition, the molecular information, combined with the imaging data, may allow for interventions that target novel brain regions that are critically involved in anxiety and depression.

b. Specifically justify the use of animals for this research. Explain why it is imperative to use animals and why non-animal alternatives such as computer simulation or in vitro systems are not possible

These studies require the ability to perform work that will help us understand how environmental challenges affect the brain and anxiety associated with brain function during development. These studies cannot be performed in test tubes because it is critical that the entire functioning brain is studied as it relates to behavior and emotion. Likewise, computer simulations cannot capture the intricacies of the functioning brain and the changes that occur during development as individuals face environmental challenges. These studies cannot be performed in humans because of the need to study individuals in a controlled environment during early development. Furthermore, the best way to understand what is causing environmental related brain changes is by using imaging methods to identify critical brain regions. This information can be used to point to the brain regions that should be sampled post mortem for analyses of the molecules underlying anxiety. Post mortem sampling cannot be done in a controlled way in humans. The important similarities in brain anatomy between rhesus monkeys and humans are critical in the decision to study this species. These similarities include how the well developed cortical regions control the deeper structures in the brain that underlie fear and anxiety. These higher processing functions are found in primate species. The social and mental processes of non-human primates are unparalleled elsewhere in the animal world, making them valuable research models for emotional and psychiatric disorders. This forms the basis as to why primates, and not rodents or any other species, have been selected for these experiments.

A live snake is necessary for the Snake Exposure test because, though inanimate objects such as rubber snakes and tape rolls elicit a fear response, they do not elicit as reliable or robust of a fear response as a live snake does in nonhuman primates.
c. Specifically justify why you chose the species cited in 9(a) for your work, such as the appropriateness of the species for your proposed work. Cost considerations are not justifications.

Rhesus monkeys have been selected because their similarities to humans in social behavior, emotion, hormonal responses and brain structure make them the best model for examining human emotion regulation as well as the risk to develop anxiety and depression. In addition, a vast body of knowledge has been collected in this primate species that has direct relevance to these studies.

Kingsnake was selected for our experiment because it is a non-dangerous, hearty species that is easy to care for.

12. Explain how the number of animals required was determined and justify that need. Include all control animals and breeding colony animals in this discussion. A table may help clarify different experimental groups or studies and the specific numbers needed for each. Include any statistical analysis used (e.g. power calculations) in determining the animal numbers.

We plan to study the development of Rhesus Monkeys who will experience differing early social and environmental conditions:

40 subjects
Animals will be placed into 2 groups of 20 animals each:
Group 1 = Mother reared animals. Animals will remain with their mothers during infancy and rearing.
Group 2 = Surrogate/peer reared animals. Animals will be reared in the nursery after birth, and paired with a conspecific at 3-6 weeks of age. This design has been based on the SOP 1.16 (Nursery Rearing Procedure). While maternal rejection or death occurs occasionally in the naturalistic setting of the laboratory, we cannot rely on the uncontrolled and unplanned nature of these events to form our study sample. This is because we need to pair two similar aged infants at the same time for them to be surrogate/peer reared and developmentally comparable. In addition to the importance of pairing similar aged animals, environment is an important factor in our study, so all infants need to have the same early environmental experience within experimental groups. This cannot be controlled in naturalistic cases where there is variability as to when the infant is taken from its mother, as well as in the reason for removing the infant, which may be due to illness, physical abuse or maternal neglect. However, if an animal is removed from its mother on day 1 of life due to maternal rejection that does not negatively influence its health, such an animal will be considered for the experimental group. Additionally, if otherwise healthy, animals brought to the nursery after a caesarean section will also be considered.

40 subjects – Mother reared infants. Animals will remain with their mothers during infancy and rearing.

40 20 subjects – Mothers of infants subjects in the mother reared group. Mother reared subjects Infants will be housed with their mothers for approximately the first 6 months of life. Assigning mothers of infants will ensure that our research is not affected by, and does not affect, other research studies.

10 subjects – Stimulus animals for behavioral tests. Animals will be used as stimulus animals to elicit a reaction from the test subjects when testing anxiety in response to conspecifics.

20 subjects – Exposure animals for stimulus animal. Animals will be used to pre-expose the stimulus animals to the testing conditions prior to conspecific testing.

1 snake – Animal will be used as stimulus for the Snake Exposure test.

Replacement Animals
10 subjects – Mothers and/or infants will be assigned if needed as replacement animals. Replacement animals will be tested from birth the same as all other animals as stated in the timeline (Question 17a).

Based on our earlier studies examining the range of individual differences of anxious temperament in monkeys, we have a good estimation of the magnitude of differences that will be seen in molecular and imaging data when comparing mother to surrogate/peer reared animals. In addition, we have performed prior work with different size experimental groups and, based on this, have a good idea of the sample size that is necessary to observe the expected differences in brain activity and molecular changes within the brain regions that underlie anxious temperament. We also have consulted Dr. in regard to sample size, experimental design and
analytic strategy in relation to our ongoing studies. In performing statistical modeling to estimate the necessary sample size for the current experiment (detailed below), we found that the estimate of 20 animals per group is similar to the sample size we have found to work in our earlier experiments. Because the differences between groups in mRNA expression are predicted to be relatively small, a pilot study with a few animals will not shed insight into potential differences between groups. In fact, a lack of significant differences in an underpowered sample could prove misleading. It is important to emphasize that, while differences in mRNA may be small, these differences may be very important from the standpoint of understanding psychopathology and in thinking about treatments aimed at novel systems. Animals will be assessed in cohorts consisting of one pair of maternal-reared and one pair of surrogate/peer-reared animals. These pairings are accounted for in the power analysis. Additionally, analyses will take into account these pairings and pair will be used as a covariate in some of the analyses. Our group has considerable experience with large-scale, multivariate analyses (1-9). Primary hypothesis testing will focus on individual group differences in anxious temperament (AT). The brain analyses will control for potential confounds (e.g., age, gray-matter probability (9) and use false discovery rate (FDR)-techniques (q<0.05, two-tailed) to correct for multiple comparisons (10). Based on our earlier studies examining the range of individual differences of anxious temperament in monkeys, we have a good estimate of the magnitude of differences that will be seen in molecular and imaging data when examining individual differences in maternally reared populations. In addition, we have performed prior work with different size experimental groups and, based on this, have a good idea of the sample size that is necessary to observe the expected differences in brain activity and molecular changes within the brain regions that underlie anxious temperament. We also have consulted Dr. [Redacted] in regard to sample size, experimental design and analytic strategy in relation to our ongoing studies. While our initial molecular experiment used 24 animals, we recently analyzed a data set from 48 animals and have realized that a sample of approximately 40 animals markedly improves the reliability of the data when examining relations between brain specific transcripts and anxious temperament. Therefore, we plan to fully phenotype 40 animals all of which were reared with their mothers and represent the wide range of individual differences in anxious temperament. Hypothesis testing will examine the impact of rearing condition on AT and AT-related brain function and structure. Effect sizes for univariate behavioral (d>1.3, α=0.05) and massively univariate imaging/genetic comparisons (d=2, α=0.0001; FDR q<0.05) were estimated from our published experiments on the effects of orbito-prefrontal cortex (OFC) lesions on AT and amygdala metabolism (2, 11). The sample size to be used has been selected based on this study and another study characterizing RNA expression in amygdala tissue collected from monkeys phenotyped for AT. Our experience supports that a minimum of 15 animals per group is necessary to detect between group differences in mRNA expression. We plan to fully phenotype 20 animals per group to ensure that quality data will be available for the post-mortem analyses of up to 20 animals per group. On this basis, the proposed sample (n=20/group) would achieve >90% power for both sets of analyses.

13. Current or pending funding for this project (add more entries as needed):

Title of Grant (1): Early Neurodevelopmental Origins of Anxiety
Funding Source (1): NIH
Grant Number (1): MH100031

Title of Grant (2):
Funding Source (2):
Grant Number (2):

14. Identify the person(s) or animal care unit responsible for daily animal care:

The UW Madison Animal Care Staff will care for all primates. Research staff will care for the snake.

15. Research/teaching staff expected to work with the animals in this study (please delete examples)

**INVESTIGATORS:** Everyone listed below must take the "Responsible Use and Care of Laboratory Animals" certification course before starting work with research animals. Protocols cannot be approved until PI and all listed personnel are certified. RARC also offers several species-specific animal handling courses and procedures training (e.g. blood draw techniques, surgery). For information, call RARC 265-2694.

<table>
<thead>
<tr>
<th>Name / Degree / Phone number</th>
<th>Will work with the following species within this protocol</th>
<th>List the year each individual began working with the species(s) and performing the procedures they will work/perform in this protocol. <strong>NOTE:</strong> For personnel who have worked with the named species less than 1 year, indicate who will train and supervise them.</th>
</tr>
</thead>
</table>
| [Redacted]                  | Rhesus Monkeys, snake                                   | Student in training  
*Research experience with primates since 2015.  
†Research experience with snakes since 2015. |
| [Redacted]                  | Rhesus Monkeys, snakes  
Snakes                        | Student in training  
*Research experience with primates since 2015.  
†Research experience with snakes since 2015. |
| [Redacted]                  | Rhesus Monkeys, snake  
Associate Scientist  
Monkeys                        | Associate Scientist  
*Research experience with primates since 2012.  
†Research experience with snakes since 2013. |
| [Redacted]                  | Rhesus Monkeys, snake  
Research Associate  
Monkeys                        | Research Associate  
*Research experience with primates since 2012.  
†Research experience with snakes since 2013. |
| [Redacted]                  | Rhesus Monkeys, snake  
Associate Research Specialist  
Monkeys                        | Associate Research Specialist  
Research experience with primates since 1994  
†Research experience with snakes since 2014. |
| [Redacted]                  | Rhesus Monkeys, snake  
Principal Investigator, Professor and Chairman of the Dept. of Psychiatry  
Has worked with primates since 1979.  
†Research experience with snakes since 2013. |
| [Redacted]                  | Rhesus Monkeys, snake  
Graduate Student  
Monkeys                        | Graduate Student  
*Research experience with primates since 2012.  
†Research experience with snakes since 2013. |
| [Redacted]                  | Rhesus Monkeys, snake  
Student in training  
Snakes                        | Student in training  
*Research experience with primates since 2015.  
†Research experience with snakes since 2015. |
| [Redacted]                  | Rhesus Monkeys  
Clinical Instructor – Research Fellow  
Monkeys                        | Clinical Instructor – Research Fellow  
*Research experience with primates since 2013. |
| [Redacted]                  | Rhesus Monkeys, snake  
Associate Scientist  
Monkeys                        | Associate Scientist  
*Research experience with primates since 2012.  
†Research experience with snakes since 2013. |
| [Redacted]                  | Rhesus Monkeys, snake  
Associate Research Specialist  
Monkeys                        | Associate Research Specialist  
*Research experience with primates since 2013.  
†Research experience with snakes since 2013. |
| Rhesus Monkeys, Snakes | Student in training  
*Research experience with primates since 2015.  
†Research experience with snakes since 2015. |
| Rhesus Monkeys, Snake | Research Program Manager 1  
Research experience with primates since 2010.  
†Research experience with snakes since 2013. |
| Rhesus Monkeys, Snakes | Student in training  
*Research experience with primates since 2015.  
†Research experience with snakes since 2015. |
| Rhesus Monkeys, Snake | Research Program Manager 3  
Research experience with primates since 2004.  
†Research experience with snakes since 2013. |
| Veterinary Staff | Experience with primates in various clinical treatments and surgical procedures.  
Experience with health and treatment of snakes. |
| Veterinary Staff and Animal Care Staff | Experience with primates in various techniques such as blood sampling and clinical treatments. Training will be provided by veterinary staff when needed. Experience with snake observation and record keeping. Experience in training staff and students in snake care and handling. |

* Trained and supervised by training and veterinary staff and Animal Care Staff and veterinary staff, full time staff and experienced students for all testing procedures.  
† Trained by RARC. Trained and supervised by Animal Care Staff.

### 16 Search for Unnecessary Duplication and Alternatives to Potentially Painful / Distressful Procedures

#### 16a. UNNECESSARY DUPLICATION

The Animal Welfare Act and USDA Animal Care Policy #12 require PIs to assure the Committee that you have considered whether or not your proposed work unnecessarily duplicates existing knowledge. **The USDA believes that database searches remain the most effective and efficient method for demonstrating compliance with the requirement to consider unnecessary duplication of research.** To satisfy this requirement provide the following information:

<table>
<thead>
<tr>
<th>Electronic databases searched</th>
<th>Years covered by search</th>
<th>Date (MM/DD/YY) of most recent search performed</th>
<th>Frequency with which searches are performed (e.g. monthly)</th>
<th>Keywords used for this search</th>
</tr>
</thead>
</table>
| PubMed                        | 1966-Present             | 9/24/15                                       | Quarterly                                             | -5-HTT Rhesus macaque infant  
- ACTH Rhesus macaque infant  
- Amygdala Rhesus macaque infant  
- Behavioral inhibition Rhesus macaque infant  
- Central nucleus of the amygdala Rhesus macaque infant  
- Cortisol Rhesus macaque infant  
- CRF 2 gene Rhesus macaque infant  
- CRF CRH Rhesus macaque infant  
- CRH2 BSL NIH risk group Rhesus macaque infant  
- Early development, Anxious temperament, Rhesus macaque infant  
- Early rearing condition, anxious temperament, Rhesus macaque infant  
- Emotion regulation Rhesus macaque infant  
- Freezing behavior Rhesus macaque infant  
- Heritability Rhesus macaque infant  
- Histology Rhesus macaque infant brain  
- HPA axis Rhesus macaque infant  
- MRI Rhesus macaque infant  
- No eye contact Rhesus macaque infant  
- Novel peer Rhesus macaque infant  
- Orbitofrontal cortex Rhesus macaque infant  
- Positron Emission Tomography Rhesus macaque infant  
- Peer rearing Rhesus Macaque Infant  
- Serotonin transporter Rhesus macaque infant  
- Snake exposure Rhesus macaque infant  
- Stress and anxiety in Rhesus macaque infant |
Please provide a short narrative below of findings from your search. If your research will duplicate existing knowledge please state why this duplication is imperative to the attainment of scientific goals of the protocol.

Narrative 1:

While peer rearing has been extensively used and has been documented to increase reactivity to stress and increase anxiety, no studies have examined the effects of this manipulation using state-of-the art imaging and molecular methods to characterize the brain changes that underlie the well-documented and important behavioral changes. By using in vivo functional and structural imaging to repeatedly assess the same animal over important early developmental periods, we will characterize the developmental effects of this manipulation on brain maturation. This will add important information to the literature by addressing how early development adversity in primates influences neural systems underlying the childhood risk to develop anxiety and depression. By assessing messenger RNA in the actual circuitry that underlies anxious temperament, we will be able, for the first time, to identify novel chemical systems that mediate the development of this at-risk phenotype.

16a 2. Alternatives to procedures that may cause MORE THAN MOMENTARY OR SLIGHT PAIN OR DISTRESS

There may be alternatives to procedures that cause more than momentary pain or distress and that will not interfere with your research. Procedures that cause only momentary pain or distress are quick and minimally invasive, such as simple injections or blood collections, and typically do not include procedures performed under anesthesia. Do any procedures you have proposed cause more than momentary or slight pain or distress?

[ ] No
[ X] Yes

If YES, USDA Animal Care Policy #12 requires PIs to assure the Committee that alternatives to procedures that cause more than momentary or slight pain or distress have been considered. To satisfy this requirement, the USDA believes that database searches remain the most effective and efficient method for demonstrating compliance with the requirement to consider alternatives to more than momentary painful / distressful procedures. Note that alternatives that do not allow the attainment of scientific goals of the research are not considered to be viable alternatives.

Use the keywords ‘refinement’ and ‘alternative’ in conjunction with each procedure that causes more than momentary or slight pain or distress and species. Note that pain management for each of these procedures should be addressed in Questions 18 and/or 27a and/or 29.

(hit “tab” in bottom right cell to add additional row)

<table>
<thead>
<tr>
<th>Electronic databases searched</th>
<th>Years covered by search</th>
<th>Date (MM/DD/YY) of most recent search performed</th>
<th>Frequency with which searches are performed (e.g., monthly)</th>
<th>Keywords used for this search (e.g., &quot;procedure + species + refinement + alternative&quot;)</th>
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<tr>
<td>PubMed</td>
<td>1966-Present</td>
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<td>-Stress reduction refinement Rhesus macaque</td>
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</tbody>
</table>

Please provide a short narrative below of findings from your search. If an alternative or refined method was found, but cannot be used in your research, explain why this is the case.

Narrative 2:
We have searched alternatives to the procedures that cause more than momentary or slight pain or distress and have found no articles that offer viable alternatives. The peer-rearing paradigm is necessary and is the least stressful alternative to achieve a long-term change in anxious disposition (see Section 11a). CSF sampling is necessary to measure brain chemicals in the developing animal that are relevant to anxiety. These measures will be important from the standpoint of possibly developing translational diagnostic and/or treatment efficacy tests in humans with anxiety. CSF is frequently sampled in patients with brain disorders as a measure to assess dysfunction of specific neurochemical systems. Skin biopsy is a common technique and is necessary to harvest fibroblasts, which are cells able to be turned into induced pluripotent stem cells (iPSCs). GABA-ergic iPSCs will be compared to similar amygdala neurons harvested at necropsy to examine the utility of iPSCs as a model for primate amygdala. There are no other reliable, less-invasive alternatives at this time.

For further guidance on conducting searches visit:
http://researchguides.library.wisc.edu/animalalternatives

16b. Occupational Health and Safety Considerations Radiation or biohazard material usage in animals: In the table below, mark YES or NO for each category as it applies to this protocol. If YES, indicate the specific materials in the right-hand column and show the status (approved or pending) of Biological Safety (OBS-2) and/or Radiation Safety (99A) protocols.

<table>
<thead>
<tr>
<th>Category</th>
<th>Used in project? (Yes/No)</th>
<th>If YES, list specific materials used</th>
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<tr>
<td>Genetically altered materials</td>
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<tr>
<td>Infectious agents:</td>
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<td>Virus</td>
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<tr>
<td>Human-derived materials</td>
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<tr>
<td>Teratogens</td>
<td>Yes</td>
<td>Isoflurane</td>
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<tr>
<td>Other</td>
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<td></td>
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<tr>
<td>Radioactive material</td>
<td>Yes</td>
<td>Fluorodeoxyglucose (18F or FDG)</td>
</tr>
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</table>

Status of OBS-2 needed for this project: (Underline below OR check here): [ ] Not applicable to this project.
PENDING APPROVED Provide OBS-2 number if approved: SC12-166R

Status of 99-A needed for this project: (Underline below OR check here): [ ] Not applicable to this project.
PENDING APPROVED Provide 99-A number if approved: 0344-01

c. Special Precautions for Personnel: If you are using any agent that could be hazardous to humans or animals, please provide any special precautions that should be followed by your lab personnel, animal caretakers, veterinarians, maintenance and/or sanitation personnel, or anyone else entering the areas where experiments are conducted or animals are housed. Include any special practices required for handling of any animal or experimental waste, animal carcasses, and cages and caging materials. Consider such requirements as masks or respirators, eye protection, lab coats, gloves, and disposal methods. Also consider posting signage for special requirements on animal room doors and/or cages.

New employees are not permitted access to the animal areas without supervision until they have successfully completed the following:
- RARC Animal User Course
- Animal Handling Risk Questionnaire
- Occupational Health enrollment and Occupation Health 101 course
- Radiation Safety 101 course
- RARC species-specific courses: Primate Orientation & Primate Health and Procedures
- Blood-borne Pathogen Training course
- Biosafety Training: 101, 104 & 201 courses
- HIPAA training
- Herpes B Virus Information and Safety Quiz
- Working Safety with Nonhuman Primates video
- AALAS Training video
- Comply with ACAPAC policy 2013-053 regarding tuberculosis testing requirements
- Watch MRI Safety video
- Read Standard Operating Procedures

Personnel are issued keys or ID cards for animal area access only after the above list is completed and are usually accompanied by trained personnel. Animals are kept in facilities designed for housing of macaques. Sanitation procedures are performed by experienced personnel. All personnel entering animal areas must wear PPE consisting of long-sleeved area-dedicated scrubs/coveralls, rubber boots or area dedicated shoes (with waterproof disposable shoe covers at the feet), a hair cover, a standard surgical mask, two pairs of latex or nitrile gloves, and a face shield. Safety glasses or goggles as well as a face shield are highly recommended at all times, but required at times of high splash potential. Forearm protection (disposable lab coat or sleeves) is highly recommended when having direct contact with nonhuman primates. Potentially infectious nonhuman primate tissue samples must be disposed of properly, per Office of Biological Safety training and recommendations. Syringes, needles, and other sharps are disposed of in biohazard sharps containers and any non-sharps which come into contact with animal body fluids are disposed of in standard biohazard bags and autoclaved or disposed of by Madison Environmental Resources Inc. (MERI). All biological materials, including tissues and used culture media, are handled as per BSL2 recommendations, and are discarded into biohazard bags and autoclaved or disposed of by MERI. Potentially infectious nonhuman primate tissue samples are transported in primary and secondary leakproof containers that include enough absorbent material to contain the total volume of the sample. These procedures closely follow the SOP’s 2.13 (Sanitation of Animal Housing Areas), 5.01 (Protective Clothing), 5.05 (Proper Disposal of Biohazardous Waste: True Sharps, Potential Sharps and Non-Sharps) and 5.06 (Transporting Blood, Tissue and Specimen Samples from Nonhuman Primates) as they pertain to research staff.

For the teratogen listed in Q16b, staff will be notified of the potential hazards and staff that are pregnant will be advised to not come in contact with the substance. To protect worker health, a scavenger system will be connected to the breathing system to safely remove waste gases (isoflurane). A F/air filter canister or similar charcoal filter will be used. Canisters will be weighed before and after procedures to ensure they are within manufacturer’s specified limits.

After completion of the radiation safety course, the University Radiation Safety Committee will approve personnel to work with radioactive compounds. All personnel using radioactive tracers operate under procedures approved by the UW Safety Department, and are monitored regularly for radiation exposure with badges and/or ring dosimeters. These short-lived tracers decay to background levels quickly. Whenever possible, human exposure will be limited by decreasing time of exposure and increasing distance from the radiation source. Areas where radiation is in use will be properly marked until background levels of radiation have been achieved by decay (usually overnight). To determine that radiation has returned to background levels, a radiation survey meter (Geiger counter) will be used. Any contaminated materials will be isolated until the radiation has decayed to background levels before being properly disposed of.

**You must address Question 17 separately for each species.**

17. **Description of Proposed Experimental Design/Studies**

   a. In this section describe the animals’ roles in your experiments—that is, the treatments and procedures the animals will receive outside of normal husbandry, from the first experimental manipulation to the final outcome. This response should provide the Animal Care and Use Committee with a clear understanding of what specifically happens sequentially to each animal or group of animals, and over what time period the procedures occur, including but not limited to:
   - definitions of all materials given to animals, including dosage range, routes, and frequency of administration;
   - blood draw methods, sites, and % volume
   - breeding procedures/methods, if this protocol is to cover an animal colony or herd;
   - the expected sequence, frequency, and duration of procedures;
   - brief description of any devices/implants animals will receive, surgical and nonsurgical;
   - the timing of any surgery within the experiment (do not repeat the surgical description you will provide in
Question 28a):
- method, frequency, volumes, and numbers of biological samples taken;
- experimental diets;
- use of toxic agents, biohazardous materials, or radioactive materials (list in Question 16b);
- social or environmental manipulation;
- methods of antibody production.

Study Overview
Animals will be studied using social and environmental manipulation. Animals will be placed in 2 groups: mother reared subjects and surrogate/peer reared subjects.

Mother Rearing
Animals that are mother reared will remain with their mother until approximately 6 months (180 days) of age. While housed with their mothers, they will be briefly separated from their mothers for testing procedures (see Section 17b, Chemical Restraint of Mothers and Hand Catching of Infants during Infant Separation). Mother reared infants will be weaned from their dams at approximately 6 months (180 days) of age and relocated in the general colony where they will be pair housed with another mother reared subject.

Surrogate/peer Rearing
Animals that are surrogate/peer reared will be removed from mothers on day 1 of life and reared in the nursery using a method that closely aligns with the SOP 1.16 (Nursery Rearing Procedure). All normal daily husbandry practices in the nursery (feeding, weighing, cleaning, moving, documentation, etc) will be completed by care staff. From the first day of life to approximately 21-42 days of age, animals will be singly housed in the incubator. The 21-42 days by themselves in the incubator is consistent with practices at other primate facilities (California National Primate Research Center, Romnek, 2009, Romnek 2011, Capitanio, 2005; and the National Institutes of Health, Dettmer, 2008, Barr, 2004, Spinelli, 2009) and is required in order to specifically monitor each animal’s food intake and output and to allow more consistent incubator temperature control until animals are able to thermoregulate themselves. While in the incubator, an upright, mobile surrogate covered with a soft material will be provided for the animal. The temperature of the incubator will be maintained using a heating pad, so the surrogate will be warm. During this period, they will be fed and weighed in accordance with the SOP 1.16 (Nursery Rearing Procedure). At approximately 21-42 days of age, animals will be moved into standard caging within the nursery and socially housed with another surrogate/peer reared animal. While socially housed in standard caging in the nursery, the feeding schedule of the youngest animal will be followed. At approximately 90-150 days of age, the animals and their existing partner will be relocated from the nursery to the general colony together. The animals will remain pair housed with their existing partner until they are re-paired and re-housed with novel surrogate/peer reared animals at approximately 6 months (180 days) of age. The housing relocation schedule of the youngest animal will be followed. Prior to relocation, veterinary and behavioral management staff will assess the animals for fitness and care staff will determine daily food intake.

Mother reared animals are the critical comparison group and it is important that they are treated in exactly the same way as the experimental group, with the exception of the rearing condition. The procedures for both groups of animals are relatively brief and, therefore, will not have any major effects on the dependent variables of interest. Brief separations are typical in developing primates and do not have long-term effects on anxious dispositions (Parker and Maestripieri, 2011). All animals from both groups will undergo brief separations for the procedures. The overall health of all animals will be monitored by the veterinary staff. If any influences of separation or other components of the study significantly affect infants’ health, veterinary staff will make decisions regarding whether they should remain in the study.

Following final testing, at approximately 1-1.5 years of age, animals will be euthanized and brain tissue will be collected for molecular analyses. Euthanizing animals at this age is necessary because the brain systems that underlie anxiety are particularly plastic during the first year of life. Our focus is on understanding the molecules that are affected by early environmental adversity and are, thus, responsible for the development of anxiety.

Testing Sequence:
From 1 – 14 weeks, animals will undergo up to 3 PET and up to 3 MRI scans. The PET scan is paired with the Human Intruder behavioral assessment during the FDG uptake period and blood sampling (PET scan paradigm). Animals will receive a PET scan at 1 – 2 weeks and a MRI at 2 – 3 weeks; a PET scan at approximately 6 weeks and a MRI at approximately 7 weeks; a PET scan at approximately 12 weeks and a MRI at approximately 13 weeks. These scans will be done within a ± 5 day window from the stated time point, depending on vet staff availability and scheduling issues related to the scanner. No animal will be scanned earlier than 1 week of age.
The repeated assessments over this time period are necessary because of the rapid developments in brain and behavior that occur during this critical period. The first set of scans (PET scan paradigm and MRI) will capture the earliest period of postnatal brain development. The time point for the second set of scans was selected because we, and others, have demonstrated that this time is associated with the beginning of the ability to immaturely respond to threat. The last time point for scanning during the first 14 weeks of life was selected because, by this time, infants have developed the ability to adaptively regulate their fear related behaviors in relation to changing contexts and have developed the capacity to perform prefrontal cognitive functions. We believe that the maturation of prefrontal cortical circuitry associated with executive function is also critical for adaptive regulation of anxiety.

From approximately 15 – 23 weeks, animals will be tested in the Human Intruder paradigm and blood will be sampled immediately following the test. On a different week during this time interval, CSF and a baseline blood sample will be obtained. A baseline sample is a blood sample collected from a monkey in a nonstressful state. Baseline blood draws are typically collected prior to a testing procedure (never following a testing procedure) or on a day with no other procedures.

At approximately 24 weeks, animals will undergo a PET scan paradigm. At approximately 25 weeks, animals will undergo a MRI scan. This time point was selected because it will allow us to understand the extent to which early brain function, in relation to individual differences in anxiety, are stable during the first half of the first year of life. This age in the monkey is analogous to a critical period in human development. The maturation of brain and behavior in monkeys of this age is equivalent to that occurring in human children at the age when they express stranger anxiety. It is also an important age from the standpoint of white matter tract development between regions of the brain involved in the regulation of emotion and anxiety.

From approximately 26 – 36 weeks, animals and their cage mates will be rated for social behavior on 2 occasions, with at least one week between tests. This test may occur at time of weaning.

From approximately 37 – 51 weeks, animals will be exposed to 3 different paradigms. During this period, animals will undergo the snake exposure test one time. Animals will be exposed to a novel conspecific on 2 occasions with at least one week between tests. The same conspecific will be used for both tests. Animals will be exposed to a playroom with their cage mate on 2 occasions, with at least 1 week between tests. All tests will be conducted on different weeks and, in total, animals will only be tested during 5 weeks of this 14 week period.

At approximately one year of age, animals will undergo a PET scan paradigm and approximately one week later will undergo a MRI scan. This time point is critical because it will allow us to assess brain function at an age that is similar to human toddlers. In human children, toddlers can be assessed to establish whether they exhibit the behaviors associated with the at-risk phenotype that we are interested in. Additionally, it is critical to collect brain data at this point to understand stability throughout the first year of development and for the subsequent analysis of the postmortem data. The scans will be used to direct brain sampling and to interpret mRNA findings as they relate to individual differences in brain function in the circuit that underlies anxiety.

From approximately 52 – 78 weeks, animals will be tested in the Human Intruder paradigm and blood will be sampled immediately following the test. On a different week during this time interval, CSF and a baseline blood sample will be obtained.

Animals will undergo skin punch biopsies (procedure described in Q28a) no earlier than 4 months of age. Up to 2 skin samples will be collected from each individual animal. If skin biopsies do not produce enough cells for the cell culture, it may be necessary to repeat the biopsy procedure up to 3 times.

Animals will be euthanized for tissue collection following final testing.

Due to the possibility of animal health or technical issues, animals may need to be retested. Situations in which a procedure may need to be repeated include: animal in need of veterinary attention, animal needs to be removed from testing due to persistent fighting and/or wounding, equipment failure, or poor scan quality. Please see corresponding Specific Methods section below for detailed information regarding the maximum frequency of testing and the minimum interval between tests should animals need to be retested. Also, due to the possibility of animal health or technical issues, the testing sequence may need to be modified. However, the maximum number of each procedure and the length of the experiment will not change. Technical issues include, but are not limited to, PET tracer production or delivery, MRI coil issues, or PET or MRI scanner issues.
## Developmental Study Timeline

### Housing Plan

- **Surrogate/peer Reared (n=20)**
  - **Birth (Day 1):** Infant removed from Dam, singly housed in incubator.
  - **3-6 weeks (21-42 days):** Pair house in standard caging within nursery with another surrogate/peer reared animal.
  - **3-6 months (16-180 days):** Replace cage with standard size colony cage.
  - **6 months (180 days):** Pair house in general colony with a novel surrogate/peer reared animal.
  - Following final testing (n=20) surrogate/peer reared animals will be euthanized for tissue collection.

### Testing Sequence

<table>
<thead>
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<th>Age in weeks</th>
<th>Age in weeks</th>
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<tr>
<td>BIRTH</td>
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<tr>
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<tr>
<td>2-3</td>
<td>MRI</td>
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<td>7</td>
<td>MRI</td>
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<tr>
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<td>MRI</td>
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<tr>
<td>15-23</td>
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<td></td>
<td>Blood &amp; CSF Sample</td>
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<td>MRI</td>
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<td>Exposure to Novel Conspecific on 2 occasions</td>
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<td>Playroom Behavior Observation on 2 occasions</td>
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<td>53</td>
<td>MRI</td>
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<td>Blood &amp; CSF Sample</td>
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<tr>
<td>52-78</td>
<td>Euthanization - Tissue Collection</td>
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### Housing Plan

- **Mother Reared (n=40)**
  - Birth: Infant remains with Dam.
  - 6 months: Wean from Dam. Pair house in general colony with another mother reared animal.
  - Following final testing (n=40) mother reared animals will be euthanized for tissue collection.
Specific methods:

Chemical Restraint of Mothers and Hand Catching of Infants for Infant Separation
Mothers housed with their infants may be anesthetized for infant separation (up to 20 milligrams/kilogram Ketamine HCL, intramuscularly) on the day of infant testing. Once the mother is anesthetized, the infant will be hand caught and placed in a transport cage. Per veterinary recommendation, anesthetizing the mother ensures the safety of mother and infant, as well as research staff during removal of the infant for testing.

Human Intruder Paradigm
This test is used to assess the defensive behavioral responses when an animal is alone and in the presence of a human. To accomplish this, an animal will be transported to a test room and placed in a large mesh test cage. The animal remains alone or a human enters the test room and makes or avoids eye contact with the subject, depending on the experimental condition (as described in Kalin et al, 1989). Behavior will be observed and encoded by research staff via live closed circuit video monitoring for a pre-defined test period of 20-90 minutes. Our group developed and introduced the Human Intruder Paradigm and we have extensively examined different modifications of the paradigm and durations of exposure to the intruder. Our data demonstrate that the duration used in this study optimally activates the brain regions of interest. Typically, prior to PET imaging, animals undergo a modification of the Human Intruder paradigm during FDG uptake (see Positron Emission Tomography [PET] imaging section below) in which a human enters the room and presents their profile to the animal (No Eye Contact or NEC condition) for approximately 30 minutes. During this time, behavior is observed and encoded via live closed circuit video monitors. At other times, animals will receive the unmodified Human Intruder paradigm in which the animal is alone (Alone or AL condition) in the test cage, immediately followed by a human entering the room and presenting their profile to the animal. Following NEC, the human intruder leaves the room for approximately 3 minutes, then re-enters the room and makes eye contact with the animal (Stare or ST condition). The ST condition is followed by another AL condition. During all of these approximately 10 minute testing periods behavior is observed and encoded via live closed circuit video monitoring. The order and duration of conditions in the paradigm may be modified if data suggests it would be more effective. For animals under 2 months of age, veterinary staff will be consulted regarding the use of a towel, heating pad or heated water pad covered with a towel, placed on the floor of the test cage if there are concerns regarding body temperature. Animals may be tested in the Human Intruder paradigm no more than once a week. Although animals are scheduled to be tested on 7 occasions, they may be tested up to 9 times per year.

Observation of Social Behavior in Test Cage and/or Playroom
Subjects and their cage mates will be transported to a test room and placed in a large mesh double cage for observation and encoding of behaviors for 30-120 minutes. This test may occur on the day of weaning. A newly weaned subject may be tested with another newly weaned subject with whom it will be paired. Animals may be tested with their cage mates on 2 occasions, with at least one week between tests. Although animals are scheduled to be tested on 2 occasions, they may be tested up to 4 times per year.

Animals will be transported to a playroom, with their cage mate, for observation and encoding of behaviors for 30-120 minutes. This test will be done to understand potential alterations in social and individual behavior when subjects are placed in a large novel environment that contains novel objects. More complex novel spaces and objects are likely to reveal more subtle behavioral effects. In contrast, the conspecific test described below is focused on understanding potential alterations in dyadic behavior when animals are confronted with a novel conspecific animal. In the extremely unlikely event that animals engage in persistent fighting or wounding, the research staff will immediately separate them and veterinary staff will be consulted. Some minor aggression is expected when newly paired animals are establishing dominance. These minor aggression bouts do not result in wounding and usually conclude quickly once hierarchy is established. The playroom is an 8’x8’x6’10” room with solid walls and a window on one side for behavioral observations. Animals may be tested in the playroom with their cage mate on 2 occasions, with at least one week between tests. Although animals are scheduled to be tested on 2 occasions, they may be tested up to 4 times per year.

Response to Conspecifics Test
This test will be performed to understand potential alterations in dyadic social behavior when experimental monkeys are confronted with a novel conspecific animal. Subjects will be introduced to a novel conspecific monkey in a large mesh double cage in a test room to evaluate behavior during social interaction for approximately 30 minutes. During the time spent with a novel conspecific, individual and interactive behavioral data will be obtained and encoded for both animals by research staff via live closed circuit video monitoring. The experimental animal will be the focal animal for obtaining and encoding behavior, but any interactive behavior between it and the novel conspecific animal will be encoded as well. In the unlikely event that animals engage in persistent fighting or wounding, the research staff will immediately separate them and veterinary staff will be consulted. Some minor
aggression is expected when newly paired animals are establishing dominance. These minor aggression bouts do not result in wounding and usually conclude quickly once hierarchy is established. Two tests will be performed with the same "novel" conspecific, at least one week apart, to examine the extent to which the subject habituates.

Prior to the experiment, the novel conspecific animals used as stimuli will be pre-exposed to 4 new animals on 4 different occasions, each for approximately 30 minutes. This ensures that, at the time of the experiment, they will have had experience with new animals. See Question 9a for the source of all animals and Question 9c for the guidelines of animal selection. Novel conspecific animals may be tested in the paradigm with experimental subjects for up to 180 minutes per day, no more than 3 days per week.

Experimental animals may be tested in the paradigm two times for approximately 30 minutes per day, at least one week apart. Although animals are scheduled to be tested on 2 occasions, they may be tested up to 4 times per year.

**Snake Exposure Test**

Subjects' food preference will be established prior to introduction to the snake exposure test. To accomplish this, an animal will be moved to a test room and placed in a small primate cage with 5 mesh sides and 1 side with bars (to allow an animal to retrieve rewards) that is connected to the Wisconsin General Testing Apparatus (WGTA).

The animal will be presented assorted food items on top of a clear plastic box. To establish a clear food reward preference, the order and time it takes to retrieve food items and the amount taken will be recorded. Food items taken the quickest and most often will be designated as an animal's preferred choice. The food preference procedure will continue until the animal has established a clear preference for at least two food items. Once a food preference has been established, the animal will progress to habituation to task training. During this procedure, the animal will be placed in the test apparatus and trained to reach for their preferred food items during a mock test session. This training will continue until the animal appears comfortable and reaches for the preferred food items greater than 70% of the time. Food preference and habituation to task training may last from 30-90 minutes/day, for up to 14 days.

During the snake exposure test, animals will be placed in the small primate cage in a test room. The testing apparatus will be placed in front of the cage and the animals will be presented with their most preferred food items placed on top of a clear plastic box that contains a stimulus such as a live snake, rubber snake, roll of tape or nothing. Our previous work has shown that each of these items elicits a response in primates, with the live snake eliciting the most reliable and robust fear response (Nelson, Shelton, Kalin, 2003). The items that are not fearful or those that elicit lesser responses are included as comparison and control measures to quantitate the magnitude of the animals' response to the live snake. Each stimulus item will be presented for no more than three minutes at one time and latency to reach for each food item will be recorded. The snake exposure test may last from 30-90 minutes/day. Although animals are scheduled to be tested on 1 occasion, they may be tested up to 2 times per year.

The snake will be housed in a 30" x 12" x 12" 20 gallon or larger terrarium with artificial grass covering the floor, kept in rm... A heat lamp will be placed on one side of the terrarium to create a heat gradient, allowing the animal to regulate its body temperature. The temperature on the side with the heat lamp will be 60-88°F and the cool side will be maintained at 70-75°F. A hide box will be provided on both the cool and warm sides of the terrarium. Water will be provided ad libitum. Research staff will feed the snake once a week with a frozen mouse that has been thawed. The terrarium will be cleaned every 2 weeks and debris will be removed as necessary. If health issues arise, the snake will be taken to the University of Wisconsin for assessment.

**Blood and CSF Sampling**

Blood and CSF will be sampled to assess the pituitary-adrenal components of anxious temperament. These include plasma levels of cortisol and CSF levels of corticotrophin releasing hormone. While hair samples can be used to assess cortisol concentrations, this method does not answer the questions of interest in this study, which require a cortisol measure that reflects an animal's state at baseline prior to testing and following testing procedures. Hair cortisol measures are a reflection of long-term cortisol exposure and do not allow for the more refined understanding of the physiological function and response of the hypothalamic-pituitary-adrenal (HPA) axis.

Blood will be sampled immediately prior to PET imaging, after the Human Intruder paradigm, and at baseline. Blood may also be collected prior to, or immediately following, MR imaging. Blood samples will be obtained using a vacutainer system or needle and syringe via femoral or saphenous venipuncture using a tabletop restraint, manual restraint, or chemical restraint (up to 20 mg/kg Ketamine HCL may be given intramuscularly, or an alternative anesthetic in consultation with a veterinarian). Pressure will be used to assure hemostasis following the draw. The
blood volume sampled will be in accordance with current RARC guidelines, which is the same as the blood volume guidelines in the [redacted] SOP 4.01 (Blood Sampling). These guidelines currently state that up to 10% of blood volume may be collected at one time and up to 20% of blood volume may be collected in any 30-day period.

While it is not our intent to sample at maximal levels, if this does occur, animals being sampled at maximal levels will be assessed by veterinary staff to determine any need for fluid replacement therapy, clinical monitoring and periodic complete blood counts (CBC). The CBC will be available to the veterinary staff for evaluation, and they will determine the need for further monitoring and/or iron supplementation.

In conjunction with blood sampling procedures, cisternal CSF (1-5 milliliters) may be obtained. For animals less than 8 months of age, up to 2 milliliters of cisternal CSF may be obtained. CSF sampling will be accomplished by anesthetizing the animal with up to 20 mg/kg Ketamine administered intramuscularly, or an alternative anesthetic in consultation with a veterinarian, shaving and prepping the site with alcohol, or other disinfection solution made for this purpose, and positioning in the lateral decubitus or ventral recumbent posture. The sample will be obtained by percutaneous puncture of the cisterna magna by a sterilely-gloved personnel who will pass a 21–26 gauge needle (or needle gauge recommended by veterinary staff) directly through the skin over the center of the atlanto-occipital (AO) joint and into the sub-arachnoid space. If needed, a supplemental dose of Ketamine (up to 20 mg/kg IM, or up to 10 mg/kg IV), or an alternative anesthetic in consultation with a veterinarian will be administered. After CSF collection, animals 8 months of age and older will receive at least one intramuscular dose of ketoprofen (2-5 mg/kg), or alternative analgesic in consultation with a veterinarian. For animals less than 8 months of age, veterinary staff may administer ketoprofen (2-5 mg/kg, intramuscularly), or an alternative analgesic, if they observe signs of discomfort. Although animals are scheduled to be sampled on 2 occasions, they may be sampled up to 4 times per year with at least one week between samples.

**Positron Emission Tomography (PET) Imaging**

PET will be used to monitor changes in brain areas associated with emotional processing, specifically, the circuitry that underlies anxiety and depressive disorders. We will use a PET procedure to image the brain’s metabolic response to exposure to one or more conditions of the Human Intruder Paradigm.

All animals undergoing PET imaging procedures will have 18F (listed in Question 16b) administered intravenously. Using a tabletop restraint or manual restraint, a catheter will be placed for tracer delivery, with preference given to the saphenous vein. This tracer will be given at a dose of up to 10 mCi, and then the catheter will be removed, and pressure will be placed at the site of injection to prevent leakage. These minute doses have no expected harmful effects and are commonly used in human PET studies. After injection of the tracer, animals will be transported to a test room and placed in a large mesh test cage. Their behavioral response to being alone in the test cage or in the presence of a human making or avoiding eye contact (Human Intruder paradigm) will be observed and encoded during the tracer’s approximately 30 minute uptake period. Veterinary staff will be consulted regarding the use of a towel, heating pad or heated water pad covered with a towel, placed on the floor of the test cage if there are concerns regarding body temperature. Following behavioral testing, animals will be anesthetized with up to 20 milligram/kilogram Ketamine HCL administered intramuscularly, or an alternative anesthetic in consultation with a veterinarian, to allow for safe transfer to the scanner, and to ensure that stress hormone levels and brain metabolism are unaffected by the transfer. When scanning all [redacted] animals will be transported in a primary transport cage within a rigid, opaque leak proof secondary container with or without a small window for animal observation. For some young animals (typically under 8 months of age), a rolled up towel may be placed in the transport. Heated water bottles covered with towels may also be placed in the transport to help maintain body temperature.

For PET imaging of all animals, atropine sulfate (0.01 - 0.3 mg/kg intramuscularly or subcutaneously) may be given to depress salivary secretion, ketoprofen (2-5 milligrams/kilogram intramuscularly) may be given and Cetacaine spray or another topical anesthetic may be used to aid the intubation process. During the scans, all animals will be fitted with an endotracheal tube, induced with 5% or less isoflurane anesthesia, and typically maintained on less than 3% isoflurane. Heart rate, respiration and oxygen saturation will be monitored continuously and recorded every 15 minutes throughout the procedure. Body temperature will be maintained with a warm air blanket and will be measured and recorded before and after imaging procedures. In addition to the warm air blanket, heated water bottles, heated water pads and/or towels may be used to maintain body temperature during imaging. An indwelling catheter may be placed, with preference given to the saphenous vein, before, during or after the scanning procedure. Warm saline (0.9% NaCl), Plasmalyte, lactated Ringer's or other isotonic fluid (approved by a veterinarian) may be administered (subcutaneously or intravenously) before, during or after imaging procedures to maintain hydration. Animals will be monitored continuously until they are able to lift their own head, then they will be monitored at least every 15 minutes until they are sitting up, after which they will be monitored every 30 minutes until fully recovered.
For PET imaging of animals less than 8 months of age, veterinary staff will perform all preparation, maintenance and monitoring of PET procedures. Glucose will be monitored and recorded before and after the scanning procedure, and at intervals during the scanning procedure if recommended by veterinary staff. Because veterinary staff will attend and directly supervise these procedures for animals of this age group, the veterinary staff will decide whether or not to administer supplemental dextrose (alone or mixed with an isotonic fluid) if necessary before, during or after the imaging procedure.

Although animals are scheduled to have 5 scans during the first year, animals may undergo up to 7 PET scans per year with at least one week between scans. Each PET scan will last approximately 60-240 minutes.

**Magnetic Resonance Imaging (MRI)**

MRI scans are performed to understand the structure of the gray and white matter of the brain as it is related to anxiety and depressive disorders. The scans are commonly used in humans and give detailed images of the anatomy of the key brain regions underlying anxiety as well as the structures connecting these key brain regions.

The animals will be transported to the MRI suite in a primary transport cage within a rigid, opaque secondary leak proof container with or without a small window for animal observation, using a cage that is dedicated to animal transport, is sanitizable, and is temperature controlled. For some young animals (typically under 8 months of age), a rolled up towel may be placed in the transport. Heated water bottles covered with towels may also be placed in the transport to help maintain body temperature.

Under our G00181 protocol, we established and confirmed methods for imaging young monkeys soon after birth. Working with veterinary staff, the parameters for anesthesia, glucose and body temperature maintenance have been established. Optimization of scanning parameters was also a focus and resulted in our ability to acquire high quality images. For animals less than 8 months of age, veterinary staff will perform all preparation, maintenance and monitoring of MRI procedures. Animals will be initially anesthetized with up to 20 milligrams/kilogram dose of Ketamine HCL intramuscularly to allow for safe transfer of the animal to the scanner. Atropine sulfate (0.01-0.3 milligrams/kilogram) may be given, ketoprofen (2-5 milligrams/kilogram intramuscularly) may be given and Cetacaine spray or another topical anesthetic may be used to aid the intubation process. During the scans, animals will be fitted with an endotracheal tube, induced with 5% or less isoflurane anesthesia, and typically maintained on less than 3% isoflurane. Heart rate, respiration and oxygen saturation will be monitored continuously and recorded every 15 minutes throughout the procedure. Body temperature will be measured and recorded before and after imaging. Heated water bottles and/or towels may be used to maintain body temperature during imaging. An indwelling catheter may be placed, with preference given to the saphenous vein, before, during or after the scanning procedure. Warm saline (0.9% NaCl), Plasmalyte, lactated Ringer’s or other isotonic fluid (approved by a veterinarian) may be administered (subcutaneously or intravenously) before, during or after imaging procedures to maintain hydration. Glucose will be monitored and recorded before and after the scanning procedure, and at intervals during the scanning procedure if recommended by veterinary staff. Because veterinary staff will attend and directly supervise these procedures for animals of this age group, the veterinary staff will decide whether to administer supplemental dextrose (alone or mixed with an isotonic fluid) if necessary before, during or after the imaging procedure. Animals will be removed from the scanner and monitored until they can be safely transported. Animals will be monitored continuously until they are able to lift their own head, then they will be monitored at least every 15 minutes until they are sitting up, after which they will be monitored every 30 minutes until fully recovered.

For MR imaging of animals 8 months of age and older, animals will be chemically restrained with Ketamine HCL (up to 20 milligrams/kilogram given intramuscularly), which will be repeated as needed approximately every 20-40 minutes. The initial dose will be given after the animal is positioned on a restraint table. After the initial dose of anesthesia takes effect, the animal will be transported to the MRI suite and scanned for approximately two hours. No animal will receive more than 40 milligrams/kilogram Ketamine HCL in a single day, unless approved by a veterinarian. Up to 0.025 milligrams/kilogram dexmedetomidine will be given intramuscularly. The dexmedetomidine will be reversed with up to 0.25 milligrams/kilogram dose of atipamezole given intramuscularly. Atropine sulfate (0.01 – 0.3 milligrams/kilograms intramuscularly or subcutaneously) may be given to depress salivary secretion. Heart rate and oxygen saturation will be monitored continuously and recorded every 15 minutes throughout the scan to evaluate the depth of anesthesia, and changes in these vital signs during the scan will be used to determine if additional doses of Ketamine HCL are needed. If heart rate decreases significantly, relative to baseline, the dexmedetomidine will be reversed using atipamezole (up to 0.25 milligrams/kilogram intramuscularly). Body temperature will be measured and recorded before and after imaging and maintained by wrapping animals in bubble wrap and/or blankets. Catheters may be placed in a peripheral vein, generally the cephalic or saphenous, for administration of appropriate fluids. Animals will be removed from the scanner and monitored until they can be
safely transported. Animals will be monitored at least every 15 minutes until they are sitting up, after which they will be monitored every 30 minutes until fully recovered.

Animals 8 months of age and older may be scanned using injectable anesthesia or with the use of gas anesthesia as is done in animals younger than 8 months. Injectable anesthesia has been routinely used successfully by this lab with animals older than 8 months. Unless recommended by veterinary staff, blood glucose will not be monitored in animals 8 months of age and over.

Although animals are scheduled to have 5 scans, animals may undergo up to 7 MRI scans per year with at least one week between scans. MRI scanning will last approximately 60-240 minutes.

**Histology**

All 40 experimental animals will be euthanized for tissue collection. It is necessary to collect brain tissue to understand the molecular alterations associated with the early environmental experiences as it relates to maladaptive anxiety. It is also important to analyze changes to morphology of key peripheral organs to understand the relation between impacts on brain development and hormonal/physiological mechanisms by which these effects may be mediated. In all animals, tissue will be collected and flash frozen for analysis of RNAs and methylation of DNA and histones. This will allow for an optimal sample size for understanding differences in RNA expression and methylation patterns. To optimize the use of the tissue, some of the sections may be fixed after brain tissue is sampled for the above analyses. Post fixation will allow for further analyses with immunostaining methods primarily focused on markers of cell types and neuroplasticity.

All compounds given to living animals, including FDG, will be pharmaceutical grade.

b. Do any animals undergo any type of restraint beyond normal housing methods? (examples of non-normal housing include metabolic crates and restraint chairs). **Underline one:** YES NO

If YES, describe the method, length of restraint, and justification for such restraint. If the design of the study requires continuous restraint for longer than 12 hours without the opportunity for exercise, be sure the justification addresses need for such an extended period and include the maximum length of time the animals will be restrained. Include any plans for providing additional enrichment and any steps taken to avoid physical discomfort during the restraint. If you are unsure whether or not your proposed methods are considered restraint, contact your Attending Veterinarian.

Conscious animals will be restrained in a tabletop restraint device (considered normal husbandry method) or manually restrained for some procedures such as blood sampling, injections and shaving. Animals at the [redacted] are acclimated to this device from the time they are infants. The animals will spend no more than a few minutes being restrained for any one procedure. When using a tabletop restraint, the animal will be transported from its home cage in a transport cage, and allowed to enter the restraint device. The gate will be adjusted to hold the animal firmly, yet comfortably, and released as soon as the procedure is completed, thereby eliminating the need for anesthesia. When using manual restraint, animals will be gently removed from their cage or transport cage and their arms and legs carefully grasped and held while the procedure is performed. This eliminates the need for anesthesia and is the most efficient restraint method for animals that are too small for the tabletop restraint device.

c. Are any animals subjected to fluid or food restriction or regulation? **Underline one:** YES NO

If YES, discuss type and length of restriction, the expected consequences of restriction on the animals’ health and well-being, and justification for such restrictions.

For the subjects’ safety and per the recommendations of veterinary staff to prevent aspiration, food will be restricted before the administration of anesthesia for imaging scans. For animals 8 months of age and over, solid food will be restricted at least 5 hours before the onset of experimental scanning procedures and for no more than 24 hours. This is consistent with the [redacted] SOP 1.11 (Food Deprivation). Because these techniques are being developed for animals under 8 months of age, veterinary staff will work to determine the safest, most effective methods of food restriction for animals of this age. If housed with mother, animals will be allowed to nurse until removal for procedures. The mother’s food will be restricted at least 5 hours, but no longer than 24 hours, before administration of anesthesia to the infant for imaging scans to ensure the infant does not ingest solid food. For animals living in the nursery, solid food will be restricted at least 6 hours and formula at least 2 hours before administration of anesthesia for imaging. For infants under 8 months of age, solid food or formula will not be restricted for more than 24 hours. No adverse consequences are expected from this restriction.

d. Will any animals require nonstandard husbandry or housing exemption (e.g. exercise exemption, modified light cycle, extended cage cleaning periods, nonstandard cage type or size, etc.)? **Underline one:** YES NO
If YES, indicate the type of nonstandard husbandry required and scientific justification for these practices.

Please see Q35a for details.

18. Will animals be subjected to more than momentary or slight pain or discomfort as a result of the experimental or other study-related procedures? Underline one: YES NO
If YES describe the analgesics you will provide. Include drug names (generic preferred), dosages, route of administration, nursing care, mechanical devices, etc.

**NOTE:** If all experimental or other study-related procedures are terminal and therefore performed only on anesthetized animals, type an X between the brackets: [ ]

In the unlikely event that our young surrogate/peer reared animals develop self-aggression, veterinary and behavioral management staff will be consulted and appropriate interventions, such as daily surveillance, behavioral assessment, extra enrichment, relocation, or re-pairing of animals, will be instituted. If the animal does not respond to these interventions, pharmacotherapy will be instituted at the discretion of veterinary staff. Specific pharmacological agents and doses will be case dependent and decided upon by the veterinary staff. If the animal does not respond to these interventions, in extreme and very rare cases, veterinary staff will consider euthanasia. There are also concerns that surrogate/peer reared animals may be more susceptible to diarrheal illnesses. As with all animals, we will monitor closely for health status and veterinary staff will be consulted regarding any concerns.

When infants are permanently removed from their mothers, animal care, behavioral and veterinary staff will be notified to assess the effect of infant removal on the mother. Care will be provided at their discretion if necessary.

**Post CSF tap pain management**
After CSF collection, animals 8 months of age and older will receive at least one intramuscular dose of ketoprofen (2-5 mg/kg), or an alternative analgesic in consultation with a veterinarian, to relieve any possible inflammation. For animals less than 8 months of age, veterinary staff may administer ketoprofen (2-5 mg/kg intramuscularly), or an alternative analgesic in consultation with a veterinarian, if they observe signs of discomfort.

**Post skin biopsy pain management**
Animals undergoing skin biopsies will receive one dose of a perioperative analgesic (0.01 - 0.03 mg/kg buprenorphine administered IM, 2-5 mg/kg ketoprofen administered IM) or an alternative as recommended by a veterinarian, with additional doses given only as directed by a veterinarian if unanticipated pain is observed after the procedure.

The snake will only be subject to routine handling, which does not constitute more than momentary or slight pain or discomfort. It will only be used as a stimulus. When used as a stimulus in the Snake Exposure test, there will be a protective barrier between the snake and the monkey.

19. Describe how frequently animals will be monitored to ensure they are not experiencing pain or discomfort from your procedures or any unanticipated illness or injury not necessarily directly related to your research. Describe the criteria or clinical signs (e.g. ruffled fur, hunched posture) that you will use to determine when euthanasia will be performed in these cases.

Post-anesthesia monitoring procedure will be specific to the anesthesia given. Animals that require injectable anesthesia will be monitored at least every 15 minutes until they are awake and sitting up, after that they will be monitored every 30 minutes until fully recovered. Animals that require Isoflurane anesthesia will be monitored continuously until they are able to lift their own head, then they will be monitored at least every 15 minutes until they are sitting up, after which they will be monitored every 30 minutes until fully recovered. Animals that are housed with their mother will not be returned to their mother until they recover muscle tension in their limbs and are able to grasp. After returning to their mother, animals will be continuously monitored until the mother picks up and holds the infant.

As part of normal animal husbandry, the [ ] has a comprehensive monitoring system which encompasses checking fecal material, food intake, behavior, weight, and general appearance, as well as any signs of injury. All animals are observed a minimum of twice per day, but usually more frequently. In the case of illness or injury, the veterinarians will decide if the animal must be euthanized.

The snake will be monitored at least once daily for signs of illness. If health issues arise, the snake will be taken to the University of Wisconsin-[ ] for assessment.
20. Describe the specific criteria for termination of animals if experiments could induce chronic disease, tumors or radiation sickness. These criteria should be described in terms of tumor size, specific animal characteristics or behaviors, weight loss changes, observed clinical signs, etc.

NOTE: If experiments are not expected to induce these conditions, please type an X between the brackets: [X] Chronic disease, tumors or radiation sickness are not anticipated.

None of these are expected.

21. Describe the methods of euthanasia used, including drugs, dosage, and any sedation. Consult the 2007 Report of the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia (www.avma.org/resources/euthanasia.pdf) or your school's Attending Veterinarian for appropriate euthanasia methods. Even if euthanasia of animals is not part of this project, complete this Question for cases of unanticipated illness or injury.

NOTE: In general, physical methods (cervical dislocation, decapitation) are recommended for use only after other acceptable means have been excluded, in sedated or unconscious animals when practical, when scientifically or clinically justified, and with Animal Care and Use Committee approval. Physical methods without pre-anesthesia require scientific justification and description of the training of personnel who will perform it.

Under the supervision of a veterinarian and pathologist the primates will initially be anesthetized using at least 15 milligram/kilogram of Ketamine HCL intramuscularly. They will then be humanely euthanized with an intravenous overdose of at least 50 milligrams/kilogram sodium pentobarbital, or equivalent as approved by a clinical veterinarian. Death will be defined by stoppage of the heart as described by a qualified and experienced person using a stethoscope to monitor heart sounds from the chest area, as well as all other vital signs, which can be monitored by observation. These are standard methods of euthanization and are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

For all animals, tissue will be collected without perfusion and flash frozen. We and others have used this method for successful analyses of RNA expression and DNA methylation. The major focus will be the measurement of RNA and methylation of histones and DNA. Some samples of tissue will be post fixed for immunostaining procedures focusing on identifying and quantifying cell types as well as markers of neuroplasticity.

If it becomes necessary, the snake will be euthanized by a method approved by a UW Research Animal Veterinarian. Death will be confirmed by the absence of a heartbeat and lack of neurological activity.

22. If the animals are not euthanized at the end of the study, what will happen to them? Include descriptions of transfer of animals to other approved animal care and use protocols, or return of animals to managed colonies or herds.

Only experimental animals (n=40, 20 mother reared animals, n=20 surrogate/peer reared animals) will be euthanized at the end of the study. Animals that are not euthanized (mothers of all experimental subjects, animals used only as cage mates, stimulus animals, and any animals that need to be dropped from the study) will be returned to the UW Madison primate colony.

The snake will not be euthanized at the end of the study, and will remain housed in RM.

23. Could any animals or animal products involved in these studies possibly be consumed by humans? Underline one: YES NO

If YES, list any drugs to be given to the animals and the recommended withdrawal times before safe consumption:

INVESTIGATOR SIGNATURE:
To the best of my knowledge, I certify that the information provided in this Animal Care and Use Protocol is complete and accurate. I understand that approval must be renewed annually, that every third year the ACUC must perform a new review of my protocol, and that I might be required to complete a newer version of the Animal Care and Use Protocol and provide additional information at the time of the triennial review.

I also understand that ACUC approval must be obtained by an amendment to this protocol before I:
- Use additional animal species, increase the number of animals used, or increase the number of procedures performed on individual animals;
A. Change procedures in any way that might be considered a significant departure from the written protocol;
B. Perform additional procedures not described in this Animal Care and Use Protocol;
C. Allow other investigators to use these animals on other protocols, or use these animals on another of my ACUC-approved protocols.

I further certify that:

1. No personnel will perform any animal procedures until they have been approved by the ACUC, via RARC. When new or additional personnel become involved in these studies, I will submit their qualifications, training, and experience to the ACUC and seek ACUC approval before they are involved in animal studies;
2. I will ensure that all personnel are enrolled in an institutional Occupational Health and Safety Program prior to their contact with animals, or have declined in writing to participate, if allowed by local policy;
3. I will provide my after-hours telephone numbers to the animal care staff in case of emergency.

I plan to follow the provisions for the care, use and treatment of animals found in the NIH "Guide for the Care and Use of Laboratory Animals," or the "Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching". I assure that these procedures do not unnecessarily duplicate previous experiments.

Signature of PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR: ________________________________

(A signature is required for submission. Either print, sign, and fax this page to 265-9040 with a cover sheet that identifies you/your protocol clearly, or paste an image of your handwritten signature here.)

Questions for Projects Involving Surgical Procedures

24. Give the names of all research staff who will perform hands-on surgery on the animals in this study. For each person listed, describe their type and length of surgical training and experience, emphasizing specific experience with surgeries to be performed as a part of this study. For personnel listed below who have less than 1 year of experience with the surgeries they will be involved with, indicate who will train and supervise them. Please delete the examples are provided in the table below for you.

<table>
<thead>
<tr>
<th>Name/Phone Number</th>
<th>Brief description of SURGICAL training/experience.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ned H. Kalin, M.D. 263-6079</td>
<td>25 yrs primate surgical experience.</td>
</tr>
<tr>
<td>[Redacted] Ph.D.</td>
<td>Surgical experience with primates since 2013. Will be supervised by Ned Kalin and/or veterinary staff. Completed the Lab Animal Surgery training course.</td>
</tr>
<tr>
<td>[Redacted] veterinary staff, and [Redacted] animal care staff under the direction of a veterinarian</td>
<td>Experience in various clinical treatments and surgical procedures.</td>
</tr>
</tbody>
</table>

25. Where will surgery be performed?

Skin Punch Biopsies
Room number(s): Room [Redacted] or [Redacted] Building: [Redacted] Procedure rooms: [Redacted]

26. How many animals listed in Question 9(a) will undergo surgery?

Up to 40 experimental animals (n=40) mother reared animals; n=20 surrogate/peer reared animals) may undergo skin punch biopsies. If replacement animals are added to the study they will also undergo skin punch biopsies.

27. Anesthetics and Paralytic Agents

a. Describe anesthetic method used, including all drugs, dosages, routes of administration and supplementation regimen. Include how anesthesia level is monitored, e.g., list the physiologic parameters that will be monitored to ensure adequate anesthesia depth for both general and local anesthesia. Documentation of the anesthesia used and the monitoring of anesthetic depth is required for all surgical procedures.

Minor Surgical Procedures
For skin punch biopsies animals will be anesthetized with ketamine (up to 20 mg/kg IM) or up to 7 mg/kg ketamine IM and up to 0.03 mg/kg dexmedetomidine IM to be reversed at conclusion of procedure by up to 0.3 mg/kg atipamezole (IV or IM) or alternative anesthetics in consultation with veterinarians. The animals will be monitored visually during the brief procedure to assure adequate plane of anesthesia and to assess that they are stable.

b. Are any paralytic agents being used? **Underline one:** YES  NO
If YES, indicate agent, justification for use, and any special monitoring techniques used to assess animal condition while under paralysis.

28. **Surgical Procedures**

a. Describe the surgical procedure(s), including narrative description(s) for the following: reason for the surgery, incision site(s), tissue isolation methods, wound closure, and an estimate of time required to complete the surgery.

**NOTE:** Aseptic procedures must be used for all survival surgery.

Minor Surgical Procedures

**Skin Punch Biopsies**

Animals will undergo skin punch biopsies to collect fibroblasts for induced pluripotent stem cell technology (iPSCs) which is an approach we will use to understand molecular mechanisms that underlie the pathological expression of anxiety. This technique uses fibroblasts that are collected from a small skin biopsy and then reverts them into stem cells. The stem cells are then differentiated into GABA-ergic neurons, similar to neurons in the amygdala, a key brain region for the expression of emotion and anxiety. Gamma-Aminobutyric acid (GABA) is a neurotransmitter (chemical messenger) that is used by cells in the amygdala and many other brain systems to communicate inhibitory signals. Deriving GABA-ergic neurons from skin cells obtained from nonhuman primates will enable us to perform in vitro studies to identify molecular alterations associated with anxious temperament. This work will be performed in collaboration with Dr. [redacted] at the UW [redacted] His laboratory has had success deriving GABA-ergic forebrain neurons that share characteristics with amygdala GABA-ergic neurons (Ma, L., et al., Cell Stem Cell, 2012).

During a minor surgical procedure up to 2 skin samples will be collected from each individual animal. The upper back or abdominal region will be used for obtaining the skin biopsies. The skin at the proposed biopsy site will be clipped, surgically prepped and may be sterile draped. A sterile circular punch biopsy instrument (6mm or smaller) will be rotated several times until a full thickness sample is obtained. A forceps and scissors may be used to lift and free the biopsy from underlying connective tissue, as needed. Punch biopsy sites may be closed utilizing absorbable (e.g. Vicryl) suture in a subcuticular pattern, may be closed using skin adhesive, or may be left open to heal by second intention. If skin biopsies do not produce enough cells for the cell culture, it may be necessary to repeat the biopsy procedure up to 3 times. This procedure generally takes less than 15 minutes to complete.

b. Describe which of the following procedures will be used to maintain a sterile field during surgery (place an X between the brackets of all that apply):

[X] sterile instruments: specify method: [ ] bead sterilizer [X] autoclave [X] describe other: gas
[X] sterile gown/garb  [X] sterile gloves  [X] sterile drapes  [X] face mask/eye protection
[ ] surgeon scrub  [ ] other (please describe):

29. **Will the animals be allowed to recover from surgery?**  (Underline one)  **YES  NO**

If YES, describe the post-anesthetic and post-surgical monitoring and care procedures, including:

• all drugs and dosages
• how body temperature will be maintained during recovery
• the plan for suture or staple removal
• who will perform the monitoring, frequency/duration of monitoring
• the parameters that will be evaluated
• method of maintaining written records of these examinations
• measures designed to alleviate post-operative discomfort

**NOTE:** Documentation of the post-operative monitoring of post-surgical animals is required!

Animals will be monitored at least every 15 minutes until they are sitting up, after which they will be monitored every 30 minutes until fully recovered. As part of normal husbandry, animals will also be monitored daily by care staff for signs or symptoms of ill health during post surgical treatment and after they return to their home cage. Care staff
will check fecal material, food intake, behavior, general appearance, as well as any signs of injury. Weight changes will also be regularly monitored as part of routine animal husbandry.

Discomfort, distress, pain or injury will be minimized by the appropriate use of anesthetics and analgesics, which will be administered in consultation with a veterinarian. Animals undergoing skin biopsies will receive one dose of a perioperative analgesic (0.01 - 0.03 mg/kg buprenorphine, IM; 2-5 mg/kg ketoprofen, IM; or an alternative) as recommended by a veterinarian. If unanticipated pain is observed after the procedure, additional doses may be given only as directed by a veterinarian. Body temperature will be monitored before and after the skin biopsy by research and/or veterinary staff and, if necessary, will be maintained with a warm air blanket, isothermal pads or infrared lights.

Individual animal treatment records and anesthesia recovery records are maintained in our log books, as well as the computerized records. All drugs, drug dosages, food and water consumption, as well as the health condition of the animal will be noted when the animal is treated.

30. Will any animal(s) be allowed to recover from more than one major operative procedure?
Underline one: YES NO

NOTE: A major operative procedure is defined as any surgical intervention that penetrates and exposes a body cavity or any procedure that produces permanent impairment of physical or physiological functions.

Animals will not undergo any major surgeries.

a. If YES, provide scientific justification for performing these procedures and list the species and number of animals:

b. What is minimum length of time between the operative procedures?

Questions for Projects Using Wild-Caught Animals
(It is the responsibility of the PI to obtain all necessary state and federal permits for work with wild animals.)

31. Do you capture wild animals or do experimental manipulations (or procedures) on animals in the wild?
Underline one: YES NO, Observation only

32. If you capture wild animals, describe how they will be trapped, what types of traps will be used, and how often traps will be checked.

33. Quarantine and Release Information
a. Describe quarantine procedures and precautions to prevent exposure of humans and other animals to zoonotic diseases.

NOTE: If animals will not be housed, please state this.

b. If animals will be released back to the wild, explain how the released animals will not present a disease exposure to wild populations and explain why this release will not expose the animal to greater risk of predation as a direct result of procedures performed or materials administered.

NOTE: If animals will not be released back into the wild, please state this.

34. If wild animals will be anesthetized and released to the wild, describe anesthetic doses, method of administering and procedures for ensuring that animals are sufficiently recovered from anesthetic to be released. Consider that prey species may have to be monitored until fully recovered to avoid predation. NOTE: If animals will not be anesthetized, please state this.

Questions for Projects Using Nonhuman Primates

35. Nonhuman Primate Enrichment
a. If nonhuman primates used in your study must be housed individually due to scientific consideration, provide that scientific rational.
Some animals may be singly housed for brief periods until a compatible housing partner is selected. If aggressive behavior is observed between partners, animals will be separated and singly housed for their own safety. Following separation, behavioral management staff, in consultation with veterinary staff at the [redacted] are responsible for determining when it is appropriate to find another partner for the animal.

Our intent is to establish standard surrogate/peer rearing conditions for the experimental group. This requires pairing with a similarly aged peer very early in life. Ideally, animals would be paired with a peer as soon as possible for the experimental purposes of our study, but infants must remain housed in the incubator in order to specifically monitor each animal’s food intake and output and to allow more consistent incubator temperature control until animals are able to thermoregulate themselves until day 21, and possibly up to 42 days to allow for appropriate pairing (described below), which is an accepted practice at other primate institutions. To control for the possible effects of early environment on brain development, it is important that all infants experience pair formation at approximately the same age. Therefore, we have chosen an age range of 21-42 days for pairing that, based on projected parturition dates, will allow for similar timing of pair formation of surrogate/peer reared animals. Animals in the surrogate/peer reared group will be singly housed in their incubators for approximately the first 3-6 weeks of life. The procedures proposed for nursery and incubator housing, as well as pair formation, are consistent with the [redacted] SOP.1.16. Our research staff will be in close consult with [redacted] veterinary staff to ensure that our procedures for nursery and incubator housing are in line with [redacted] nursery rearing practices. During the time spent in the incubator, animals will be provided with an upright surrogate covered with a soft material that is able to move back and forth at the infant’s discretion. To reiterate, the 21-42 days alone in the incubator is consistent with practices at other primate facilities (California National Primate Center; Romnee, 2009; Romnee 2011; Capitanio, 2005; and the National Institutes of Health; Dettmer, 2008; Barr, 2004; Spinelli, 2009) and is required because the infants must develop the ability to thermoregulate and self-feed.

b. Provide scientific rationale for any restrictions to environmental enrichment. Include the specific restriction(s) such as: puzzle feeders, cage perch, wooden chew sticks, food treats (bananas, carrots, oranges, other fruit or vegetables), etc.

Environmental enrichment will not be restricted, but will be coordinated with ongoing experiments, may be coordinated between the two experimental groups to ensure consistency between groups. An upright surrogate covered with a soft material that is able to move back and forth at the infant’s discretion will be provided while infants are housed in the incubator. Once in standard caging, environmental enrichment will be provided in coordination with the environmental enrichment received by the mother reared animals. Due to the importance that surrogate/peer reared infants are treated as similarly as is possible to the mother reared infants, with the exception of the rearing condition, surrogate/peer reared animals will be exempt from socialization sessions in a socialization cage listed in [redacted] SOP.1.46.
INVESTIGATOR SIGNATURE:
To the best of my knowledge, I certify that the information provided in this Animal Care and Use Protocol is complete and accurate. I understand that approval must be renewed annually, that every third year the ACUC must perform a new review of my protocol, and that I might be required to complete a newer version of the Animal Care and Use Protocol and provide additional information at the time of the triennial review.

I also understand that ACUC approval must be obtained by an amendment to this protocol before I:
- Use additional animal species, increase the number of animals used, or increase the number of procedures performed on individual animals;
- Change procedures in any way that might be considered a significant departure from the written protocol;
- Perform additional procedures not described in this Animal Care and Use Protocol;
- Allow other investigators to use these animals on other protocols, or use these animals on another of my ACUC-approved protocols.

I further certify that:
- No personnel will perform any animal procedures until they have been approved by the ACUC, via RARC. When new or additional personnel become involved in these studies, I will submit their qualifications, training, and experience to the ACUC and seek ACUC approval before they are involved in animal studies;
- I will ensure that all personnel are enrolled in an institutional Occupational Health and Safety Program prior to their contact with animals, or have declined in writing to participate, if allowed by local policy;
- I will provide my after-hours telephone numbers to the animal care staff in case of emergency.

I plan to follow the provisions for the care, use and treatment of animals found in the NIH "Guide for the Care and Use of Laboratory Animals," or the "Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching". I assure that these procedures do not unnecessarily duplicate previous experiments.

Signature of PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR: ____________________________

Attending Veterinarian (SVM ONLY) _____________________________________________