The University of Mississippi Medical Center

Animal Activity Protocol

IACUC - Institutional Animal Care and Use Committee
Telephone [redacted] / Facsimile [redacted]
iacuc@umc.edu

Contents

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To be completed by IACUC

Protocol Number: **1512A**	Date: **05/20/2020**	Classification: **D**

1. Principal Investigator

Name	[redacted]		
	☒PhD ☐ MD ☐ Other:		
Title	**Professor**		
Dept.	**Psychiatry & Human Behavior**		
Phone #	[redacted]	**Office Location**	[redacted]
email	[redacted]	**Emergency #**	[redacted]

Note: The emergency number should be a number at which the PI can be contacted on nights and weekends.

2. Other Personnel

All listed personnel must complete IACUC required training, including completion of Occupational Health forms and submit a Training Requirements Registration form prior to working with animals and receiving access into the Center for Comparative Research (CCR).

You may authorize personnel to submit modifications to this protocol by checking the box for signing privileges.

Name	Title	Ext/Cell	Email	Signing Privileges
[redacted]	Professor	[redacted]	[redacted]	☒
[redacted]	Postdoctoral fellow	[redacted]	[redacted]	☒
[redacted]	Associate professor	[redacted]	[redacted]	☐
[redacted]	Assistant professor	[redacted]	[redacted]	☐
[redacted]	Instructor	[redacted]	[redacted]	☐
[redacted]	Postdoctoral fellow	[redacted]	[redacted]	☐
[redacted]	Researcher II	[redacted]	[redacted]	☐
[redacted]	Graduate Student (PIN/Psychiatry)	[redacted]	[redacted]	☐

[redacted]	Graduate Student (PIN/Psychiatry)	[redacted]	[redacted]	☐
[redacted]	Head of UMMC Behavioral Core	[redacted]	[redacted]	☐
[redacted]	Lab Manager – Freeman lab	[redacted]	[redacted]	☐
[redacted]	SURE Student	--	[redacted]	☐
[redacted]	Resercher II		[redacted]	Added 8/10/21
[redacted]	Researcher II		[redacted]	Added 9/17/20
[redacted]	Resercher II		[redacted]	Added 5/11/21
[redacted]	Researcher II		[redacted]	Added 5/13/21
[redacted]	INBRE student		Added 5/26/21	Departure 7/30/21
[redacted]	Researcher II		[redacted]	Added 6/8/21
[redacted]	Researcher II		[redacted]	Added 6/14/21
[redacted]	Researcher II			Added 8/31/21
[redacted]	Med Student		[redacted]	Added 9/3/21
[redacted]	Researcher II	[redacted]	[redacted]	Added 11/4/21
[redacted]	Graduate Student		[redacted]	Added 02/01/22
[redacted]	Researcher II	[redacted]	[redacted]	Added 4/21/2022
[redacted]	Post- Doc Fellow	[redacted]	[redacted]	added 5/18/22
[redacted]	Researcher II	[redacted]	[redacted]	Added 8/18/22

3. Project Title:

Behavioral pharmacology studies in monkeys: chronic administration and dependence

4. Proposal is **3 year Full Submission Renewal (must attach Appendix K)**

5a. Outside Contracts

Will any components of this study involve live animals maintained at another institution?

☒ No

☐ Yes (if yes, provide information on the level of involvement)

5b. Animal Behavior Core

Will this study use the Animal Behavior Core (ABC)?

☒ No

☐ Yes – Requires review and approval by ABC Director. See Appendix L.

6. Funding Source

☒ Extramural/Intramural Funding

Title	Anxiolytic and abuse-related effects of BZ ligands		
PI	[redacted]		
Funding Agency	NIH/NIDA		
Status	☐ Submitted ☒Funded	Grant Number	R01 DA011792
Covered Dates	6/1/1998-12/31/2019		

Title	Tolerance and Physical Dependence after Chronic Benzodiazepine Treatment		
PI	[redacted]		
Funding Agency	NIH/NIDA		
Status	☐ Submitted ☒Funded	Grant Number	R01 DA043204
Covered Dates	7/1/17-6/30/22		

Title	Opioid and benzodiazepine co-abuse in nonhuman primates		
PI	[redacted]		
Funding Agency	Alkermes		
Status	☐ Submitted ☒Funded	Grant Number	PW014-2018
Covered Dates	01/01/2019 – 12/31/2020		

Title	EEG Telemetry in Monkeys: Potential Markers of Benzodiazepine Action		
PI	[redacted]		
Funding Agency	NIH/NIDA		
Status	☐ Submitted ☒Funded	Grant Number	R21 DA046778
Covered Dates	06/01/2019 – 05/31/2021		

☐ Department – List Department: Click here to enter text.

☐ Other (*Example: Divisional funds which you have control over, start-up funds*)
Explain:

7. Dates of Study

Anticipated start date of study: 6/1/2020

All investigators must adhere to a federally mandated three-year cycle of full protocol review, even if a funding period exceeds three years in duration.

8. Source of Animals

Will any animals be obtained from non-commercial sources? ☒No ☐Yes
If Yes, list:

Note: Animals from non-commercial sources must have their health status evaluated by a CCR veterinarian prior to their arrival at UMMC. This question does not relate to

the acquisition of animals from other UMMC investigators. If animals are transferred from a UMMC source, an Animal Transfer Form must be completed and approved for each transfer.

9. Animal Requirements

For **New** submissions complete **Table A.**
For **3 Year FSR** submissions complete **Table B**.

Animal numbers MUST be calculated for a period not to exceed three (3) years from the start of the study.

A. New:

Species	Strain/stock	Sex	Source	Total for 3 years	Average daily census

(Insert additional lines as needed)

Note: If using nonhuman primates, complete Appendix A.

B. 3 Year FSR: **For a 3-year renewal, number of animals needed to complete the studies in this protocol. This must include the number of animals to be received plus the number of animals currently on campus to be carried over from the previous version of this protocol.**

Example: You need 100 animals to complete your study and you have 20 animals currently in house to carry over to this is protocol.

Total Needed for 3 years		*Total Carried Over*		*Total Requested*
100	-	20	=	80

You will be approved for 100 animals to complete the study (number to be justified in question #17) of which you already have 20, so you will have 80 animals available to order.

Species	Strain/stock	Sex	Source	Total Needed For 3 years	Total Carried Over	Total Requested (Needed – Carried Over)	Average daily census
NHP	Rhesus Macaque	M/F	National Primate Centers (e.g. CNPRC, Yerkes, etc.)	45	6	39	10-35

(Insert additional lines as needed)
originally approved for 45; 5/20/20

Note: The number of animals available for ordering will be the difference between total animals needed minus carryover animals.

C. List any unusual phenotypes or abnormalities associated with the animals (including sublines) listed above (i.e., prone to diarrhea, decreased appetite, patchy hair loss, increased sensitivity to pain, slow wound healing, etc.).

10. Breeding program

Will animals be involved in a breeding program at UMMC or will time-pregnant animals be used?

☒ No

☐ Yes (if yes, provide information in Appendix B)

11. Potential Hazards

		Yes	No	Pending
A	**Chemical toxins used in animals?**	☒	☐	
	Reviewed by Environmental Health & Safety?	☐	☐	☐
B	**Radioisotopes used in animals?**	☐	☒	
	Reviewed by Radiation Safety?	☐	☐	☐
C	**Use of laser, CT, x-ray, or fluoroscopy?**	☐	☒	
	Reviewed by Radiation Safety?	☐	☐	☐
D	**Biohazards used in animals?**	☒	☐	
	Reviewed by Institutional Biohazard Committee?	☒	☐	☐
E	**Human cells used in animals?**	☐	☒	
	Reviewed by Institutional Biohazard Committee?	☐	☐	☐

If YES, provide specific details of specialized animal husbandry, care, cleaning, or decontamination procedures**, especially identifying responsible parties**.

Although biohazards are not used in the animals, the monkeys themselves represent a biohazard risk in that they carry macacine herpesvirus 1, or Herpes B virus. In order to reduce the risk of exposure, proper PPE is worn at all times in monkey areas. This includes a lab coat, face mask, face shield, gloves, pants and closed-toed shoes. In the event an exposure does occur, a biohazard protocol is in place such that the person must scrub the affected area with a betadine pad for 15 minutes (for scratches, cuts, bites, etc.) of flush the affected area for 15 minutes (e.g., eye & mouth exposures). Following this, blood is taken from both the exposed person and the monkey and sent off to test for the Herpes B virus, with a follow-up blood test occurring 2 weeks later. **The lab has obtained IBC approval for these procedures, and signed informed consent forms for all lab members are on file in the IBC office as well as within the lab.**

12a. Animal Husbandry

	Standard	Nonstandard
Feeding	☐	☒
Watering	☒	☐
Caging	☒	☒
Room/Environment	☒	☐
Altered light cycle	☒	☐

Note: Provide complete explanation and justification for any **nonstandard animal husbandry** (e.g. metabolic caging, restraint chairs, transport devices, singly housed animals, altered light cycle). Protocols listing non-standard husbandry must provide complete details of the cleaning and sanitation, **especially identifying responsible parties**:

Feeding: Monkeys will be fed a prescribed amount of monkey chow by lab personnel listed on the protocol Monday-Friday (not including holidays). CCR personnel feed the monkeys the same amount of monkey chow on weekends and holidays. Amount of chow for each monkey will be determined in consultation with veterinarians to be that which maintains healthy weights in rhesus monkeys and also allows for criteria-level performance on behavioral tasks. For monkeys on studies that involve food reinforcement or drug/food choice, the amount of food consumed within-session will be subtracted from their daily food ration that is fed post-session. This allows for the maintenance of healthy body weights regardless of how much food is consumed within-session. Physical exams (including determination of weight and body temperature and hands-on assessment of body condition to prevent major fluctuations in weight, examination of eyes/ears/teeth/tongue/lips for lesions associated with the Herpes B virus, etc.; hands-on palpation of abdomen for presence of masses, examination of incision sites, catheter exit sites, and pretreatment drug injection sites for adverse consequences) will be conducted every 1-2 months by listed personnel. When in study, monkeys will be weighed a minimum of every 2 weeks. Monkeys will be lightly sedated with ketamine (~10 mg/kg, i.m. or i.v.), or other sedative-anesthetic as administered by CCR staff, for these exams. Physical examinations are conducted for several reasons, such as the potential for early detection of indicators of health problems (e.g., dehydration, insufficient feed, parasitism, etc.); and to obtain recent accurate weights necessary for calculation of doses for experimental drugs/compounds.
Under exceptional circumstances (e.g. 2020 COVID 19 pandemic), Lab staff will work with CCR in order to guarantee the animals will be fed daily either by CCR (if Lab staff is not allowed on campus) or by lab staff (if CCR is unable to feed the animals).

Caging: Some studies will be conducted in the monkey's individual living quarters. Monkeys will self-administer intravenous (i.v.) infusions of drugs/compounds. The i.v. catheter exits the monkey's back or is attached to a subcutaneous vascular access port and is threaded through a tether attached to a swivel which can be inserted into a self-administration panel on the side of the home cage. Monkeys cannot be pair-housed while on home-cage self-administration studies due to the risk of damage to the tether, catheter, etc. (see Appendix A for more information on pair-housing exemptions), and the self-administration panel inserted into one side of the cage prevents visual contact with other cages/monkeys on that side of the monkey. The monkeys do still have visual, auditory, and

olfactory contact with others in the room via the front of their cage unit as well as the side opposite the self-administration panel. Personnel listed on the protocol will be responsible for all cage-associated experimental equipment.

12b. Singly Housed Animals

Will animals be singly housed?

☐ No

☒ Yes – Please provide justification for single housing. **NOTE: If using non-human primates you must complete Appendix A.**

Appendix A attached for singly-housed non-human primates.

13. Housing

Will animals be housed outside of the CCR for greater than 12 hours?

☒ No

☐ Yes Where?

Note: If yes, provide complete explanation and justification for any **decentralized animal housing.**

14. Objectives in lay terminology

In **non-technical**/lay terminology, what is the **objective of the experiments** proposed in this Animal Activity Protocol? (i.e. ***Response should be written in non-scientific language, as though explaining the study to a high school student***.)

- In non-technical**/lay terminology**, what is the objective of the experiments proposed in this Animal Activity Protocol?
- Why are the experiments proposed?
- What knowledge do you hope to achieve?
- What is the potential relevance (e.g. benefits) of experimental findings to human or animal health, advancement of knowledge, and/or the good of society?

Generally, **single sentence explanations** for these types of questions will suffice.

Valium and related drugs, called “benzodiazepines”, are commonly prescribed for the treatment of anxiety and sleep disorders. Benzodiazepines often are taken every day for long periods of time, referred to as “chronic treatment”. With a long enough period of chronic treatment, some of the effects of benzodiazepines disappear (“tolerance”) and a syndrome will develop referred to as “physical dependence”. Physical dependence, or sometimes just called “dependence” is evident only when the benzodiazepine is removed and “withdrawal” signs emerge. Tolerance and dependence are linked—with tolerance, patients may start to increase their dosage, which leads to more intense dependence. Individuals often will abuse benzodiazepines in order to avoid withdrawal.

While benzodiazepine dependence is not life threatening, it does limit the clinical usefulness of these drugs and may lead to addiction. In people, withdrawal signs consist of nausea, tremors (trembling, especially in hands), anxiety (feelings of stress and worry) and in the most severe cases, mild seizures. In rhesus macaques, the withdrawal signs we have

quantified (see [redacted], *Psychopharmacology*, 2020, *in press*) are nose rub (potentially a sign of nausea/stomach upset), vomit/retch, procumbent (lying down on the cage floor, which monkeys don't often do), tremors, and rigid posture ("freezing", not moving at all; occurs briefly, typically < 1 min). As with human patients, seizures can occur but generally only when taking the drug for a long time (many months to years) and at much higher doses than proposed for this protocol (in our preliminary studies in 10 monkeys, no seizures were observed).

This research program is designed to understand why tolerance and dependence to chronic benzodiazepine treatment occurs, and if we can "re-design" benzodiazepines to lack these properties. We have preliminary findings that suggest different versions of the "GABA-A" protein, which contains the site of action for benzodiazepines, may control tolerance and dependence. The studies described in this protocol are designed to investigate a series of hypothesis revolving around this central premise.

The monkeys in these studies will receive chronic treatments with commonly-used (and abused) benzodiazepine-type drugs, such as Xanax® (alprazolam) and Ambien® (zolpidem). Rather than take the chronic drug away to induce withdrawal, we have developed a method by which we can administer a very short-acting blocker (flumazenil) which displaces the benzodiazepine from its site of action, in other words, "pharmacologically" removing the drug. Withdrawal occurs rapidly and for a very short period of time (15-30 minutes), reducing distress to the animal. Because of this, we will conduct withdrawal tests for 30 minutes maximum. After this test period, we will re-start chronic drug, which will immediately alleviate any remaining withdrawal effects.

Our primary scientific approach will be treat chronically with novel drugs with specific properties, which will allow us to test hypotheses about mechanism of action. We also will be able to give unique blockers with different properties to mimic withdrawal. We will use this information to inform our medicinal chemist collaborators (University of Wisconsin-Milwaukee) on ways to build better benzodiazepines with reduced tolerance and dependence liabilities.

15. Rationale

A. What is the rationale for using animals rather than using non-animal models?

The work described in this protocol cannot be conducted in humans because of the experimental nature of compounds that have not been evaluated in any form in human subjects. The research also cannot be conducted solely using tissue samples or biological material (e.g., organs-on-chips) because a primary goal of the research is to determine how drugs/compounds alter and control behavior in models predictive of effects in humans. Computer modeling does not provide meaningful information for this type of research, since modeling depends largely on a priori information that is not available without first conducting the types of studies described in this protocol.

B. What is the rationale for using the particular animal species and/or strain noted in Item 9?

Rhesus macaques are ideally suited for preclinical research on drug/alcohol addiction, and for many questions in addiction research are considered the "gold standard" species, particularly by federal regulatory agencies such as the FDA and DEA. This species has been used in behavioral pharmacology research for over 40 years and has provided valid and

reliable models of multiple aspects of substance use disorders. Because of the extensive use of rhesus macaques in neuroscience research, there is a large body of scientific information which provides indispensable comparative information for proper interpretation of our research. Finally, our new initiatives in tolerance and dependence are empowered by the observation that tolerance and dependence, including withdrawal syndromes, are essentially identical in human subjects and rhesus monkeys (for review, see Griffiths & Weerts, 1997, Psychopharmacology 134:1-37; Mintzer & Griffiths, 2005, Psychopharmacology 178: 259-67; Mintzer et al., 1999, Psychopharmacology 147: 200-9).

Other species used in benzodiazepine/GABA pharmacology research include rats and mice. Rodent species are useful for many studies in this area, but there are profound differences in both pharmacokinetic and pharmacodynamic effects of these drugs in rodents vs. primates. Perhaps the most important reason for choosing NHPs over rodents is the recent discovery that GABA-A receptor subtype distribution in rhesus monkey brain is much closer to human brain than either rat or mouse brain (Sperk et al. 2020, J Comp Neurol in press). Other species used in biomedical research such as dogs, cats, pigs, birds are rarely used in this field and would require decades of background research prior to conducting the type of research proposed in this protocol.

Monkeys will be obtained from National Primate Research Centers, with the Tulane National Primate Research Center as first choice (the PI has a joint appointment with Tulane, and this is the closest NPRC to UMMC). If no suitable monkeys are available from the NPRC system, we will explore commercial sources.

16. Brief Outline

Provide a general description of the animal procedures included in the experimental design. This description should allow the IACUC to understand the experimental course of an animal from its entry into the experiment to the endpoint of the study.

- **Briefly outline** the proposed animal manipulations and provide a time-line of events.
- **Note that specific details about methods and procedures will be required in the appropriate appendix (see list below)**
- **Complete** only those **appendices that apply** to the animal manipulations in your experimental design.
- If possible, flow charts and/or time lines should be included to clarify the timing of procedures which are to be performed.

Verbatim descriptions from a grant submission are not acceptable and will not be reviewed.

The proposed animal manipulations consist of 4 phases: 1) Pre-chronic tests; 2) Chronic treatment; 3) Tolerance & precipitated withdrawal tests; 4) Post-chronic tests. Daytime activity/sleep monitoring using Actiwatches (actigraphy) attached to the monkeys' jackets or primate collars will be conducted throughout all these phases.

1. Pre-chronic tests. The dose- and time-dependent observable behavioral effects of the chronic drug and a blocking agent ("antagonist") will be assessed before initiating chronic treatment. First, a dose of drug will be evaluated once/day, generally at 12:00 noon, to provide a significant period of time post feeding (feeding can engender a range of species-

specific effects). Test sessions will be planned with at least 2 drug-free days in between determinations. Behavioral observation periods (5 minutes each) will be taken at regular intervals (e.g., 0, 7.5, 15, 30, 60, 120, and 240 min post injection).

In some studies, monkeys will be trained to self-administer the benzodiazepine midazolam (or another benzodiazepine, e.g., alprazolam) i.v. in daily sessions. In these sessions, the monkey will have access to two levers, one in which lever pressing will result in drug and the other in which lever pressing will result in food (1-gram pellets, BioServ). As described above, these pellets will be subtracted from daily food allotments, since they are nutritionally matched with the regular diets. Available injections of midazolam will be capped at ~30-50 per day, at doses that are well below those that induce dependence, in order to avoid difficulties with interpreting the results.

Once these tests are completed, all monkeys will undergo a 1-month drug-free "washout" period. Although benzodiazepines are not associated with toxicity, we propose to monitor, with the assistance of CCR veterinary staff, clinical indicators of health prior to entering the next phase of the study.

2. Chronic treatment. Chronic treatment with drug will consist of an i.v. injection of the chronic drug, injected every nth hour (doses and time intervals will be determined during Pre-chronic tests). Behavioral observations will occur daily at 12 noon. Feeding and maintenance of the cage/equipment will occur the same time each day in the AM. Water will be available ad libitum throughout the experiment.

For studies involving self-administration, the sessions will be started again prior to chronic treatment, and will remain ongoing during chronic treatment. The chronic treatment regimen will be halted before the daily self-administration session, and started immediately after the session (sessions last ~2 hours).

3. Tolerance and precipitated withdrawal tests. After 30 days of chronic drug, tests for tolerance and precipitated withdrawal will be scheduled. The tests conducted during the pre-chronic phase will be repeated, only with chronic drug treatment prior to and after the tests. Each observation test will involve suspending chronic treatment 4 hours prior to the test session, followed by an experimenter-delivered injection of drug or antagonist at noon. Five-min observation sessions will occur at regular intervals (0, 7.5, 15, 30 min post-injection). Chronic drug treatments will be re-initiated after the 30 minute observation period. The same dose ranges used during the pre-chronic observation tests will be evaluated.

For studies involving self-administration, tests with other drugs will occur no more than twice per week (usually Tuesdays and Fridays). Studies for precipitated withdrawal will not be included in these experiments, since behavior would likely be absent.

4. Post-chronic tests. We anticipate that the tolerance and precipitated withdrawal tests will require ~60 days to complete. After the ~90 total days of chronic drug exposure and all tests are complete, we will replace drug with vehicle, starting on approximately day 91. This cessation of drug treatment is a test of "spontaneous withdrawal", i.e., withdrawal that emerges due to the absence of chronic drug treatment. Based on our preliminary studies, withdrawal signs were not recorded following chronic alprazolam treatments by approximately day 5 after cessation of treatment. A checklist of withdrawal signs is filled out at the same time each day by trained observers—this serves as our dependent measure of benzodiazepine withdrawal. In order to reduce the time in spontaneous withdrawal, we will monitor the monkeys 3 times per day (AM, noon, PM) for withdrawal signs. If 4 of the 5 signs

observed in our initial studies are recorded (see [redacted], Psychopharmacology 2020, *in press*) we will reinstate the chronic drug treatment, and administer anti-emetics in consultation with veterinary staff. We anticipate that all effects on behavior induced by the drugs will return to the levels observed during the pre-chronic tests, i.e., tolerance and withdrawal will be reversible.

Specific groups ("cohorts"): The project consists of 4 cohorts, up to n=10 monkeys each: (1) chronic alprazolam cohort; (2) chronic clonazepam cohort; (3) chronic zolpidem cohort; (4) chronic HZ166 cohort. As these are repeated measures designs, all monkeys within a cohort will experience, whenever possible, all test drugs/compounds, however, we request the flexibility to make the determinations for drug assignments based on the evolution of the studies. Note that this approach constitutes a reduction in animal number since one subject can be used to test a number of drugs. Also note that different groups of monkeys will be used for each cohort.

Timelines: We are proposing two Specific Aims for these studies. We anticipate Specific Aim 1 to be completed during Project Years 1 and 2. We anticipate Specific Aim 2 to require Project Years 3, 4, and 5 to complete. All estimates include time allocated for procurement of monkeys and typical down-time for catheter placement/replacement.

Design and Time Line Chart:

	Pre-Chronic	Chronic	Tolerance & Acute Precipitated Withdrawal	Taper or Spontaneous Withdrawal	Post-Chronic
Acute	T1-Tx		T1-Tx		T1-Tx
Chronic		C1-C4			
Time Estimates	~9 weeks	30 consecutive days	~9 weeks	1-3 weeks or to effect	~9 weeks

T1= alprazolam
T2= clonazepam
T3= zolpidem
T4= HZ166
T5= flumazenil
Tx= experimental compounds

C1= alprazolam
C2= clonazepam
C3= zolpidem
C4= HZ166

Tx ("experimental compounds") refers to both novel agonists and antagonists. In addition to tests with alprazolam, clonazepam, zolpidem, HZ166, flumazenil, we have planned to test novel agonists (e.g., TP003, L-838,417) which are not predicted to precipitate withdrawal (i.e., they will result in "cross-tolerance"). We will also test the alpha1-selective antagonist, BCCT, which we predict will have effects the same as flumazenil, and the alpha5-selective antagonist, XLi-093, which we predict will have no effects. Other drugs will be evaluated in

collaboration with our Subaward collaborator, [redacted] and colleagues, U Wisconsin-Milwaukee.

We will obtain an additional N=5 female monkeys for a pilot study, conducted as generally described in the Timeline. However, we will train these monkeys for awake blood sampling prior to the pre-chronic phase, and will establish menstrual cycles using the Oregon National Primate Research Center's fee-based service for evaluating sex hormones (which has 24-h turnaround capability). The overall sequence will consist of: (1) sampling/vaginal swab training; (2) menstrual cycle determination with daily draws; (3) pre-chronic studies, based on menstrual cycles (blood draws every 3 or 4 days); (3) chronic observation studies (blood draws every 3 or 4 days); (4) post-chronic studies (blood draws every 3 or 4 days). Although plans may change based on initial results, we intend to limit testing to different alprazolam doses and precipitated withdrawal tests with flumazenil (no spontaneous withdrawal tests will be conducted). Over years 3-5, we will repeat the experiment described above in this cohort with zolpidem or HZ166 as the chronic ligand, using the same limited testing of the chronic drug, flumazenil, and no spontaneous withdrawal. Altogether, these data will provide a preliminary assessment of the extent menstrual cycle phase enhances or attenuates tolerance and/or withdrawal.

17. Justification of animal number

Explain and **justify** how the number of animals requested was determined. (Flow diagrams/tables to define animal use are encouraged. **Statistical support should be included.** This number should support the request made in the *Total for 3 years* column in #9 and be consistent with the outline in #16).

A sample size of N=10 will be used in each separate experimental group, except for our pilot group for hormonal studies (N=5). Based on our preliminary data, power analyses indicated a sample size of N=5 as sufficient for identifying tolerance and withdrawal (alpha= 0.05, power=0.80; G*Power version 3.1.9.2). The larger sample size will allow us to account for any attrition and to address individual differences in tolerance and withdrawal differences, if they arise. Our pilot group will consist of 5 monkeys, given the exploratory nature of this proposed study.

To determine treatment effects, repeated-measures analysis of variance (ANOVA) will be used to evaluate statistical significance when warranted. For all studies, multiple comparisons will be assessed for statistical significance using a priori Bonferroni t-tests, when applicable. Comparisons of drug potencies will be conducted by computing ED50 values (dose engendering 50% of the maximum effect) using non-linear regression analysis techniques ([redacted]2000). For all tests, the alpha level will be constrained to $p \leq 0.05$.

18. Location & transportation

A. Indicate room(s) where animal procedures will be conducted.

Room Number	Procedures performed
[redacted]	Home-cage behavioral tasks will be conducted in these rooms.

B. Studies involving animal transportation to locations other than the housing area must identify the animal transport device, the nature of the shrouds used to cover the transport device, and describe the route of transport. **Include transport within the CCR (e.g. IVIS, surgery room).**

Monkeys will be transferred from housing areas in the [redacted]) to the nearby surgical suite. A cart will be used to transport the anesthetized monkey through the hall.

19. Euthanasia

A. At what point in the proposed experiments will animals normally be euthanized, (experimental end-points)? Or at what point will any individual animal be euthanized?

Euthanasia is not a part of the proposed experiments and will performed only as necessary (e.g., due to terminal illness).

Based on our experience with benzodiazepine-dependent monkeys, the animals can be used for other studies. A washout period of approximately 2 months is more than sufficient for the monkeys to not show any signs of tolerance and/or dependence. We have seen no evidence that dependence/tolerance develops quicker with re-exposure, and there is no evidence for this in the extant literature.

Monkeys from the cohorts are suitable for use in our other programs that include observation studies following acute drug exposures, self-administration, operant responding maintained by food, etc. Prior to use in another study, the monkeys will undergo a dose taper and a 2-month "wash out" with behavioral assessment of withdrawal. No "re-assignment" will occur without the animal showing no withdrawal signs for at least 1 week.

B. What humane endpoints or criteria will be used to determine if an animal is to be euthanized prior to, rather than at, the anticipated end-point of an experiment? Note: Contact CCR, [redacted], for recommendations on the assessment criteria.

Benzodiazepines and the related compounds described in this proposal are non-toxic by pharmacological/pharmaceutical standards and are well known to be some of the safest drugs known to clinical medicine. In this regard, doses of benzodiazepines >1000-fold the effective doses in tests of anxiolysis and sedation do not result in death in laboratory animals (in fact, LD50 doses cannot be calculated for most of these drugs). Due to this safety profile, humane endpoints for euthanasia are not required for administration of these drugs or the related compounds.

The studies in this protocol all require chronic intravenous catheters. We have considerable experience (>20 years) in the implantation and maintenance of these catheters. Complications with catheters do arise that are variable in frequency across monkeys, catheter placements, and/or time. The complications usually involve infections or thrombosis-induced clogging of the catheter. Regarding possible infections, the incision site is checked (if not obscured by the jacket) visually at least 2-3 times per week, by the PI's staff. For those in standard caging, catheter exit sites are inspected at a minimum of once every 2 weeks when the animal is sedated for changing the home cage by the PI's staff. Any evidence of swelling, exudates, or redness will be reported to the CCR veterinary staff for assessment and treatment, if necessary.

Clogged catheters due to thrombogenic events are typically detected prior to an experimental session, when the catheter is flushed to assess patency. Clogged catheters are

replaced with a new catheter, if possible. Our primary preventative measure is frequent flushing with sterile saline containing heparin (40-150 U/ml). Clot formation resulting in cardiac dysfunction can occur, and is usually detected as a change in weight and appetite, sometimes accompanied by swelling in the extremities. These events will be reported to the CCR veterinary team for cardiac assessment and the decision to euthanize is made in consultation with the PI.

Losing weight/not eating would be highly unusual under the conditions of the proposed studies, and would trigger consultation with the CCR veterinary staff for concerns about illness not related to the drug treatments. If recommended, the monkey will be removed from the study by instituting a dose taper—depending on where the animal is during chronic treatment. Because withdrawal signs only emerge after 30 days of chronic treatment, dose tapers will only be instituted at 30+ days, unless the monkey unexpectedly shows signs of withdrawal at earlier time points. This can be achieved in ~1 week: e.g., if treated with 1.0 mg/kg/day chronic alprazolam, doses can be decreased according to the schedule (mg/kg/day) 1.0, 0.5, 0.5, 0.25, 0.25, 0.12, vehicle. The PI's staff that are trained in behavioral observation will monitor the animal daily—if withdrawal signs emerge, the dose will be doubled again and a slower taper instituted (e.g., decreasing dose every 3 days). Sedation by ketamine injection is safe at any time of the study and can be used for physical exam, tests, etc. If catheter removal is necessary, the dose taper can be achieved with intramuscular injections at longer intervals (e.g., 6-7 hours between injections, performed by the PI and staff).

C. Will natural death (or death due to manipulations) be used as an endpoint?

☒No ☐Yes – if "Yes", explain and justify.

N/A

20. Euthanasia Procedures

What procedures will be used to euthanize the animals? Note: Secondary methods are required to ensure death. (Consult the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition for appropriate methods of euthanasia or contact the CCR.)

Euthanasia is performed by veterinarians in the CCR. Monkeys are first anesthetized with ketamine (10-20 mg/kg) then administered a commercial euthanasia solution, such as Fatal Plus, to effect. The veterinarian confirms euthanasia by ausculting for a loss of heartbeat, checking peripheral reflexes, etc. Necropsies are typically performed post-euthanasia by veterinarians. In addition to providing information about the health of the animal prior to euthanasia and allowing for tissue collection, this acts as a secondary form of euthanasia in ensuring the animal is deceased.

Assurances

1. Have all personnel received a medical evaluation from UMMC Student/Employee Health and updated Occupational Health Information annually?

   ☐No ☒Yes

2. Have all personnel listed on this protocol been informed and understand their role in the experiments?

☐No ☒Yes

3. Review of the available resources and previous experiments have determined that the proposed activity is not unnecessarily duplicative of previously reported activities.

☐No ☒Yes

USDA Policy #12, "Consideration of Alternative to Painful/Distressful Procedures": states the following: ***The Animal Welfare Act (AWA) regulations require principal investigators to consider alternatives to procedures that may cause more than momentary or slight pain or distress to the animals and provide a written narrative of the methods used and sources consulted to determine the availability of alternatives, including refinements, reductions, and replacements.***

List each potentially painful or distressing procedure included in these protocol:

Intra-muscular injections	Vaginal swabs (female NHPs)
Blood draws	Surgical implantation of I.V. Catheter
Benzodiazepine dependence	Chronic benzodiazepine administration
Spontaneous Withdrawal testing	Precipitated Withdrawal Testing

To comply with Policy #12, investigators are required to conduct literature searches using **two different search engines (see below)** addressing each of the procedures listed above. Specific procedures listed may be utilized as key terms.

Additional assistance may be obtained by contacting the Rowland Medical Library reference desk at ext. [redacted]. See IACUC Guidance on Minimizing Pain and Distress in Animals and Searching for Alternatives.

Helpful Databases

(Please note: PubMed and Medline are the same and cannot both be used.)

☒Medline/PubMed (http://www.ncbi.nlm.nih.gov/pubmed)
☐Toxnet (http://toxnet.nlm.nih.gov)
☐AWIC (http://awic.nal.usda.gov)
☐Agricola (http://agricola.nal.usda.gov)
☒Scopus (http://www.scopus.com/home.url)
☐Other (Click here to enter text.)

		Period of years	Potentially painful or		Indicate which mandate each search addressed

Name of the database	Date of search	covered by the search	distressing procedures addressed	Key words and/or search strategy used	Replacement of animals	Reduction in numbers of animals used	Refinement to minimize pain or distress	Lack of unnecessary duplication
Pubmed	3/1/20	Past 10 years	i.m. injection	Rhesus Monkey + Intramuscular injection	☐	☐	☒	☒
Pubmed	3/1/20	Past 10 years	i.m. injection	Rhesus Monkey + Intramuscular injection + alternative	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	IV self-administration	Rhesus Monkey + self-administration + animal model	☐	☐	☒	☒
Pubmed	3/1/20	Past 10 years	IV self-administration	Rhesus Monkey + self-administration + animal model + alternative	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Blood draws	Rhesus Monkey + blood draw	☐	☐	☒	☒
Pubmed	3/1/20	Past 10 years	Blood draws	Rhesus Monkey + blood draw + refinement	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Benzodiazepine drugs	Rhesus Monkey + benzodiazepine	☐	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Benzodiazepine dependence	Rhesus Monkey + chronic + benzodiazepine dependence	☐	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Benzodiazepine dependence	Rhesus monkey + model + benzodiazepine dependence	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Spontaneous withdrawal	spontaneous withdrawal + benzodiazepine	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Spontaneous withdrawal	spontaneous withdrawal + animal model	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Spontaneous withdrawal	spontaneous withdrawal + alternative	☒	☒	☒	☒

Pubmed	3/1/20	All available years	Spontaneous withdrawal	spontaneous withdrawal + refinement	☒	☒	☒	☒
Pubmed	3/1/20	All available years	Vaginal swab	Rhesus monkey + vaginal swab	☐	☐	☒	☒
Pubmed	3/1/20	All available years	Vaginal swab	Rhesus monkey + vaginal swab + animal model	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Catheterization Surgery	Rhesus monkey + catheterization surgery + refinement	☐	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Catheterization Surgery	Rhesus monkey + catheterization surgery + alternative	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Chronic administration	Rhesus monkey + chronic administration + animal model	☒	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Chronic administration	Rhesus monkey + chronic administration + benzodiazepine	☐	☒	☒	☒
Pubmed	3/1/20	Past 10 years	Chronic administration	Rhesus monkey + chronic administration + alternative	☒	☒	☒	☒
Pubmed	3/1/20	All available years	Precipitated withdrawal	Rhesus monkey + precipitated withdrawal + animal model	☒	☒	☒	☒
Pubmed	3/1/20	All available years	Precipitated withdrawal	precipitated withdrawal + alternative	☒	☒	☒	☒
Scopus	3/1/20	All available years	i.m. injection	Rhesus Monkey + Intramuscular injection	☐	☐	☒	☒
Scopus	3/1/20	All available years	i.m. injection	Rhesus Monkey + Intramuscular injection + alternative	☒	☒	☒	☒
Scopus	3/1/20	All available years	IV self-administration	Rhesus Monkey + self-administration + animal model	☐	☐	☒	☒
Scopus	3/1/20	All available years	IV self-administration	Rhesus Monkey + self-administration + animal model + alternative	☒	☒	☒	☒
Scopus	3/1/20	All available years	Blood draws	Rhesus Monkey + blood draw	☐	☐	☒	☒

Scopus	3/1/20	All available years	Blood draws	Rhesus Monkey + blood draw + refinement	☒	☒	☒	☒
Scopus	3/1/20	All available years	Benzodiazepine drugs	Rhesus Monkey + benzodiazepine	☐	☒	☒	☒
Scopus	3/1/20	All available years	Benzodiazepine dependence	Rhesus Monkey + chronic + benzodiazepine dependence	☐	☒	☒	☒
Scopus	3/1/20	All available years	Benzodiazepine dependence	Rhesus monkey + animal model + benzodiazepine dependence	☒	☒	☒	☒
Scopus	3/1/20	All available years	Benzodiazepine drugs	Rhesus monkey + self-administration + benzodiazepine + alternative	☒	☒	☒	☒
Scopus	3/1/20	All available years	Spontaneous withdrawal	spontaneous withdrawal + benzodiazepine	☒	☒	☒	☒
Scopus	3/1/20	All available years	Spontaneous withdrawal	spontaneous withdrawal + animal model	☒	☒	☒	☒
Scopus	3/1/20	All available years	Spontaneous withdrawal	spontaneous withdrawal + alternative	☒	☒	☒	☒
Scopus	3/1/20	All available years	Spontaneous withdrawal	spontaneous withdrawal + refinement	☒	☒	☒	☒
Scopus	3/1/20	All available years	Vaginal swab	Rhesus monkey + vaginal swab	☐	☐	☒	☒
Scopus	3/1/20	All available years	Vaginal swab	Rhesus monkey + vaginal swab + animal model	☒	☒	☒	☒
Scopus	3/1/20	All available years	Catheterization Surgery	Rhesus monkey + catheterization surgery + refinement	☐	☒	☒	☒
Scopus	3/1/20	All available years	Catheterization Surgery	Rhesus monkey + catheterization surgery + alternative	☒	☒	☒	☒
Scopus	3/1/20	All available years	Chronic administration	Rhesus monkey + chronic administration + animal model	☒	☒	☒	☒
Scopus	3/1/20	All available years	Chronic administration	Rhesus monkey + chronic administration + benzodiazepine	☐	☒	☒	☒

Scopus	3/1/20	All available years	Chronic administration	Rhesus monkey + chronic administration + alternative	☒	☒	☒	☒
Scopus	3/1/20	All available years	Precipitated withdrawal	Rhesus monkey + precipitated withdrawal + animal model	☒	☒	☒	☒
Scopus	3/1/20	All available years	Precipitated withdrawal	precipitated withdrawal + alternative	☒	☒	☒	☒

Narrative

Below, provide a brief summary of any articles that were identified in the search and how these studies relate to the current animal protocol. The narrative must discuss what efforts were made to REDUCE animal number and REFINE experimental procedures to reduce or eliminate pain and distress to the experimental animals, as well as whether there are alternatives that could REPLACE the use of animals. Interaction with peers and educational materials may be used to supplement discussion of literature searches.

Summary of articles:

The literature searches described above indicate that addiction researchers are using self-administration models of reinforcement to investigate the behavioral pharmacology/abuse- related effects of drugs. However, relatively few researchers have been evaluating the abuse-related effects of benzodiazepines in rhesus monkeys (e.g., ~2% of total "Rhesus Monkey + self-administration + animal model" hits were associated with benzodiazepines, with all hits being publications from the [redacted] laboratory). The literature searches also emphasize that we seem to be the only laboratory currently investigating dependence following chronic treatment with benzodiazepine drugs. Search for the terms “Rhesus Monkey + chronic + benzodiazepine dependence” had 12 results, with the most recent publication before our 2020 paper ([redacted] *Psychopharmacology*, 2020, *in press*) dating from 2013 and being on nicotine dependence. These numbers indicate that benzodiazepine self-administration and chronic treatment with benzodiazepine drugs to study dependence, as proposed here, are not duplicative and represent an unmet need, given that drug self-administration is the "gold standard" of abuse liability assessment.

Because withdrawal is a fundamentally important—yet poorly understood—aspect of benzodiazepine dependence, whole, intact, behaving organisms are required in order to study these phenomena fully and appropriately. Searches for refinements and alternatives to procedures for studying these phenomena were of utmost importance, and yielded no significant results that would reduce the number of subjects necessary nor the species used any further.

These searches also clearly emphasize that there are no alternatives to the proposed procedures. Physical dependence studies, as well as other cognitive/operant/observable behavior studies, require intact, behaving organisms. Search hits that included the term “alternative” were from studies that used these techniques to supplement behavior data, not to replace it; or were on an unrelated topic that happened to use these words/phrases.

Based on the literature and our initial results ([redacted]. *Tolerance and dependence following chronic alprazolam treatment: quantitative observation studies in female rhesus monkeys, Psychopharmacology (Berl). 2020, in press*) we can empirically define "momentary" with respect to our standard blocker for precipitated withdrawal studies (flumazenil). In this regard, signs of withdrawal (nose rub, vomit/retch, procumbent, tremor, rigid posture) are at near control levels 15 minutes post i.v. injection, and absent 30 minutes after i.v. injection. In our preliminary studies, spontaneous withdrawal emerges on day 2 of cessation of chronic treatment and is confined to days 2 and part of 3.

In order to reduce this amount of exposure to potential distress and to refine our procedure, we will modify our procedure so that during precipitated withdrawal tests, we will reinstate the chronic drug treatment at 30 minutes, with an anti-emetic drug administered at 15 minutes (in consultation with CCR veterinary staff). For spontaneous withdrawal, we will (a) increase the number of observation periods to 3 per day and (b) reinstate chronic drug as soon as 4 of the 5 signs of spontaneous withdrawal are recorded. Using this approach, we anticipate that most monkeys will be reinstated with chronic drug and out of withdrawal on day 2 of drug cessation.

Reductions in animal number:

Our primary strategy to reduce the number of monkeys is by using a within-subjects experimental approach or mixed-factor approach with within-subjects factor. For within-subjects designs each animal serves as its own control. This approach permits scientifically meaningful results to be obtained with fewer animals than would be required with other types of designs (e.g., an exclusively between-subjects approach). A second strategy that we use is to, whenever possible, re-use monkeys in our studies. Thus, once a particular experimental phase is completed; we will test the same monkey in subsequent experimental phases. To our knowledge based on the extant literature, and based on our preliminary studies, if monkeys are dose tapered sufficiently there is no change in the rate of dependence development or intensity of dependence.

Refinements to methods to reduce distress:

Unlike typical procedures that are used in our lab, dependence and withdrawal are planned endpoints of the studies proposed herein. We have selected experimental parameters to reduce or minimize distress or adverse side effects associated with benzodiazepine dependence, and importantly we have included monitoring/behavioral scoring periods by technicians trained to recognize species-typical and drug-induced behaviors with the goal of monitoring these effects multiple times over the course of the day to ensure the health and wellbeing of the animals during experimental time points at which they may be dependent or experiencing withdrawal symptoms. Should significant, severe symptoms develop that cause concern (seizures), a CCR veterinarian will be contacted to provide additional assessment and consultation, as well as treatment if needed.

The reference to mild withdrawal pertains to the intensity of the withdrawal signs, either individually or as a whole. In monkeys, the predominant signs are nose rub, vomit/retch, procumbent, tremors, rigid posture, which are analogous or the same as signs observed in humans (nausea and vomiting, fatigue, tremors). Please note that "mild" in this case also refers to comparisons with withdrawal elicited by other drugs of abuse, such as opioids and

alcohol (the latter being life-threatening).

Animal **Replacement**:

Because we are studying whole-organism behavior/cognition/species-typical behavior, we cannot conduct our studies with tissue or cell lines, and there are no currently available replacements to the use of animals in experimental settings by use of computer simulation. In fact, computer simulation generally relies—and is only as good as—data generated by the types of studies in this protocol. Regardless, we will consider any alternatives should they become available.

Training and Qualifications

- PI

Name► [redacted]
Animal research experience ► 25+ years of experience with NHPs

Qualifications to perform specific procedures

Specific procedure(s) that the PI will perform personally	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] has 25+ years of experience conducting behavioral and pharmacology research with non-human primates, and developed the current research program contained in this protocol. He has experience with all procedures involved in running this lab.
Behavioral testing	
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	

- Other research personnel

Name► [redacted]
Animal research experience ► 25+ years of experience with NHPs

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] has over 25 years of experience conducting behavioral and pharmacology research with non-human primates, and has experience with all procedures involved in this protocol. [redacted] is a trained primatologist, receiving her Ph.D. from [redacted] at U Massachusetts – [redacted] is a leading primatologist, having been trained by [redacted].
Behavioral testing	
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	

NHP Chairing	

Name► [redacted]
Animal research experience ► 15+ years of research experience, over 10 with NHPs.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] has 10+ years of experience working with NHPs, and currently conducts research in his own NHP laboratory at UMMC. He has experience with all relevant procedures, and may step in to assist the [redacted] lab from time to time as needed given the closeness of all NHP laboratories in both physical space and areas of research focus.
Behavioral testing	
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ► 13+ years of research experience, 7+ years of NHP experience.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] has over 7 years of experience working with NHPs at UMMC. She was trained by [redacted] and [redacted], and currently has her own NHP research lab. She has experience with all relevant [redacted] procedures and may step in to assist from time to time as needed given the closeness of all NHP laboratories in both physical space and areas of research focus.
Behavioral testing	
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ► 20+ years of rodent research experience, 13+ with NHPs.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] has over 13 years of experience working with NHPs, including time spent working with [redacted] and [redacted] at NEPRC. Prior to moving to the US, [redacted] worked at the Swiss Federal Institute. She has been thoroughly trained on all relevant procedures performed in the [redacted] lab.
Behavioral testing	
Observations	

Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ► 7+ years of rodent research experience, 6+ years of research experience with NHPs

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] is a post-doctoral fellow working in the [redacted] lab. In addition to previous rodent research experience, she received a Ph.D. in Psychobiology from Universidade Federal de Sao Paulo with an Exchange Program at the Yerkes National Primate Research Center, Emory University, where she worked with NHPs prior to coming to UMMC. She therefore had a developed NHP research skill set prior to UMMC. She has been trained and is proficient on all NHP-related procedures performed in the [redacted] lab.
Behavioral testing	
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ► 8+ years of research experience, 5+ years with NHPs.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	Trained to assist in surgery.
Behavioral testing	[redacted] is a post-doctoral fellow working in the [redacted] lab. In addition to previous behavioral research with rodents and pigeons, she is also a board-certified behavior analyst. She has been thoroughly trained on any NHP procedures relevant to her work, and is primarily responsible for behavioral observations.
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	To be trained

Name► [redacted]
Animal research experience ► 2+ years of research experience with NHPs & rats
Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	To be trained
Behavioral testing	[redacted] is a research tech that has been working with the [redacted] and [redacted] labs for 1 year (and spent the previous 1+ years with us
Observations	

Catheter flushing/maintenance	as a volunteer and SURE student), so he has been trained on all basic relevant tasks in the lab. He will be trained on surgeries and chairing in the near future.
Enrichment & Feeding	
NHP Chairing	To be trained

Name► [redacted]
Animal research experience ► No previous animal research experience, has been in the [redacted] labs for ~4 months

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	To be trained
Behavioral testing	[redacted] is a research tech that has been working with the [redacted] and [redacted] labs for ~3 months, so she has been trained on all basic relevant tasks in the lab. She will be trained on surgeries and chairing in the near future.
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	To be trained

Name► [redacted]
Animal research experience ► 2+ years of research experience (rats & NHPs, [redacted] lab)
Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	To be trained.
Behavioral testing	[redacted] is a graduate student working in the [redacted] and [redacted] labs. He previously worked as a volunteer and SURE student in the [redacted] lab (primarily with rats), and will require training on all NHP-related tasks. He has been trained to do basic enrichment and feeding, and on behavioral observations and behavioral testing, and will eventually be trained on surgery and chairing.
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	To be trained

Name► [redacted]
Animal research experience ► 4 years of experience working with CCR, 4 years of experience working in the [redacted] laboratory.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	To be trained by [redacted] staff – will only be involved in [redacted] surgeries under special circumstances.
Behavioral testing	[redacted] is the lab manager for the [redacted] and [redacted] labs, and given the closeness of all NHP labs he may at times assist
Observations	

Catheter flushing/maintenance	on procedures or tasks related to [redacted] NHPs. He has experience working for CCR (husbandry) prior to working for the [redacted] and [redacted] labs.
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ► Current graduate student in the [redacted] lab – research experience with both rats and NHPs.

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] is a graduate student in the [redacted] lab. The primary focus of his current work is with rats, but he does do some NHP studies. Given the closeness of the [redacted], [redacted] and [redacted] labs, he may at times assist with NHP-related tasks such as feeding or monitoring. He will only be asked to help with tasks he has been thoroughly trained on by his own lab (typically feeding/enrichment, occasionally assisting on surgery, etc.).
Behavioral testing	
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ► Approx. 1 year NHP experience ([redacted] lab tech)

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] is a research tech for the [redacted] lab. Given the closeness of the primate labs, she may at times assist with NHP procedures and tasks in [redacted] rooms (e.g., train on observations, assist on surgeries, enrichment/feeding). Training will be provided by the [redacted] lab, and she will only be asked to help out in rare instances and on tasks that she has been deemed fully proficient by her own lab on.
Behavioral testing	
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ► Approx. 1 year NHP experience ([redacted] lab tech)

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] is a research tech for the [redacted] lab. Given the closeness of the primate labs, she may at times assist with NHP procedures and tasks in [redacted] rooms (e.g., train on observations, assist on surgeries, enrichment/feeding). Training will be provided by the [redacted] lab, and she will only be asked
Behavioral testing	
Observations	

Catheter flushing/maintenance	to help out in rare instances and on tasks that she has been deemed fully proficient by her own lab on.
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ► MSRP student, no previous experience

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	[redacted] is an MSRP student with no previous NHP research experience. He will primarily be focused on rodent work within the [redacted] lab, but may assist in some general NHP tasks in order to gain experience. He will need to be trained on all tasks and procedures. Given the closeness of the [redacted] and [redacted] labs, he may train in both labs.
Behavioral testing	
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	

Name► [redacted]
Animal research experience ►Head of UMMC Animal Behavior Core

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Sleep Scoring	The [redacted] lab (specifically [redacted]) has recently developed a set of sleep scoring and EEG telemetry research studies. Given [redacted] many years of experience with sleep research, he may be involved in portions of this work, be present for EEG surgeries, etc., to provide valuable input.
EEG Telemetry	

Name► [redacted]
Animal research experience ► 1+ summers in SURE program w/ [redacted] lab

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	To be trained.
Behavioral testing	[redacted] is a SURE student who worked with the [redacted] lab summer 2018 and has returned for Summer 2019. He has been trained on all basic NHP tasks (enrichment, feeding, etc.), and is currently training in observations.
Observations	
Catheter flushing/maintenance	
Enrichment & Feeding	
NHP Chairing	To be trained

Name► [redacted]
Animal research experience ► 1+ summers in SURE program w/[redacted]

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	SURE student/undergrad – will not train on Sx.
Behavioral testing	[redacted] is a SURE student who worked with the [redacted] lab summer 2018 and has returned for summer 2019. He has been trained on basic NHP tasks (enrichment & feeding, behavioral testing, etc.). He is primarily a student in the [redacted] lab but may assist with [redacted] work.
Observations	
Enrichment & Feeding	
Catheter flushing & Maintenance	To be trained (Primarily a [redacted] student, so may not train on this)
NHP Chairing	To be trained (Primarily a [redacted] student, so may not train on this)

Name► [redacted]
Animal research experience ► None (SURE Student – Summer 2019)

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Catheter surgeries & Post-surgical monitoring	SURE Student – will not train on Sx.
Behavioral testing	[redacted] is a SURE student working in the [redacted] lab. She has been trained on enrichment & feeding, and is currently training on behavioral testing, observations, and catheter maintenance by [redacted] and [redacted].
Observations	
Enrichment & Feeding	
Catheter flushing & Maintenance	
NHP Chairing	To be trained

Name ► [redacted]
Animal research experience ► none

Qualifications to perform specific procedures

Specific procedure(s) that this individual will perform	Experience with each procedure in the species described in this Protocol
Monkey catheter surgeries & post-surgical monitoring	None
Monkey behavioral testing	None
Monkey behavioral observations	None
Monkey catheter flushing/maintenance	None
Monkey Enrichment and feeding	None
Monkey chairing	None

Rat catheter surgeries & post-surgical monitoring	None
Rat behavioral testing	None
Rat feeding and weighing	None
Rat and monkey drug administration (oral, i.m., s.c., i.p.)	None

- **Training to be provided.** List here each procedure for which anyone is shown as "to be trained", and describe the training. For each procedure, describe the type of training to be provided, and give the name(s), qualifications, and training experience of the person(s) who will provide it. If no further training is required for anyone, enter "N/A"

Surgical Training: Currently, [redacted] is trained to perform IV Catheter surgeries (in addition to: [redacted], [redacted]). [redacted] is currently training to perform IV catheter surgeries. Training typically involves the trainee assisting an experienced surgeon and gradually completing more and more steps of the surgery under the direct supervision of one of the trained surgeons. This supervision and training will continue until trainees can complete an entire surgery start to finish without assistance from the trained surgeons. The CCR veterinary staff oversees and are available to assist and monitor surgical training.

NHP Chair Training: [redacted] is currently the most experienced at NHP chairing in the [redacted] lab, and therefore any training on this procedure will take place with her. Training a new individual to chair monkeys involves training in positive reinforcement and shaping of behavior, as well as safety precautions given that it involves taking a monkey out of its home cage. Trainees will begin by observing [redacted], and fade in the amount of physical assistance they provide while [redacted] fades out her own part in the chairing until the trainee is able to complete the process on their own.

Behavioral Testing and Observations: Behavioral testing and observation studies involve learning a variety of skills including behavioral coding, using MedPC and other computer programs, and a knowledge of how to hook up certain sets of equipment, such as self-administration panels. Any persons requiring training will work closely with an individual who is already proficient on the task. For observation training, individuals must meet 90% reliability criteria (i.e., observation data must match 90% of the trained observer's data) in order to be considered trained.

[redacted] and [redacted] will train [redacted] in all monkey-related procedures listed in protocols 1389B and 1512A, including behavioral observations, PPE usage, drug mixing, equipment set-up, computer programming, drug administration, aseptic surgery technique, catheterization surgeries, catheter maintenance, chairing, etc. [redacted] has worked with monkeys conducting similar studies to those that [redacted] will run for over 6 years, and [redacted] has been fully trained in most procedures since she joined our lab in June, 2021.

[redacted] will train [redacted] in all rat-related procedures listed in protocol 1393B, including handling, drug mixing, computer programming, etc. [redacted] has worked with rats and other laboratory animals for more than 10 years, and has conducted experiments similar to those that [redacted] will run for more than 6 years.

Certification of the Principal Investigator:

Signature certifies that the Principal Investigator will conduct the project in full accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations, and UMC policies governing the use of live vertebrate animals for research and teaching purposes. The procedures involving animals will be conducted by trained or experienced personnel or under the direct supervision of trained or experienced persons. It is understood that IACUC approval is valid for a period of 12 months following the date of original approval and must be renewed annually for continued approval. I understand there is a 3-year requirement for full protocol rewrite. It is further understood that should this project be submitted for external funding, the information presented on the UMMC Animal Activity Protocol form accurately reflects the animal use in the full grant application.

X [redacted]

Signature of Principal Investigator (Paste digital copy of signature)

Approval by the Attending Veterinarian:

Approval by the Institutional Animal Care and Use Committee:

Assigned To	Title	Due Date	Status	Related Content	Outcome
☐	[redacted]	Please approve FSR_ 1512	Completed	FSR_ 1512	Approved
☐	[redacted]	Please approve FSR_ 1512	Completed	FSR_ 1512	Approved

Appendix A	**Non-Human Primate Environmental Enhancement/Enrichment**

This appendix must be appended to each protocol involving the use of nonhuman primates.

Nonhuman primates must have their physical environments enhanced/enriched by providing means of expressing non-injurious, species-typical activities. The *Animal Welfare Act* (9 CFR 3.81) states that research facilities "must develop, document, and follow an appropriate plan for environment enhancement adequate to promote the psychological well-being of nonhuman primates".

The default position of USDA and OLAW is that non-human primates must be socially housed. The Guide (2011) states, "*… nonhuman primates should normally have social housing (i.e., in compatible pairs or in larger groups of compatible animals)".* Exemptions to the social housing requirement must be based on **strong scientific justification** approved by the IACUC or for a specific veterinary or behavioral reason.

The Center for Comparative Research provides an active plan of environmental enrichment that includes cage complexities (tunnels, barrels), social interaction, fruit/vegetable supplements, foraging, and manipulative devices/toys. Unless otherwise, specified, the CCR will provide all available forms of enrichment.

1. Enrichment Techniques

Are there any enrichment forms/techniques that are included in this protocol?

☐No ☒Yes

2. Description

Describe the above techniques.

The CCR provides an active plan of environmental enrichment. Acceptable forms of enrichment for this protocol include: social interaction (in the form of visual, auditory and grooming interactions), fruit/vegetable supplements, foraging, and manipulative devices/toys. CCR will provide all items of physical enrichment (e.g. devices/toys, cage bedding for forage). Laboratory staff will provide audio/visual enrichment (e.g., radio, television) and palatable enrichment (e.g., food treats). Enrichment will be documented in writing by the person actually giving it to the monkey. The form for documentation is on the room door.

3. Exemption from Enrichment

Are there any forms of enrichment/enhancement that should not be used in this study?

☐No ☒Yes

4. Justification for Exemption

If Yes, provide complete justification for this exemption.

Exemption from social/pair housing:

The studies will be conducted in monkeys in their individual living quarters. For these studies, the cages have been modified so that experimental equipment can be mounted to a side. Monkeys will self-administer intravenous (i.v.) injections of drugs/compounds. The i.v. catheters exit the monkey's back and is threaded through a tether attached to a swivel on the side of the cage. Monkeys wear custom jackets to protect the catheters; however, another monkey in the cage very likely would interfere with the jacket, tether, and/or swivel in such a way as to put the catheter at risk of damage or even removal. Moreover, the presence of other monkeys would interfere with the performance of the monkey in the tasks, or alter dramatically the behavior of the monkey during observation sessions, thereby seriously compromising the validity of the research. In most cases, however, individual cages will be grouped together in colony rooms in order to allow visual, auditory, and olfactory contact with other monkeys. Tactile contact between cages is also available depending on the compatibility of individual monkeys.

Exemption from other forms of enrichment:

Monkeys in this protocol are given foraging treats and room enhancements (radio with music, interaction with humans, etc.). However, given the behavioral nature of our protocol, and the specialized home cages used for some studies, we request an exemption from enrichment during experimental sessions that would interfere with the conduct of the research. There is ample scientific evidence that the availability of alternative behaviors during an experimental session can alter drug taking (e.g., Nader and Woolverton, Psychopharmacology 105:169, 1991). Therefore, enrichment needs to be removed from home cages during some behavioral sessions so that manipulation of enrichment devices cannot interfere with the behavior being studied, i.e., manipulation of levers. During this period, the environment is enriched by the opportunity to manipulate levers to receive rewards.

Appendix B Time-Pregnant/Breeding Programs

Complete Appendix B for all proposals planning on establishing a breeding colony or for those studies utilizing time-pregnant animals. Studies incorporating breeding programs or offspring from time-pregnant animals will be required to report annual production (number of offspring used) at the time of IACUC protocol annual renewal.

1. Description

a. Provide a specific description of the type of breeding program to be utilized (monogamous pair, "trio" breeding: 2 females and 1 male, "harem" breeding: up to 4 females and 1 male, etc.).

b. The *Guide for the Care and Use of Laboratory Animals* sets minimum space requirements for breeding animals. *See chart below.

If you wish to request a deviation from the minimum requirement provide justification based on performance standards (e.g., health, reproduction, growth, behavior, activity, and use of space) and special needs determined by the characteristics of the animal strain or species (e.g., obese, hyperactive) and experimental use (e.g., animals in long-term studies may require greater and more complex space).

c. For all mating schemes other than pair breeding, pregnant females must be separated prior to birth of the litter unless an exemption is justified. If using trio or harem breeding, please describe how/when dams will be separated to ensure that overcrowding does not occur.

d. All litters must be separated at 21 days of age unless an exemption is justified. Please describe specific plans for weaning.

2. Personnel Responsible

Identify personnel responsible for the breeding program, including weaning and documentation of program.

3. Records

Please describe the record-keeping system that will be used and how breeding, health and maintenance of the colony is recorded.

4. Adults

a. How many adults will be utilized in this breeding program over the 3 year period?

b. How many breeding pairs/groups will be utilized at one time (may be explained with a range)?

c. How many breeding cycles will be utilized or what is the maximum length of breeding (e.g., 3 breeding cycles or 1 year)?

5. Final Disposition

What is the final disposition of these adults at the conclusion of their breeding program?

6. Offspring

How many offspring are anticipated from each breeding or time-pregnancy?

7. Final Disposition

What is the final disposition of any offspring not utilized in the experimental program (e.g., euthanasia, replacement of retired breeders, transferred to another protocol)?

8. Genotype

Describe the sample collection method used for genotyping animals, including age at time of genotyping. Include tissue sampled in Appendix D.

9. Phenotype

Will any offspring have any known or anticipated clinical health concerns (immunocompromised, severe diabetes, ataxia, prone to dermatitis, etc. see also #9.c.)?

*Guide for the Care and Use of Laboratory Animals: Eighth Edition
http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf

TABLE 3.2 Recommended Minimum Space for Commonly Used Laboratory Rodents Housed in Groups*

Animals	Weight, g	Floor Area/Animal,[a] in.^{2} (cm^2)	Height,[b] in. (cm)	Comments
Mice in groups[c]	<10	6 (38.7)	5 (12.7)	Larger animals may require more space to meet the performance standards.
	Up to 15	8 (51.6)	5 (12.7)	
	Up to 25	12 (77.4)	5 (12.7)	
	>25	≥15 (≥96.7)	5 (12.7)	
Female + litter		51 (330) (recommended space for the housing group)	5 (12.7)	Other breeding configurations may require more space and will depend on considerations such as number of adults and litters, and size and age of litters.[d]
Rats in groups[c]	<100	17 (109.6)	7 (17.8)	Larger animals may require more space to meet the performance standards.
	Up to 200	23 (148.35)	7 (17.8)	
	Up to 300	29 (187.05)	7 (17.8)	
	Up to 400	40 (258.0)	7 (17.8)	
	Up to 500	60 (387.0)	7 (17.8)	
	>500	≥70 (≥451.5)	7 (17.8)	
Female + litter		124 (800) (recommended space for the housing group)	7 (17.8)	Other breeding configurations may require more space and will depend on considerations such as number of adults and litters, and size and age of litters.[d]

Appendix C Surgery & Management of Surgical Pain and Distress

1. **Complete description of surgical procedures** – List details for each surgical approach noted in question #16.

Surgical site preparation
Surgery is performed in a fully-equipped operating suite (CCR surgical suites, [redacted]) using appropriate anesthetic agents and analgesics. Monkeys are initially given atropine (to reduce secretions) and ketamine in their home cage prior to surgery, then moved to the surgical prep room. Aseptic preparation of the surgical site (limb or neck) and midscapular exit site occurs in the prep room. Hair is removed using clippers followed by a disposable razor, if needed. The surgical site is prepared with three alternating applications of surgical scrub (betadine or chlorhexidine) and 70% alcohol or similar. After arrival in the operating room, a final scrub is performed and sterile drapes are placed. Inhaled isoflurane in oxygen is administered for the duration of the surgery.
Surgical approach

FOR CATHETER SURGERIES:
Chronic intravenous catheters are implanted following the general procedure described by Carey & Spealman, Current Protocols in Pharmacology, pp. 10.5.1 – 10.5.15 (1998), updated by [redacted] et al., Current Protocols in Pharmacology, Unit 9.2.1 (2005).
For the catheter implantation, a small skin incision is made over the femoral, jugular, or brachial vein of the anesthetized monkey. The vein is exposed by blunt dissection, tied off to prevent blood loss, and an incision is made in the vein to accept one end of a polyvinyl (or other medical grade material) catheter. The catheter is passed through the vein to the level of the right atrium and secured to the vein and adjacent muscle by sutures. This placement of the cathether to the right atrium is achieved by measuring and cutting the catheter based on the type of vein and the size of the monkey (measurement made from incision site to center of sternum). Once in place, the catheter is observed for several minutes to ensure that blood does not advance into the lumen, which would indicate that the catheter is too close to the heart. If this occurs, the catheter is removed, shortened, and re-implanted. Once the catheter is determined to be the appropriate length and is placed, it will be secured to the muscle at the incision site via two cuffs placed on the catheter line. Pulling back rather than removing and cutting would result in the catheter cuffs being out of place. Once the catheter is secured, the distal end of the catheter is passed subcutaneously through a hollow probe to exit the body in the midscapular region. From this point, the NHP may be fitted with a jacket and the catheter threaded through a tether attached to said jacket, or a port will be implanted (see below).

PORT IMPLANTATION FOR CATHETER SURGERIES: After the hollow probe has been removed, the catheter is gently pulled to ensure the excess in the limb or neck is eliminated without disturbing the prep or contaminating the catheter. A sterile drape is placed over the catheter and exit site, and a non-sterile person turns the monkey back over so that the incision site can be accessed for suturing. The surgeon then will change into clean, sterile gloves in order to suture the incision and complete the subcutaneous vascular access port placement. After suturing of the incision on limb or neck is complete, a non-sterile person will turn the monkey back to a more sternal position to allow easy access to the back, and the back is re-prepped with betadine or chlorhexidine. New sterile drapes are placed to establish a sterile field around the exit site. A small skin incision is made starting at the point where the catheter exits the skin either lateral or medial to the exit point, depending on where the exit point is in relation to the spine, so that the port resides next to the spine on the side where the catheter was implanted. The incision is made perpendicular to the spine, with a catheter loop (4-6 cm long) placed cranial and the port caudal to the incision. The catheter is attached to
the port, and the port is flushed with 2-3 ml of heparin lock flush solution using a Huber needle, and the port is placed in the subcutaneous pocket. After the port has been anchored to the underlying tissue at two positions on the skirt to keep it from flipping following surgery, the incision in the back is closed. An even more thorough explanation of details and step-by- step procedures is listed in our Port surgery SOP, which is attached to this protocol.

Wound closure method, materials, and removal plan

Catheter surgeries: The incision is sutured using a combination of sub-cuticular and external suturing techniques (e.g., sub-cuticular continuous suture followed by horizontal mattress sutures). An appropriate absorbable material is used for all suturing (e.g., Maxon/Biosyn, vicryl). External sutures may be done with non-absorbable material which will be removed one week post-surgery. In consultation with the CCR veterinary staff, we will limit the use of external sutures as much as possible. Catheters are flushed regularly with saline/heparin solution (100-150 u/mL) and sealed with stainless steel obturators or knots when not in use.
Either a port is surgically implanted in the monkey's back or the monkey wears a custom-fitted nylon mesh jacket at all times to protect the catheter. Catheters are expected to remain patent for 6 to 36 months, although they may fail sooner or last far longer than that.

Catheters that become occluded or dislodged will be replaced by re-implanting a new catheter in the original vein. In our experience, the vessel remains viable only when a catheter is in place—we are sometimes able to replace a non-functioning catheter. However, once the catheter is removed, the vessel is not viable in the majority of cases. If the catheter cannot be replaced, the monkey is sutured as described above and another vein will be catheterized after a minimum of 1 week recovery period between surgeries. Up to 8 vessels (2 internal jugular veins, 2 external jugular veins, 2 brachial veins, 2 femoral veins) are used for these surgeries, depending on the disposition of the monkey. In general, a monkey will have catheter placements in all 8 sites over the experimental life of the animal, although there is considerable variability in catheter life. Sometimes the catheter is broken at the exit site and will, in the majority of cases, reenter the body. If this occurs, the monkey is prepared as described above for aseptic surgery, but placed on the abdomen. After surgical preparation of the area around the last exit site, a small incision is made, and the catheter is re-exposed using blunt dissection. A sterile metal connecting pin is used to attach additional catheter material as needed. For this procedure, the monkey's pre- and post-operative care is identical to catheter implantation.

We have not typically limited the number of replacement surgeries, primarily because they tend to be self-limiting. In our experience, the vast majority of replacements can occur only once, after which the vein is no longer viable. Also, we typically do not limit the maximum amount of time that a catheter remains in place. It is the exception, rather than the rule, that catheters remain patent for more than 12 months, and there is considerable variability in how long catheters remain patent. Our studies can be relatively long in duration, and it is advantageous to have catheters last as long as possible in order to (a) limit the number of surgeries, and (b) limit the amount of time the monkey is off study.

Vascular Access Ports (Catheter surgeries): Both incisions (limb/neck and back are sutured using the same suturing techniques described above for catheter surgeries. The subcutaneous vascular access port should be removed/replaced any time there are signs of irritation or inflammation around the port area or along the

catheter track. Signs which may warrant port movement include: thinning of skin over the port, increased redness, swelling, warmth on or around the port or catheter track; signs that the animal is scratching or picking at the port or catheter track. As long as there are no signs of catheter infection, ports can be
replaced without necessarily having to replace the catheter. A 1-week recovery period is provided between port removal and new port placement, with the catheter being placed subcutaneous until the placement of a new port. For these procedures, the monkey is prepared and monitored pre- and post-operatively as stated above for catheter surgeries.

2. Provide a complete formulary of medications related to surgical procedures:

	Agent	Dose	Route	Frequency /Duration	Pharmaceutical Grade
Pre-anesthetic	Atropine	0.02-0.05 mg/kg	i.m.	Once	☒Yes ☐No
Pre-anesthetic	Ketamine	10-20 mg/kg	i.m.	Once, then as needed	☒Yes ☐No
Pre-operative analgesic	Buprenorphine SR (or buprenorphine)	BUP SR: 0.045 mg/kg BUP: 0.005-0.03 mg/kg	BUP SR: s.c. BUP: s.c. or i.m.	BUP SR: Once BUP: Every 8-12hr for 72 hours	☒Yes ☐No
Pre/post-operative analgesics	Carprofen	2.0-4.0 mg/kg	s.c. or p.o.	Every 24hr for 3 days post-op	☒Yes ☐No
Anesthetics	Isoflurane	1-2% (in O_2)	Inhaled	Throughout procedure	☒Yes ☐No
Fluid/blood replacement	Sterile saline	0.9%	i.v.	As needed	☒Yes ☐No
Flushing of catheter/port during surgery	Sterile saline containing heparin	40-150 U/ml	i.v.	As needed	☒Yes ☐No

For non-pharmaceutical-grade compounds:

a. **Justify** the need to use the non-pharmaceutical-grade compound(s) (e.g., veterinary or human pharmaceutical-grade product is not available).

N/A

b. Discuss steps taken to ensure the health and welfare of the animals. Examples may include grade, purity, sterility, pH, pyrogenicity, osmolality, stability, formulation, compatibility, storage, side effects, and pharmacokinetics of the compound(s).

N/A

3. Anesthesia

a. Who will conduct the anesthesia procedure(s)?

CCR veterinary staff (veterinary technician or veterinarian)

b. Describe experience and training with anesthesia.

CCR veterinary technicians are certified veterinary technicians and/or have been extensively trained by CCR members who have significant experience with this procedure.

c. What criteria will be used to assess anesthetic depth and how will this be monitored?

Monitored by CCR vet staff (Criteria used: heart rate, respiratory rate, muscle tone, oxygen saturation, body temperature).

4. Aseptic Technique

a. What procedures will the surgeon use to prepare himself/herself for aseptic surgery?

Surgeons will prepare hands and arms with aseptic procedure (scrub in, use aseptic hand spray in room after scrubbing). Sterile surgical gowns will be worn, as well as sterile gloves. Face masks, eye protection, hair bonnets and shoe covers also will be worn. Assurance of proper technique will be assessed by the CCR veterinary staff prior to the initial surgery.

b. How will the instruments be prepared for aseptic surgery? (Sterile instruments must be used for each animal.)

All instruments and surgical packs will be autoclaved as appropriate. Equipment for surgery that is not appropriate for autoclaving (e.g., polyvinyl chloride catheter material) will be sterilized via the Ethylene Oxide (EO) sterilization unit housed within the UMMC dental school. Whenever possible, commercially-available sterile instruments and equipment will be used.

5. Location of Procedures

Where will the surgical procedures be conducted?

CCR surgical suites ([redacted])

6. Post-procedural Care

a. Who will conduct and document post-procedural animal care (post-op analgesia, nursing care, etc.)? *Documentation will be checked at IACUC semi-annual inspection.*

Laboratory staff and CCR staff will conduct post-op monitoring in accordance with the CCR post-op monitoring form.

b. Include a plan of monitoring frequency, duration and intervals of post-op analgesia, nursing care, etc.

Monkeys are monitored continuously until extubation by CCR staff, after which lab staff monitor continuously until the monkey is sitting up on their own and responsive to their environment. Lab staff will administer post-operative

drugs (antibiotics, analgesics) over the next 3-7 days in accordance with CCR recommendation(s). Analgesics and antibiotics are administered in close collaboration with the veterinary staff.

c. What is the expected time from end of procedure until animal(s) are returned to home environment?

No more than 1 hour.

7. Emergency Contacts

Provide emergency contact information (pager/phone number) for evenings or weekends concerning post-operative complications.

[redacted]

Appendix D Collection of Biological Samples from the Live Animal

Biological samples include blood collection, urine collection, ascites, tail tips for DNA, cerebrospinal fluid, biopsy, etc. Appendix D is completed for all sample collections from live animals, including under terminal anesthesia. Appendix D is not required for samples taken after euthanasia.

1. Indicate the body fluid or material to be collected.

Blood and vaginal discharge

2. Indicate the method and site of collection.

The PI's staff will conduct the awake blood draws, including the training, in consultation with the veterinary staff. Blood will be collected from the femoral or saphenous vein using vacutainers and a new puncture for each sample. In some cases, monkeys will be lightly sedated with ketamine for the collection of samples. For the majority of samples, awake sampling will be required since (a) ketamine may interfere with the experimental endpoints and (b) behavioral tasks will occur soon after sampling. The monkeys first will be trained to present a leg for awake sampling by using the squeeze-restraint mechanism in the cage, as well as gentle handling, with food as reward. The method of "successive approximations" will be used, in which components of the task will be successively reinforced (e.g., sitting in front of the cage door result in an apple slice, then allowing the trainer to touch the leg will be required

for an apple slice, and so on). Clickers can be used as an auditory cue for leg presentation. Using this technique, we have routinely performed awake blood draws on consecutive days for up to 1 month

Vaginal discharge will be collected for evaluation of cells consistent with different phases of the menstrual cycle. Awake sampling will be required for the same reasons as described above for blood samples. The monkey will be trained to present her hindquarters. We will also use the method of “successive approximations” to train animals to present their hindquarters, then allowing the trainer to touch the skin near the vaginal opening, and so on).

3. Indicate the volume of fluid or amount of material to be collected.

Up to 3 mls of blood per draw and a single swab with a cotton tipped applicator for the vaginal swab. For blood collection, blood volume will be adjusted as not to exceed a maximum blood volume of 10ml/kg collected over a 28-day period.

4. Indicate the frequency of collection.

Blood samples are required for analyzing progesterone and estradiol determination, which is used to determine different phases of the menstrual cycle in female rhesus monkeys, may be collected for the duration the subject is in the study (i.e., our collection may be continuous and may occur with all subjects in the approved protocol). Samples may be collected daily for at least one complete cycle (~28 days). After this initial precise determination, sampling during a cycle may occur every 2 to 4 days. Further determination of the entire cycle may be necessary periodically throughout the study (approximately every 6 months) and at the end of a study to assess potential drug effects and/or changes in cycling. We will ensure that daily draws will not exceed 10% of an animal’s circulating blood volume within a 2-week period (see [redacted] Psychopharmacology, 2020, in press). If at any point, the maximal volume is met, we will not collect further samples until the 2-week period is over.
Of note, throughout all experiments, blood volume will be adjusted as not to exceed a maximum blood volume of 10ml/kg collected over a 28-day period.

For the purposes of pharmacogenomics, up to two samples will be collected per NHP, a minimum of two weeks apart. Only one sample will be taken in the majority of cases. A second blood sample may be taken if the first is contaminated or in some other way unusable.

Vaginal swabs may be collected for the duration the subject is in the study (i.e., our collection may be continuous and may occur with female subjects in the approved protocol). Vaginal swabs may be collected on a daily basis to track different phases of the menstrual cycle. These samples may be used to correlate with progesterone and estradiol levels or may be used to track the menstrual cycle in the absence of progesterone and estradiol depending on the experimental endpoint.

5. Will the animal(s) be anesthetized or sedated during this procedure?

☐No ☒Yes

If No, describe restraint method. (Note: If methods require a prolonged period of restraint, Appendix G is required.)

Restraint will be limited to the squeeze back mechanism of the home cage. In our experience, well-trained monkeys may not require this restraint, although the mechanism is always brought forward at least half way as a safety consideration.

Blood samples will typically be taken without anesthesia, however, if an animal is sedated for reasons already described in the protocol (i.e., to fix a catheter issue, for a health check, etc.) on the same day a blood draw is needed, the blood may be taken while the animal is sedated.

If Yes, list agents used for anesthesia and anaglesia:

Agent	Dose	Route	Frequency/Duration	Pharmaceutical Grade
Ketamine	10-20 mg/kg	i.m. or i.v.	Once/sample	X Yes ☐No
				☐Yes ☐No
				☐Yes ☐No
				☐Yes ☐No

For non-pharmaceutical-grade compounds:

c. **Justify** the need to use the non-pharmaceutical-grade compound(s) (e.g., veterinary or human pharmaceutical-grade product is not available).

N/A

d. Discuss steps taken to ensure the health and welfare of the animals. Examples may include grade, purity, sterility, pH, pyrogenicity, osmolality, stability, formulation, compatibility, storage, side effects, and pharmacokinetics of the compound(s).

N/A

Appendix E Antibody Formation /Hybridoma & Ascites

1. Indicate what antigen will be used: Click here to enter text.

2. Indicate what vehicle/adjuvant will be used: Click here to enter text.

   a. Initial immunization: Click here to enter text.
   b. Subsequent immunizations: Click here to enter text.
   c. Anticipated complications/side effects: Click here to enter text.

3. Indicate sites for immunization: Click here to enter text.
4. Describe skin or animal preparation prior to injection: Click here to enter text.
5. Indicate route of administration: Click here to enter text.
6. What is the total and per site injection volume? Click here to enter text.
7. What is the frequency/duration of immunization (e.g., 1 injection every 2 weeks for 3 injections)? Click here to enter text.

ASCITES PRODUCTION

Fluid accumulation associated with ascites/hybridomas should not become greater than 10% of body weight. Animals should be euthanized if they become moribund.

8. Indicate the maximum volume of ascites fluid to be collected per sampling (ml/mouse) and the method of collection (skin prep, gauge needed, gravity vs. suction, etc.)

9. Indicate the number of fluid collections and anticipated frequency of collection.

10. Describe procedures used to care for and monitor the health of animals with ascites and the point of euthanasia.

Consult: http://oacu.od.nih.gov/ARAC/documents/Adjuvants.pdf
http://oacu.od.nih.gov/ARAC/documents/Ascites.pdf

Appendix F Administration of Drugs/Test Compounds

All agents given to the animals **must** be listed in this section with the exception of veterinary pharmaceuticals (antibiotics for treatment, anesthetics, and analgesics for treatment). Those will be listed in Appendix C.

NOTE: A pharmaceutical-grade compound (PGC) is defined as any active or inactive drug, biologic or reagent, for which a chemical purity standard has been established by a recognized national or regional pharmacopeia (e.g., the U.S. Pharmacopeia (USP), British Pharmacopeia (BP), National Formulary (NF), European Pharmacopoeia (EP), Japanese Pharmacopeia (JP), etc.). These standards are used by manufacturers to help ensure the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy.[1]

The Food and Drug Administration (FDA) maintains a database listing of FDA approved commercial formulations for both FDA approved human drugs (the Orange Book) and veterinary drugs (the Green Book).

Provide the following information:

Agent	Dose	Volume	Vehicle	Route	Frequency	NDC or CAS#	Hazard?	Pharmaceutical Grade
Ketamine HCl (anesthetic, experimental drug as a negative control)	0.3-20 mg/kg	≤ 0.2 ml/kg	water	i.m., i.v	As an anesthetic prior to blood draws, surgeries or catheter maintenance/cage changes; as an experimental drug no more than twice/week (doses <1.0 mg/kg)	0856-2013-01	Yes	☒Yes ☐No
Alprazolam (Benzodiazepine agonist)	0.01 – 10 mg/kg	0.01-3.0 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	92623-85-3	Yes	☐Yes ☒No
Diazepam (Benzodiazepine agonist)	0.01-0.5 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	61786-0782-08	Yes	☒Yes ☐No
Diazepam (Benzodiazepine agonist)	0.1-10 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	439-14-5	Yes	☐Yes ☒No
Clonazepam (Benzodiazepine agonist)	0.001-1 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	1622-61-3	Yes	☐Yes ☒No
Zolpidem (Benzodiazepine agonist)	0.1-30 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	82626-48-0	Yes	☐Yes ☒No
L-838,417 (subtype-selective benzodiazepine agonist)	0.3-3 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	286456-42-6	Yes	☐Yes ☒No
TP 003 (subtype-selective benzodiazepine agonist)	0.01-30 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	628690-75-5	Yes	☐Yes ☒No
Experimental GABA-A/ benzodiazepine agonists (e.g., QH-ii- 066; NE-594; HZ-166; JY- XHe-053; YT- III-	Doses ranges for all compounds are 0.1-30 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	N/A	Yes	☐Yes ☒No

31; PWZ-029)								
Flumazenil (Benzodiazepine antagonist)	0.01-10 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	78755-81-4	No	☐Yes ☒No
Experimental GABA-A/benzodiazepine antagonists (e.g., XLi-093; BCCT; 3-PBC; DM-D-053)	Dose ranges for all compounds are 0.3 – 30 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	N/A	Yes	☐Yes ☒No
Experimental GABA-A/benzodiazepine inverse agonists (e.g., RY-23), PWZ-029 (mixed agonist/inverse agonist)	RY: 0.03-1 mg/kg; PWZ: 0.001-1.0 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	N/A	Yes	☐Yes ☒No
Midazolam HCl (Benzodiazepine agonist)	0.1-1.0 mg/kg/injection	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	00409-2308-01	Yes	☒Yes ☐No
Midazolam maleate (Benzodiazepine agonist)	0.1-1.0 mg/kg/injection	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	59467-94-6	Yes	☐Yes ☒No
Clonazepam (Benzodiazepine agonist)	0 – 10 mg/kg	0.01-0.1 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	~30 injections/ day	1622-61-3	Yes	☐Yes ☒No
Methohexital (Barbiturate)	0-3 mg/kg	≤ 2 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	Daily and single-injection on test day	18652-93-2; 151-83-7	Yes	☒Yes ☐No
Almorexant (non-selective orexin receptor antagonist for sleep studies)	0.1-100	≤ 2 ml/kg	Any vehicle listed in protocol	i.m., i.v., p.o.	≤6 injections/ day, no more than 5 times/week		Yes	☐Yes ☒No
Vehicles								
Sterile water	Up to 100% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No

0.9% sterile saline	Up to 100% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No
Jell-O, juice, or equivalent	Up to 100% of a solution	≤ 2 ml		p.o.	No more than 5 times/week		No	☐Yes ☐No
Sodium benzoate	Up to 100% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No
Benzyl alcohol	Up to 100% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No
Benzoic acid	Up to 100% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No
Propylene glycol	Up to 100% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No
Ethanol	≤ 20% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No
Tween 80	≤ 10% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No
β-cyclodextrin	Up to 50% of a solution	≤ 2 ml		IV, IM, SC	IV: ≤30 injections/day IM & SC: ≤6 injections/day, no more than 3 times/ week		No	☐Yes ☒No

NDC# is preferred over CAS#, if available. The NDC# will be on the bottle or box if the substance is a pharmaceutical. If there is no NDC# then include the CAS#. CAS# and hazard information can be obtained from the MSDS sheet through the UMMC Intranet (http://www.umc.edu/intranet/index.php). Choose the "MSDS On-Line" link under "Hot Spots".

1. Describe any potential adverse side effects that may result in the animal from the administration of this material. If agents are unknown or their potential side effects are not documented, provide a reasonable estimate of the effects of the general class of chemicals (e.g., compound may have sedative properties, compound will likely produce diarrhea, etc.).

Physical dependence and tolerance are planned experimental endpoints of chronic treatment studies included in this protocol, and precipitated withdrawal will be tested. Subjects will be observed by laboratory staff for designated time periods during withdrawal and chronic drug treatment will resume after the time period has elapsed. In the event of serious withdrawal symptoms, midazolam or diazepam (1-3 mg/kg, i.m or i.v., to effect) will be administered to alleviate symptoms. Although both pharmaceutical-grade diazepam and midazolam are available in the lab, pharmaceutical-grade diazepam and midazolam doses will not be high enough to alleviate withdrawal symptoms. For this reason, non-pharmaceutical grade diazepam or midazolam is mixed in the appropriate concentrations to have the intended effect.

GABA-A/benzodiazepine agonists (e.g., diazepam, triazolam, etc.) could cause temporary and mild sedation and/or ataxia. If deemed hazardous, these effects are reversible with administration of the antagonist flumazenil (0.3 – 3 mg/kg, i.m. or to effect).

At doses above those proposed for use in our studies, GABA-A/benzodiazepine inverse agonists can be proconvulsant. Animals will be observed routinely by laboratory staff during and after the experimental session for any signs of convulsant activity. These effects are reversible with administration of diazepam (1 – 3 mg/kg, b.i.d., i.m., or to effect).

Ketamine will be administered at doses and concentrations, and by routes of administration, within proven safe parameters in monkeys and is not expected to produce any adverse side effects that would require medical attention or endanger the health of the animal.

In the case of adverse effects for any compound or drug, a CCR veterinarian will be contacted to provide additional assessment and consultation, as well as additional treatment if needed.

2. For each hazardous material, a Hazard Use form must be completed and attached it to the protocol. How many Hazard Use forms are included?

We list drugs and test compounds in our protocols as hazardous due to the fact that they may be toxic or hazardous at extremely high doses, but they are not hazardous or toxic at the concentrations used in our protocols. No appreciable amounts will be present in bedding, and no non-standard husbandry practices are required.
As requested, we are including five (5) Hazard Use Forms: ketamine (sedative drug), methohexital (barbiturate), almorexant (orexin receptor antagonist), a single form incorporating all Benzodiazepine agonists, and a single form incorporating all Benzodiazepine antagonists.

As requested, we are including five (5) Hazard Use Forms: ketamine (sedative drug), methohexital (barbiturate), almorexant (orexin receptor antagonist), a single form incorporating all Benzodiazepine agonists, and a single form incorporating all Benzodiazepine antagonists.

<link to Hazard Use form>

3. For non-pharmaceutical-grade compounds:

    a. **Justify** the need to use the non-pharmaceutical-grade compound(s) (e.g., veterinary or human pharmaceutical-grade product is not available).

Our studies require formulations compatible for i.v./i.m. administrations. To our knowledge, acceptable pharmaceutical grade formulations are available for midazolam and ketamine – and we use these in our research. On the other hand, triazolam and zolpidem are available in pharmaceutical grade, tablet formulations. However, these cannot be dissolved appropriately for preparation of i.v./i.m. formulations due to the inactive ingredients required to prepare a tablet or capsule formulation. Flumazenil and diazepam are available in an appropriate formulation (i.e., liquid), but the available concentrations (e.g., 0.1 mg/ml) are too low to meet our needs (i.e., to administer this pharmaceutical grade formulation, too high volumes would be required). Pharmaceutical-grade diazepam will be used whenever the concentration/dose allows. Finally, a good number of the compounds we use are experimental in nature. In many cases, veterinary or human pharmaceutical grade products are not available. In others, the compounds are the product of our collaboration with [redacted], a medicinal chemist from the University of Wisconsin-Milwaukee.

    b. Discuss steps taken to ensure the health and welfare of the animals. Examples may include grade, purity, sterility, pH, pyrogenicity, osmolality, stability, formulation, compatibility, storage, side effects, and pharmacokinetics of the compound(s).

We strive to acquire compounds with ~99% purity from commercial sources or our academic collaborator, prepare the solutions with precision instruments (and with trained personnel), and filter-sterilize all solutions with 200 µm filters into autoclaved containers.

Reference: UMMC Chemical Safety Manual
http://ehs.umc.edu/documents/ChemicalSafetyPolicy2010.pdf

Please remember that the use of any hazardous material in animal rooms requires that a sign be posted in that room and on the cages containing the hazard in accordance with the policy on Signage for Hazardous Studies.

[1] AAALAC Frequently asked questions about Non-Pharmaceutical Grade Compounds

Appendix G Prolonged Physical Restraint

Physical restraint is the use of manual or mechanical means to limit some or all of an animal's normal movement for the purpose of examination, collection of samples, drug administration, therapy, or experimental manipulation. Examples of prolonged physical restraint include: chairing of nonhuman primates, chronic harness restraint of metabolic animals, and tube restraints for rodents. For additional information, consult the IACUC's policy statement on Prolonged Physical Restraint.

1. **Justify** the need for prolonged physical restraint.

Self-administration and observation studies are conducted in the home cage. In order to administer investigational drugs, the animals are implanted with a chronic indwelling intravenous catheter. In order to protect the catheter, we must employ a vest and tether system (Lomir, Inc). Importantly, from the animal perspective, we use a 4 ft flexible stainless steel tether. This allows the monkey full range of motion to all aspects of the cage while still protecting the catheter, but is not long enough to pose danger to the animal. The vest is non-allergic nylon and well-tolerated by the monkeys. From the human perspective, intravenous administration of investigational drugs/compounds can be performed from outside of the cage and away from the monkey, reducing the potential for scratches and other injuries.

2. Describe the restraint device.

All monkeys wear nylon vests while catheterized. For home cage-based studies, a 4- ft stainless steel tether will be attached to the vest and to a swivel device on the side of the cage.

3. Describe the details of how the animal(s) will be adapted to the restraint device.

Acclimation to jacket and tethers in the home-cage system occurs over a period of approximately one month. We proceed in stages – first allowing the monkey to habituate to the jacket and then adding the tether later (for the monkeys requiring the tether system).

4. a. What is the duration of a restraint period?

Self-administration and observation studies are conducted in the home cage, and the cages have been modified to accept the swivel end of the tether. Thus, the animals are tethered 24 hr/day.

b. How frequently will an animal receive the restraint (e.g., daily, once per week, every month)?

24 hours/day for the home-cage system.

5. Are animals monitored during the restraint period? ☐No ☒Yes

How often?

Animals are monitored on a daily basis by laboratory staff. On days on which the experiment is conducted (typically Monday through Friday, excluding holidays), animals are observed a minimum of four times/day.

6. Are there any anticipated problems as a result of the restraint device (e.g., skin lesion from harness, moist dermatitis, etc.)?

There is the possibility of skin lesions developing under the vest (e.g., in the shoulder or belly area). We minimize this possibility by adjusting the vest at the neck and belly to be as loose as possible, yet still protect the catheter. In extreme instances, we have had custom vests made for monkeys by Lomir. Monkeys will be checked for lesions by laboratory staff every time they are sedated; minimally every two weeks coinciding with cage change out.

Appendix H Multiple Survival Surgical Procedures

A major surgical procedure is defined as a surgical intervention that penetrates or exposes a body cavity (peritoneal, thoracic, cranium), produces substantial impairment of physical or physiologic functions, or involves extensive tissue dissection or transection (Guide, 2011). Multiple procedures are those whereby an animal will regain consciousness after each procedure. Procedures must be described in Appendix C. A surgery followed by a second procedure where the animal is euthanized is not considered multiple surgical procedures.

Surgeries performed on the animal prior to the animal's arrival at UMMC (e.g., ovariectomy procedure performed by vendor) must be considered. For additional information consult the IACUC's policy statement on Multiple Major Surgical Procedures.

1. **Justify** the need for multiple major surgical events in a single animal.

2. What is the time interval between the surgical events?

Appendix I Food and/or Fluid Regulation

The Guide (2011) states: "Regulation of food or fluid intake may be required for the conduct of some… research protocols. The regulation process may entail ***scheduled access*** to food or fluid sources, so animal consumes as much as desired at regular intervals, or ***restriction***, in which the volume of food or fluid consumed is strictly monitored and controlled." The least restriction necessary to achieve scientific

objectives while maintaining animal well-being should be used. For additional information consult the IACUC's policy statement on Food and/or Fluid Regulation.

1. Will ☒FOOD or ☐FLUIDS be ☒**scheduled** or ☒**restricted**?

Justify the need to schedule or restrict food and/or fluid.

In experiments involving food delivery, stable daily performance is maintained by restricting access to monkey chow in the animal's living quarters. Performance is maintained by commercially-available food pellets which are flavored (e.g., banana, fruit punch) and nutritionally balanced, which we have found to provide the best level of motivation for our studies while allowing us to maintain a relatively mild degree of food restriction. During initial training, body weights are reduced to approximately 90% of free-feeding values. Once subjects respond reliably under the schedule of food delivery, home cage food availability is increased to the maximum allotment that can be given without resulting in degraded performance during experimental sessions. Fruit, vegetables, and vitamins are given as supplements at least twice weekly. Over the course of the experiments, we anticipate that body weights can be maintained at 85-95% of free-feeding values.

Rhesus monkeys can be maintained indefinitely at such weights with no untoward effects or risks to health. Calorie restriction up to 30% has been shown to increase life expectancy in certain species of laboratory animals and to reduce the incidence of type 2 diabetes, endometriosis, cardiovascular disease, and other diseases of aging in rhesus macaques (Kemnitz et al, ILAR Journal, 2011). As a result, we do not expect any significant negative health effects from the relatively mild restriction described here.

Food scheduling is typically carried out to maintain health of the monkeys. Weight gain and obesity can have profound effects on the disposition of benzodiazepines, which are characteristically highly lipophilic. We also wish to keep weights to appropriate levels due to the unknown consequences of rhesus monkeys having chronic venous catheters but experiencing cardiovascular events due to obesity.

When conducting food restriction, the guidelines from the Association of Primate Veterinarians on food restriction for nonhuman primates in biomedical research will be followed. Food restriction will be monitored as follows:

Daily:

• The amount of food provided and the amount of food consumed at each meal.
• The amount of food reinforcements offered and consumed.
• Behavior of the animals. Any stress-related or abnormal behaviors (e.g. self-injury) will be discussed with the veterinary staff and [redacted], who is a trained primatologist and performs routine behavioral assessments in our colony in consultation with veterinary staff.

Biweekly

• The animal's body weight will be obtained biweekly (every other week) during physical examinations.
• Whenever possible, the weight will be obtained at the same time each day, prior to

experimentation and prior to feeding and watering.
• Whenever possible, animals will be weighed on the same scales to ensure consistent readings.
Monthly/Quarterly
• Body condition will be evaluated and scored at each physical examination, either by the laboratory staff or by CCR veterinary staff.
Semi-annually/Annually
• Clinical chemistry profiles (serum chemistry and complete blood count with differential) will be reviewed at regular intervals as determined by the veterinarian in consultation with the PI.

Food restriction will be monitored by filling out food restriction monitoring records on days when food restriction is being conducted (see attachment). Records will be available for review by the IACUC and veterinary staff. Records will include:
• The proposed individual full ration of food.
• The degree of restriction from full ration.
• The duration of the restriction and results of monitoring parameters such as: body weight, BCS, behavioral assessments, and laboratory data.
• The individual animal's preferred food reinforcements.
• The results of behavioral training and testing such as poor, satisfactory, or good, including the length of time required to acquire specific skills.

2. Check all methods that will be used to ensure adequate nutritional intake and hydration.

METHOD		FREQENCY OF CHECKS
Body weight	☒	Minimum of every 2 weeks (when on study)
Urine output	☐	
Fecal output	☐	
BUN	☐	
Hct	☐	
Food intake	☒	Daily
Other	☒	Physical exam – at least monthly

3. Restriction protocols typically base the restriction amount relative to a baseline, (free-choice consumption) parameter (body weight, intake amount). What will this restriction amount use as the baseline?

We routinely monitor weights at least monthly, and the weight data will be used over a period of 3-6 months prior to the initiation of the studies to determine a baseline.

What is the maximum restriction for any animal?

85% of free-feeding weight.

4. Growing animals must be frequently re-assessed to ensure normal growth patterns. If not using mature animals, what provisions will be made for these animals to

assure that their nutritional needs are maintained?

There is a large range of ages represented in our monkey colony. In order to ensure the health and wellbeing of each monkey, we use body condition scoring and weights to assess individual changes over time. We also will use somatometric measurements (measurements in cm around the waist, sternum, and head-to-rump) in to assess individual changes over time, if warranted (e.g., the animal is younger, e.g., 5 years of age).

5. Describe the protocol for regulating food and/or water intake.

Food restriction will only occur with self-administration studies. Initial training of food-reinforced behavior and maintenance of stable daily performance is facilitated by restricting access to monkey chow in the animal's living quarters. During initial training, body weights are reduced to 85 - 90% of ad libitum values by gradually reducing the number of daily chow. Once subjects respond reliably under the schedule of food delivery, home-cage food availability is increased gradually to the maximum allotment that can be given without resulting in degraded performances during experimental sessions. Fruit, vegetables and other food-based enrichment items are given as supplements. We will monitor weights regularly to ensure that animals remain in the desired window. Monkeys will be weighed every 2 weeks during cage change to limit risk to personnel. Body weights are not permitted to fall below 85% ad libitum values. Amount of food is adjusted as necessary to maintain appropriate body weights. On days on which experiments are conducted, animals are observed at least four times daily by trained laboratory personnel.
Any tests involving precipitated or spontaneous withdrawal will result in increased frequency of weighing and health exams. None of these tests are long-term (one day for precipitated withdrawal, ~7 days for spontaneous—these tests occur only when a new chronic drug is initiated), so we do not anticipate weight loss (or gain) due to withdrawal tests. If a >10% change is observed, the monkey will be removed from the dependence protocol by decreasing the dose of chronic drug in ½ dose intervals and monitored by lab and CCR staff.

6. How long will animals be on the regulation protocol?

Indefinitely, for the duration of their participation in the study. However, actual restriction level (85-95%) will depend upon the animal.

7. Will animals have any access to unrestricted food or water at any time?

Food will be regulated continuously to maintain healthy body weight and condition. Fluid will be available ad lib for monkeys in the home cage.

8. Who will be responsible for administering and documenting the regulation?

Personnel on this protocol (administering/documenting) and CCR personnel (documenting, if appropriate).

Note: NPO procedures for pre-surgical fasting are not included in this consideration. NPO procedures shall not extend for greater than 24 hours; if surgical delays are

encountered, the animals should be fed and re-fasted prior to the next scheduled procedure.

Appendix J Animal Pain and/or Distress

The management of post-procedural pain or distress is typically addressed with the use of appropriate pharmacologic and non-pharmacologic methods (see Appendix C). Appendix J should be completed if there are any procedures that are proposed that may cause more than momentary, slight pain or distress during which the appropriate sedatives, analgesics, or anesthetics will be **withheld** or in **which chronic pain or distress is induced**. Proposals which incorporate animal manipulations or procedures which may create more than momentary pain and distress (noxious injections, tumor growth, sequelae to compound administration, etc.) should also be addressed. For additional information consult the IACUC's policy on Animal Pain and/or Distress.

1. **Justify** the scientific need to withhold appropriate drugs or induce the pain/distress.

The purpose of this grant is to identify mechanisms of action underlying benzodiazepine tolerance and withdrawal (i.e., physical dependence). In order to empirically demonstrate physical dependence, it is necessary to induce and quantify a withdrawal syndrome. That said, we wish to make the case that any symptoms associated with benzodiazepine withdrawal is "mild", i.e., of low intensity, and that in the vast majority of tests, will be of a brief enough period of exposure to be considered "momentary". The observations supporting this position are outlined below:

1. Precipitated withdrawal will be evaluated over a maximum of 30 minutes: Based on preliminary findings (see [redacted], Psychopharmacology (Berl) 2020, in press), our standard blocker for evaluating withdrawal, flumazenil, results in withdrawal signs that last 15 to 30 minutes. These signs consist of nose rub, vomit/retch, procumbent, tremors (each approximately 25%) and rigid posture (approximately 2-3%). Maximum withdrawal signs (cumulative counts of each behavior) occurs immediately after i.v. flumazenil injection and rapidly dissipates (see Figure 1). We have changed our procedure so that the chronic drug is reinstated at 30 minutes after flumazenil treatment. In consultation with the veterinary staff, we will administer an anti-emetic at that time. Collectively, the withdrawal experienced by the monkey under these conditions is relatively brief and well within the range of observable effects that could be observed in non-dependent and/or naïve animals under non-experimental conditions.

2. Spontaneous withdrawal will be determined "to effect", rather than over a set period of time. Rather than the 2-week period proposed originally, we will monitor the animals after chronic drug cessation until 4 of the 5 observable effects detected originally with chronic alprazolam are observed, after which we will reinstate the chronic drug treatments. We also have increased the number of observation periods to 3 times per day. Based on preliminary data, we anticipate withdrawal signs to

emerge on day 2 of drug cessation, and we anticipate withdrawal testing will halt for most monkeys on day 2. In consultation with the veterinary staff, we will administer an anti-emetic at that time. For the 3-year period of this protocol, two spontaneous withdrawal tests will be conducted. Although withdrawal experienced by monkeys under these conditions will be longer relative to precipitated withdrawal, these tests will be extremely infrequent, and withdrawal-related effects will be well within the range of observable effects that could be observed in non-dependent and/or naïve animals under non-experimental conditions.

3. Precipitated withdrawal experiments have been conducted in human subjects under very similar experimental conditions (see [redacted], Psychopharmacology (Berl) 2020, in press). Drs. Miriam Mintzer and Roland Griffiths at Johns Hopkins University's Behavioral Pharmacology Research Unit have published multiple papers in refereed journals in which volunteers (in one case healthy normal volunteers) were treated chronically with diazepam (oral), followed by i.v. flumazenil tests for withdrawal (see [redacted], Psychopharmacology (Berl) 2020, in press). These human studies provide strong evidence that symptoms associated with benzodiazepine withdrawal is mild, safe, and well-tolerated by the majority of individuals.

For all withdrawal tests (precipitated or spontaneous), we will notify the CCR veterinary staff of the day and time the test will occur. Anti-emetic treatment will be initiated along with reinstatement of the chronic drug exposure. For example, ondansetron at 0.1 – 0.2 mg/kg can be given i.v., i.m., or s.c. (if given IV it will be administered slowly). The anti-emetic agent and dose ranges used will be chosen and monitored in collaboration with the veterinary staff.

Similarly, the veterinary staff will be notified and will be consulted during dose tapers, in case withdrawal signs arise. We have carefully chosen our parameters to reduce the likelihood of seizures occurring, but in the unlikely event that a seizure does occur, we will immediately reinstate the chronic drug and, in consultation with the veterinary staff, provide supplemental i.v. injections of diazepam to effect, if necessary. Anti-emetics will be administered as described above.

It is noteworthy that we have not observed any effects in addition to the 5 referred to above (nose rub, vomit/retch, procumbent, tremors, rigid posture). We have not observed effects that clearly point to headaches or depression (which may occur in humans); however, there is no consensus or rigorous evidence-based system for detecting either phenomenon in monkeys. Our behavioral scoring sheets include an "other" category that we will monitor closely, and in our biweekly discussion groups on scoring we will specifically ask the observers if "new and/or unusual" behaviors are observed during tests.

2. What is the duration of time that an animal may experience this pain/distress?

For antagonist precipitated withdrawal, the duration of time is no more than 30 minutes. For spontaneous withdrawal, withdrawal emerges on day 2 of chronic drug cessation. As described above, as soon as 4 of 5 withdrawal signs are recorded (during 3 observation

sessions/day), the chronic drug will be reinstated.

3. Describe non-pharmaceutical means to alleviate pain/distress (soft bedding, social housing, supplemental heat, etc.).

The primary signs of benzodiazepine withdrawal in rhesus macaques are nose rub, vomit/retch, procumbent, tremors, and rigid posture. We will closely monitor weights and body condition in consultation with the CCR staff. Our experience has been that antagonist-precipitated withdrawal does not alter eating or drinking, but spontaneous withdrawal can reduce eating (but not drinking) for approximately 1 day.
Supplemental fluids and preferred foods can be tolerated in most cases of withdrawal.

4. Describe situations where an animal may be removed prematurely from a study.

The most noteworthy situation for removing an animal from a study is if a catheter becomes non-functional. If this happens, then the monkey may enter into withdrawal, depending on the timing of the event. If a catheter becomes non-functional, we will remove the animal from the study and, in collaboration with CCR veterinary staff, conduct a physical exam under sedation (ketamine, i.m.). If any signs of withdrawal are observed, we will initiate a "dose taper" procedure, in which a benzodiazepine such as diazepam (1.0 mg/kg, i.m., or to effect, b.i.d.) will be administered via the i.m. route and the dose decreased every third day over approximately 2 weeks (an example of a dose taper regimen [doses in mg/kg]: 1.0, 1.0, 0.5, 0.5, 0.25, 0.25,
0.125, 0.125, 0.062, 0.062, etc.). Gradually tapering down the dose is a well- established method for alleviating dependence and minimizing withdrawal.
Observations will occur throughout this period, and once all signs of withdrawal are absent and the animal is drug-free for at least 1 week, the subject will be returned to the study, in consultation with CCR veterinary staff.

5. Describe those procedures whereby animals are likely to experience more than momentary pain or distress as a result of manipulations or procedures (noxious injections, tumor growth, sequelae to compound administration, etc.).

As stated above, there is no evidence that humans or nonhuman primates experience pain as a result of benzodiazepine withdrawal. Withdrawal will be evaluated quantitatively in dependent monkeys by administering an antagonist (precipitated withdrawal) or, on rare occasions, withholding the chronic drug (spontaneous withdrawal).

6. Will any anesthetics, analgesics, or tranquilizing drugs be used to reduce this pain or distress?

During precipitated withdrawal tests with a blocking agent (antagonist), the observation session will be terminated after 30 min by returning the monkey to the chronic drug regimen. Anti-emetic agents will be administered as described above (e.g., i.v. ondansetron). During spontaneous withdrawal, chronic drug will be halted and withdrawal signs are expected to emerge on day 2. Once 4 of 5 signs emerge, chronic drug will be reinstated and anti-emetic agents administered. If, however, a monkey shows signs of weight loss or any other signs of distress, we will contact CCR veterinary staff and determine whether the monkey needs removal from the study on a case-by-case basis.

Appendix K Progress Report

1. Give a brief description of the work performed on these projects in the past 3 years. If progress did not occur or was less than expected, please give a brief explanation.

In the past 3 years, we have completed a study investigating the mechanisms underlying tolerance and dependence following chronic benzodiazepine treatments by using quantitative behavioral observation. In that study, we evaluated the ability of chronic treatment with a commonly prescribed benzodiazepine, alprazolam, to induce tolerance to sedative effects and physical dependence using a novel set of behavioral measurements in rhesus monkeys.

We are now in the process of planning for a second study incorporating drug self- administration into the chronic benzodiazepine treatment, as described in the present protocol.

2. List any publications, abstracts, and/or presentations coming directly from the work performed on these projects in the past 3 years.

[redacted]. Tolerance and dependence following chronic alprazolam treatment: quantitative observation studies in female rhesus monkeys. *Psychopharmacology*, 2020, *in press*. doi: 10.1007/s00213-019-05447-1.

3. Answer the following questions in regard to the last year of the previous version of this protocol.

I. Animals

1. Have any unanticipated (morbidity, mortality, inability to collect data) events occurred in the past year?
   ☐ Yes ☒ No
2. Has any mortality occurred prior to the anticipated end-point of an experiment or as a result of surgical manipulation?
   ☐ Yes ☒ No
3. Have any animals been euthanized prior to the anticipated end-point of an experiment?
   ☐ Yes ☒ No
4. Did any animals show signs of morbidity or sickness following experimental manipulation other than what was detailed in the protocol?
   ☐ Yes ☒ No

If yes to 1 -4, answer #5.

5. Describe any unanticipated events (morbidity, mortality, inability to collect data) and any identified contributing factors (e.g., recurring postoperative complications, excessive or unanticipated mortality rate, unplanned event that causes the removal of an animal(s) from an experiment for a period of time, loss of implant, etc.).

N/A

If the protocol involves breeding:

Breeding: Animals born over the past year as part of this protocol

Species	Strain	# of pups born in last year	# of pups used in the last year for experiments

What was the final disposition of any pups not used for experiments?

N/A

II. Personnel

1. During the past year did any Occupational Health & Safety "incidents or accidents" *(needle sticks, animal bites, cuts, burns, etc.)* occur that involved personnel participating in the conduct of this study?

   ☐ Yes ☒ No

2. If yes, describe the event and identify any contributing factors:

N/A

3. What treatment measures were taken:

There have been no NHP exposure events within the past year. However, should one occur all lab members have been trained to follow UMMC's NHP biohazard exposure protocol, which involves scrubbing the exposed area for 15 minutes, drawing blood from both the individual who was exposed and the animal to ensure that there is no presence of the Herpes B virus, and taking retroviral medication (e.g., Valtrex).

Appendix L Behavioral Training and Testing

Useful Resources:

NIH Publication: *Methods and Welfare Considerations in Behavioral Research with Animals NIH Publication No. 02-5083, March 2002*
http://www.nimh.nih.gov/researchfunding/animals.pdf

American Physiological Society Publication: *Resource Book for the Design of Animal Exercise Protocols*, Feb. 2006
http://www.the-aps.org/pa/action/exercise/book.pdf

1. What form(s) of behavioral training/testing will be used?

Operant drug self-administration, observation of species-typical behavior

2. Describe how the behavioral training/test is conducted (include descriptions of the devices, preliminary animal training, fluid/food restriction, reward/ positive reinforcement, duration of trial, frequency of behavioral testing, etc.).

1. Self-Administration

Training: Monkeys are first habituated to a custom-designed nylon jacket, attached to a tether and swivel (see Appendix G for details). Prior to testing, all monkeys are implanted with chronic i.v. catheters, as described in detail in Appendix C. Monkeys initially are trained to respond under a fixed-ratio 1 schedule of food (1-3 pellets, BioServ) or i.v. benzodiazepine injection (e.g., midazolam or alprazolam). Once stable responding is established (± 20% variation over 3 days with no consistent trends), choice sessions will be implemented in which one lever is paired with food delivery and the other lever is paired with benzodiazepine delivery. Daily experimental sessions will be signaled by the illumination of stimulus lights and will consist of five components, each made up of up to 10 trials. Each component will be separated by a timeout (5 min or longer if required) in order to minimize drug accumulation due to repeated injections in a single session. Once a trial is initiated, monkeys choose between the food-paired lever vs. the drug-paired lever. A trial will end with food or drug delivery or the expiration of a limited hold of 20 min, at which time the stimulus lights will be extinguished. For most studies, the dose will be increased across the 5 components. Other studies may hold the dose constant, but the response requirement will be doubled from an initial requirement of 10 - 100 per injection (e.g., response requirements of 10, 20, 40, 80, 160).
Pre-Chronic testing: The acute and chronic behavioral effects of drugs or experimental compound will be determined using quantitative observational techniques and the choice procedure as described above. Prior to self-administration training, the dose- and time-dependent observable behavioral effects of benzodiazepine-type drugs and the non-selective antagonist flumazenil will be assessed. First, a dose of drug will be evaluated once/day, generally at 12:00 noon, to provide a significant period of time post feeding (feeding can engender a range of species-specific effects). Test sessions will be planned with at least 2 drug-free days in between determinations. Behavioral samples will be taken at 0, 7.5, 15, 30, 60, 120, and 240 min post injection. Vehicle (usually a propylene

glycol/water solution) and 0.01 – 1.0 mg/kg, i.v. of drug will be tested based on our preliminary data. Flumazenil (alone) will be evaluated in a similar manner, with the same time points and with 2 days in between tests. Once these tests are completed, all monkeys will undergo a 1-month drug-free "washout" period.

Chronic treatment: Chronic treatment with alprazolam, clonazepam, zolpidem, or HZ166 will consist of automatic injections every nth hour for 30 days. Behavioral observations will occur daily at 12 noon. Feeding and maintenance of the cage/equipment will occur the same time each day in the AM. Water will be available ad libitum throughout the experiment.

Tolerance and Precipitated Withdrawal testing: After 30 days of chronic alprazolam, tests for tolerance and precipitated withdrawal will be scheduled. The tests conducted during the pre-chronic phase will be repeated, only with chronic drug treatment prior to and after the tests. Each observation test will involve suspending chronic treatment 4 hours prior to the test session, followed by an experimenter-delivered injection of alprazolam or flumazenil at noon. Five-min observation sessions will occur at 0, 7.5, 15, 30, 60, 120, and 240 min post-injection. Chronic drug treatments will be re-initiated after this 4-h block of time. After the observation tests, we will re-initiate self-administration. Chronic drug injections will occur prior to and after the ~2 hour choice session.

Post-Chronic testing: We anticipate that the tolerance and precipitated withdrawal tests will require ~60 days to complete. After the 90 total days of drug exposure and all tests are complete, we will replace drug with vehicle, starting on day 91. Vehicle substitution will be scheduled for a minimum of 2 weeks, and daily observation sessions will re-main as PM sessions. Based on our preliminary data, we anticipate spontaneous withdrawal to emerge on the 2nd day of the drug-free period and to rapidly decline across the next week. Once no evidence of withdrawal is evident or after the 2-week period, whichever occurs first, the vehicle injections will be stopped and tests conducted during the pre-chronic phase will be repeated. We anticipate that all effects on behavior induced by the drugs will return to the levels observed during the pre-chronic tests, i.e., tolerance and withdrawal will be reversible.

2. Behavioral Observation Procedure:

Species-Typical Behaviors: Monkeys are first habituated to a custom- designed nylon jacket, attached to a tether and swivel (see Appendix G for details). Prior to testing, all monkeys are implanted with chronic i.v. catheters, as described in detail in Appendix C. The behavioral effects of each compound will be determined using quantitative observational techniques. The basic design is as follows: (1) Acute dose-response and time-response determinations for compounds alone; (2) tests of compounds in combination, based on the effects of the compounds alone. A range of species typical behaviors, as well as behaviors associated specifically with BZ administration, will be recorded by trained observers. All observers will be

unaware of the goals of the study as well as the compound under investigation. Observers will be trained using a standard procedures manual and will complete at least 20 hrs of scoring prior to participating in the study. Reliability will be checked every 3 months to insure consistent data across observers and time. Using this scoring system, the presence of a behavior is noted during each 15-second interval and the number of 15-second intervals during a 5 minute session is observed is recorded. Thus, the maximum score for each behavior during one modified frequency sample is 20.

Sedation Measures: We have developed a scoring system for sedation based on standards used for anesthesia of human patients used by the American Society of Anesthesiologists (ASA 2002). Based on these standards, two categories are included in the behavior scoring session: Moderate sedation and deep sedation. These measures include an evaluation of responding to external stimuli during the modified frequency scoring session. The evaluation of responsiveness is conducted at the beginning of each 60-second block of time during the scoring session (i.e., maximum of 5 evaluations during one session). If a behavior referred to as “sleep/rest posture” is observed and the animal does not attend to the activities of other monkeys in the room, the observer will speak the monkey’s name in a normal tone, walk at a normal pace towards the cage, and then move the lock used to secure the door of the cage (a feature present on all cages). The different types of response to external stimuli are described as “responds readily”, “delayed response” or “no response”. If the monkey opens its eyes and initiates movement in less than 3 sec, the observer will stop the evaluation and score “sleep posture”. If the monkey attends slowly (i.e., ≥ 3 seconds following stimuli) the observer records “delayed response” which is part of a decision rule for “moderate sedation”, whereas “no response” identifies “deep sedation”. The interaction of these response-to-external stimuli criteria and the scoring of behavior allow for easier interpretation of atypical situations, e.g., a monkey in a normal sleep-associated posture but unresponsive would be scored as “deep sedation” based on the “no-response” criterion being met. Behavioral profiles will be determined after monkeys habituate to the human observer (typically 1-2 weeks). After catheter implantation, baseline control data will be obtained following saline or vehicle injections.

Withdrawal Measures: Behaviors associated with BZ-induced withdrawal (preliminary data; e.g., Weerts et al. 1998a) will be included in the observation tests. These behaviors include vomit/retch, nose rub (wiping of nose with hand or arm), tremors (typically rapid shaking of limbs and/or digits), and seizures. Two postural effects will be included that were often observed during preliminary studies: Procumbent (animal lying on cage floor but responsive ac- cording to rest/sleep posture criteria, yet does not get up) and rigid posture (monkey seemingly “frozen” in place with limbs and/or head in atypical positions).

3. Activity monitoring:
Activity will be monitored noninvasively using Actiwatches. Actiwatches are small,

lightweight accelerometers that quantify and record movements. They will be placed in a protective case attached to commercially available nonhuman primate collars or to the pocket/extension in the back of the monkey's jacket under light ketamine anesthesia (10 – 20 mg/kg, i.m). Neither the case nor the monitor is in direct contact with the animal. This method has been used extensively to quantify activity in nonhuman primates over extended periods of time with no adverse effects. The collar, Actiwatch case or Actiwatch device do not impede the animal's movement in any manner. Both collar and case are typically well-tolerated by monkeys. For Actiwatch maintenance, the animal will be lightly sedated (ketamine: 10 – 20 mg/kg, i.m.) and the Actiwatch removed in order to download data, change batteries, and reprogram. Actiwatches can record from 45 to 180 days, depending on data collection settings, without needing to be removed. We expect that no subject will be anesthetized more frequently than once per month to remove or place a monitor, although they may be removed earlier for health (e.g. skin irritation beneath the collar), equipment (e.g. loosening of screws holding the Actiwatch case to the collar), or experimental (e.g., cessation of data recording) reasons. Some subjects will wear those monitors continuously.

3. If an unexpected problem or event occurs in the performance of the above described behavioral training/testing procedure(s) that directly impacts the live animal, what steps will be taken to ensure appropriate treatment is provided?

Should an unexpected problem or event occur, the laboratory staff will sedate the monkey (if possible) and CCR veterinary staff will be contacted immediately. Note that all studies will take place in the home cage, therefore direct contact with the monkey is minimal.

4. Will animal be observed/attended throughout the duration of the trial/test?
   ☒No ☐Yes
   If No, provide rationale.

In virtually all of our studies, animals are observed during designated observation periods, as well as pre- and post-session by trained observers from the laboratory staff. Other observers are restricted to what is absolutely necessary, since the presence of additional observer(s) would change the behavior and add an uncontrolled variable to the experiment.

5. Describe any unique post-trial animal husbandry that may be required (e.g., dry/warm environment for animals in the Morris Water Maze, soft padding for animals on the Rod Test, etc.).

No unique or special post-trial animal husbandry is necessary.

6. List personnel involved with the actual training and indicate his/her level of knowledge as it relates to the training/testing used in the lab.

[redacted] – 25+ years' experience
[redacted] – 27+ years' experience
[redacted] – 11+ years' experience

[redacted] – 6+ years' experience
[redacted] – 6+ years' experience
[redacted] – 12+ years' experience
[redacted] – 5+ years' experience

7. Where will the test(s) be conducted?

All testing will occur in the CCR – rooms [redacted]

8. Will the Animal Behavior Core (ABC) be used for this testing?
   - ☒ No
   - ☐ Yes – Use of the ABC requires review and approval by the Core Director.

X_____
ABC Director (Paste digital copy of signature)

Attach copies of ABC SOPs that will be used for this study.

Appendix N Use of Expired Medical Materials or Devices

The use of expired medical materials and/or drugs may be allowed for non-survival procedures. The attending veterinarian and the IACUC are responsible for ensuring that proposed animal activities avoid or minimize discomfort, distress, and pain to the animal. These responsibilities cannot be met unless the veterinarian and the IACUC maintain control over the use of expired medical materials.

All anesthetics, for survival and acute procedures, analgesics, emergency drugs, and euthanasia agents must be in date.

All pharmaceuticals and medical materials (e.g. drugs, antibiotics, fluids, saline bags, disinfectant solutions, catheters, sutures, etc.) used in survival procedures must be in date.

For additional guidance see the IACUC Policy Statement *Use and Maintenance of Expired Medical Materials (Pharmaceuticals and Devices)*

1. List and describe expired medical materials and/or expired medical devices to be used and describe intended use of each item. *NOTE: All expired medical materials or devices must be clearly labeled, "Expired, for conditional use only"*.

2. Please provide a justification for the use of the expired items.

3. Describe if sterility will be required, and if so, how proper sterility will be assured.

4. Identify the room and exact location where expired items will be stored.
NOTE: Items must be kept in a separate location (cabinet, shelf, box) and must be clearly labeled, "Expired, for conditional use only".