East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

February 19, 2014

Krista McCoy, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. McCoy:

Your Animal Use Protocol entitled, "Do Prenatal Sex Hormones Influence Development of Autism-Like Behavior?" (AUP #D300) was reviewed by this institution's Animal Care and Use Committee on 2/19/14. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2013

Project Title:

Do prenatal sex hormones influence development of autism-like behavior?

	Principal Investigator	Secondary Contact
Name	Krista A. McCoy	Bevin Blake
Dept.	Biology	Biology
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For IACUC Use Only

AUP # D300			
New/Renewal New 1/31/14			
Full Review/Date	DR/Date		
Approval Date 2/19/14			
Study Type hormones			
Pain/Distress Category D			
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC	EHS 2/19/14
OHP Enrollment			progesterone, testosterone, estradiol
Mandatory Training			
Amendments Approved			

Dogstable + Haworth Science

single housing - temporary

I. Personnel

A. Principal Investigator(s):

Krista McCoy

B. Department(s):

Biology

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Dr. Krista McCoy, Ph.D.	PI	Yes	~20 years of experience working with fresh and saltwater fish, amphibians, reptiles, birds and rodents. I have taken the AALAS learning module for Xenopus laevis as well as Introduction to Mice and Working With the Laboratory Mouse. I have also attended a rodent handling course through University of Florida and been trained by their vet staff to conduct cervical dislocations. In addition to my animal training, I also have formal hazardous waste training.
Bevin Blake	Graduate student	Yes	Bevin is new to animal research, but has taken ECU's rodent handling class and the AALAS learning modules: Introduction to Mice and Working With the Laboratory Mouse. She will

			be trained on additional specific procedures by K. McCoy
Ciro Amato	Graduate student	Yes	Two years of research experience working with marine wildlife. One year of experience handling dogs, cats, birds and mice in a veterinary setting. Ciro has taken ECU's rodent handling class and survival surgery class. In addition, he has taken the AALAS learning modules: Introduction to Mice and Working With the Laboratory mouse.
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II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)
Do not cut and paste the grant abstract.

Prenatal hormone signaling has powerful and long lasting effects on brain organization. There is evidence that excessive androgen signaling during critical periods of fetal development might contribute to the development of neurodevelopmental disorders such as autism spectrum disorder (ASD). Increased levels of prenatal testosterone (T) are associated with autism and autism-related traits. The extreme male brain (EMB) theory of autism states that autism is the result of abnormal brain development due to high levels of T, which results in exceedingly male-directed brain development.

Juvenile play behavior is important for normal cognitive and social development. Children with autism are unable to successfully engage in social play with peers. In fact, this disruption in normal play patterns can feed back on autism and exacerbate the symptoms. Like ASD, prenatal exposure to endocrine disrupting chemicals (EDCs) can disrupt normal social play in both rodents and humans. Play behaviors are also known to be programmed pre- and neonatally in rodents by the actions of hormone receptors. Thus, the prenatal programming for ASD and play behaviors may be linked.

We propose an integrative approach to test the EMB theory through investigating the link between prenatal hormone receptor signaling and its effects on juvenile play behavior, autistic-like behavior, and masculinized behavior (spatial learning and reproductive behavior). This work will improve our understanding of the mechanisms underlying the effects of prenatal hormone receptor signaling on brain development and subsequent changes in social behavior that are indicative of increased risk for ASD.

Rats are a useful model for understanding how the prenatal environment influences behavior at different periods in life. They are a particularly relevant model organism for understanding autism; the rich repertoire of social behaviors makes it possible to use rat models to study neurodevelopmental disorders characterized by social deficits. Furthermore, behaviors we wish to assess (including social play, spatial learning, and reproductive behaviors) have been well studied in this model organism.

Pregnant dams will be given subcutaneous injections of estradiol, 5α dihydrotestosterone, or a control on gestational days 14-19. After birth, litters will be culled to two males and two females. On post natal day (PND) 21, pups will we weaned and play behavior observations will begin. Play behavior testing will be conducted in order to assess whether or not the treatment conditions had an effect on juvenile play. This behavioral test will consist of pairing same sex littermates together in a neutral play box and recording their behaviors. Pups will be returned to their home cage and cared for until the next behavioral test (~35 days later).

On PND 55, one female and one male pup will be randomly assigned to the sociability and social approach test and the remaining female and male will be assigned to the open field test. The sociability and social approach test will assess whether or not the treatment condition altered the animal's social behavior. The sociability and social approach test will consist of placing the test animal in the testing apparatus (for which is has been habituated), which will contain a stranger animal in a smaller cage and an empty cage. The test animal's behavior and time spent investigating the stranger animal versus the empty cage will be recorded. Normal individuals are expected to be curious and inspect the new animal. The open field test will be used to assess the animals' reaction to a sudden change in environment. The open field test will consist of placing the test animal in the open field arena and recording their behavior. Rats will be returned to their home cage and cared for until the next behavioral test (~50 days later).

On PND 110, reproductive behavior and water maze tests will begin. The reproductive behavior test will assess whether embryonic hormone treatment altered reproductive behavior. The reproductive behavior test will consist of independently pairing male and female test individuals with a sexually primed female and observing behavior. The water maze test will assess whether spatial learning was altered by the treatment condition. The water maze test will consist of placing the test animal in a pool of water and recording their trajectory and swimming time as the animal navigates towards the hidden platform. Animals will be trained to learn where the platform is by using visual cues on prior to testing.

B. Ethics and Animal Use

B.1. Duplication

Does this study duplicate existing research? No

If yes, why is it necessary? (note: teaching by definition is duplicative)

Click here to enter text.

B.2. Alternatives to the Use of Live Animals

Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? No

If yes, please explain why you cannot use these alternatives.

Click here to enter text.

B.3. Consideration of Alternatives to Painful/Distressful Procedures

a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

Dams will be injected subcutaneously once per day for five days (on embryonic days 14.5-19.5). Subcutaneous injections are rarely painful (Wolfensohn and Lloyd, 1994). However, after injections, females will be presented with wet (basic) chow in an effort to reduce stress.

The hormone treatments are expected to alter the dams' sex hormone concentrations but should not make the females ill.

Offspring will complete the Morris Water Maze test. Our animals might experience temporary distress during the test, which will require swimming in a pool of warmed water to navigate towards a platform. If an animal takes longer than 90 seconds to complete the task, they will be guided by an observer to the platform. Animals will have sufficient rest time in between swimming trials. Animals completing the water maze will be carefully monitored by an observer and removed from the water if any signs of distress or potential drowning occurs. Animals will be gently dried with a towel after each trial

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	1/16/13	Click here to enter text.
Database(s) searched:	Google Scholar	Click here to enter text.
Time period covered by the search (i.e. 1975-2013):	All years	Click here to enter text.
Search strategy (including scientifically relevant terminology):	Alternatives to subcutaneous injections	Click here to enter text.
Other sources consulted:	Pubmed.gov	Click here to enter text.

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers

to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

We have read the following sources to help us address the three R's (Replacement, Reduction, and Refinement): http://www.nc3rs.org.uk/page.asp?id=7, http://www.nal.usda.gov/awic/pubs/enrich/rodents.htm, http://or.ucsf.edu/larc/10666-DSY.html

Refinement: Subcutaneous injection is the least stressful method that we are aware of to provide our treatments in a controlled manner. Although protocols to train animals to eat dosed food items are available we cannot guarantee that our rats will eat the entire food item and if it is not completely consumed that dam would have to be sacrificed. We will also provide animals with enrichment items to help alleviate boredom. Refinement is further detailed below in the section on refinement techniques (III 3 b).

Replacement: Live animal research is necessary for this type of research because it includes evaluating whole animals affects and behavioral assays. These behavioral assays are integral to the study design and must be completed in order to test our research questions.

Reduction: We are confident that we will not need as many samples as our power analyses suggest. We will, therefore, conduct our experiment in two time blocks where the second block will be run only if needed to increase sample size. In addition, as another way to reduce future use of animals, when dams and offspring are euthanized, several organs of interest will be collected and utilized. Other organs may be affected by the treatment (brain, genitals, kidney, and liver) and will be collected, properly fixed and stored in an -80 freezer. They will be held for future examination and potential collaboration.

C. Hazardous Agents

1. Protocol related hazards (chemical, biological, or radiological): Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	**Radiation**	Click here to enter text.	Choose an item.
Ionizing radiation	**Radiation**	Click here to enter text.	Choose an item.
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	**IBC**	Click here to enter text.	Choose an item.

Toxins of biological origins (venoms, plant toxins, etc.)	**IBC**	Click here to enter text.	Choose an item.
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	**IBC**	Click here to enter text.	Choose an item.
Human tissues, cells, body fluids, cell lines	**IBC**	Click here to enter text.	Choose an item.
Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	**IBC**	Click here to enter text.	Choose an item.
Oncogenic/toxic/mutagenic chemical agents	**EH&S**	Click here to enter text.	Choose an item.
Nanoparticles	**EH&S**	Click here to enter text.	Choose an item.
Cell lines, tissues or other biological products injected or implanted in animals	**DCM**	Click here to enter text.	Choose an item.
Other agents	**EH&S**	Estradiol, 5-alpha dihydrotestosterone estradiol benzoate, progesterone	Choose an item.

2. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

No

III. Animals and Housing

A. Species and strains:

Rattus norvegicus, Sprague-Dawley rat

B. Weight, sex and/or age:

Adult male and female rats, pregnant dams, and offspring. Offspring will be reared through sexual maturity.

C. Animal numbers:

1. Please complete the following table:

Total number of animals in treatment and control groups	Additional animals (Breeders, substitute animals)	Total number of animals used for this project
30 females 300 pups	5 male breeders 10 incidental females (see below)	345 animals

2. Justify the species and number (use statistical justification when possible) of animals requested:

Because our work is attempting to model effects of prenatal chemical signaling on human neurological development and behavior, rodents are among the "lowest" animals that we can use. Most fish, amphibians, birds, and reptiles do not display quantifiable play and social behaviors, so they do not function as suitable models for our research questions. Of the mammals, rodents are especially appropriate because previous research has phenotyped behaviors in rodents that are relevant to ASD (i.e. repetitive grooming, reduced sociability).

Statistical justification: We will replicate each treatment in each experiment at least 5 times and up to 10 times. Our experiment will be conducted in two time blocks with a sample sizeof five in each block. A sample size of ten will provide at least a statistical power of 0.85, anticipated effect size of 1.25, with a probability level of 0.05.

Further details of the animal numbers being requested are detailed below in section IV A.

3. Justify the number and use of any additional animals needed for this study:

Click here to enter text.

a. For unforeseen outcomes/complications:

There is a small probability that certain females could have small litters or litters that are sexually biased. If this happens our design will require that we treat another pregnant dam so that we have the appropriate sex ratio for our study. Additional females have been included in the table above (III 1) to account for this issue.

b. For refining techniques:

Refinement: Because rats are social animals females will be housed together until the day of first injection (14.5). After treatment begins females will be housed with a female of the same treatment or singly in anticipation of the birth of her pups. Females will not be housed singly for more than 7 days. To reduce injection stress, moist basic chow will be given to the rats as a "reward" to help alleviate any mental or physical stress. In the event that any rat is showing signs of distress that cannot be ameliorated with moistened chow diet we will ask for a veterinarian consultation. If an animal requires euthanasia it will be euthanized with isoflurane and decapitation will ensure death.

c. For breeding situations, briefly justify breeding configurations and offspring expected:

Two sexually mature females and one male will be housed together, individuals will not exceed 400g in weight. If a male exceeds 400g, only one female will be housed with him. Vaginal plugs will be checked every morning. When a plug is encountered the female will be considered pregnant, and the embryos will be given the designation of embryonic day (E) 0.5. Pregnant females will be removed, and a new breeder female will be added to the male's cage. This breeding configuration will allow us to obtain the correct number of pregnant dams per treatment.

Females will be housed singly with their young. It is expected that each female yields a litter with at least four males and four females. However, some of the genitalia and anogenital distances will be ambiguous due to the prenatal hormone treatment. Therefore, pups will be ear punched to obtain tissue samples in order to assign genetic sex to each individual. Once genetic sex is determined, and at weaning, litters will be culled to two females and two males. Tissues from culled offspring will be used for physiological/mechanistic work. This design will allow us to study both the behavior and physiology of animals of the same treatment, thus reducing the number of dams required and increasing our productivity from this experiment. The remaining four offspring will be weaned and housed together based on sex.

d. Indicate if following IACUC tail snip guidelines: N/A
(if no, describe and justify)

Click here to enter text.

4. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? No **(if yes, describe)**

Click here to enter text.

5. Are there any deviations from standard husbandry practices?

YES **If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**

1) No animals may receive any food other than the standard rat diet. Rationale: it is unknown how supplemental foods may impact developmental and behavioral parameters.
2) Personnel from K. McCoy's laboratory will be responsible for all injection procedures. Rationale: this requires more time and effort than we feel is reasonable to ask of animal care personnel.
3) Personnel from K. McCoy's laboratory will pair breeder animals and monitor females for mucous plugs and signs of pregnancy

6. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study? Yes **(If yes, describe housing and justify the need to singly house social species):**

Stud males will be housed in groups of two three according to weight. Cages will be monitored for aggression and animals will be separated if necessary. Pregnant females will always be paired with other pregnant females until the first injection (on embryonic day 14.5). We cannot co-house females from different treatments because feces and urine from one treatment might contaminate the cage and therefore the animal of the other treatment (e.g., DHT to control). In most cases, females will be able to be housed with another co-treated female until just prior to birth. Just before birth, females will be housed singly to give birth.

As a standard protocol, pups will be housed individually for a brief period of social isolation (24 hours) prior to play behavior observations in order to increase the likelihood of play behaviors and other relevant social interactions. This is a standard method employed when studying play behavior in rodents and is not known to have significant adverse effects.

7. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? Yes

(If yes, describe and justify the need to withhold enrichment)

We cannot use plastic enrichment items because they can be made of endocrine active materials, but alternate enrichment will be acceptable. Rats will be given enrichment activities including enviro-dri and wooden chew blocks to improve living conditions and reduce stress. To provide stimulation several dry food items will be scattered in the bedding.

8. If wild animals will be captured or used, provide permissions (collection permit # or other required information):

N/A

9. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.

Ragsdale Annex and S112A Howell Science Center

IV. Animal Procedures

A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.

We will test the extreme male brain theory of autism in order to achieve a better understanding of how the prenatal hormone environment can contribute neurodevelopmental disorders.

There is one experiment planned that will follow three treatment groups from conception to adulthood and measure behavior at three different points in time. Females will be time mated, and the observation of vaginal plugs will be recorded as gestational day (GD) 0.5. Pregnant dams will be randomly assigned to three treatment groups (max n=10/treatment) and subcutaneously injected with a vehicle control, DHT, or estradiol. Injections will be given on GD 14-19 (Figure 1).

Prior to the day of birth, females will be housed singly. Between days 10 and 15 Pups will be ear punched for identification purposes and the tissue samples will be collected for genotyping. The tissue sample is necessary in order to determine the genetic sex of each individual via PCR. At weaning, litters will be culled to two females and two males. Culled pups will be deeply anesthetized and blood will be collected via cardiac puncture. After blood collection they will be humanely euthanized (see below), perfused with fixative, and tissues of interest will be preserved.

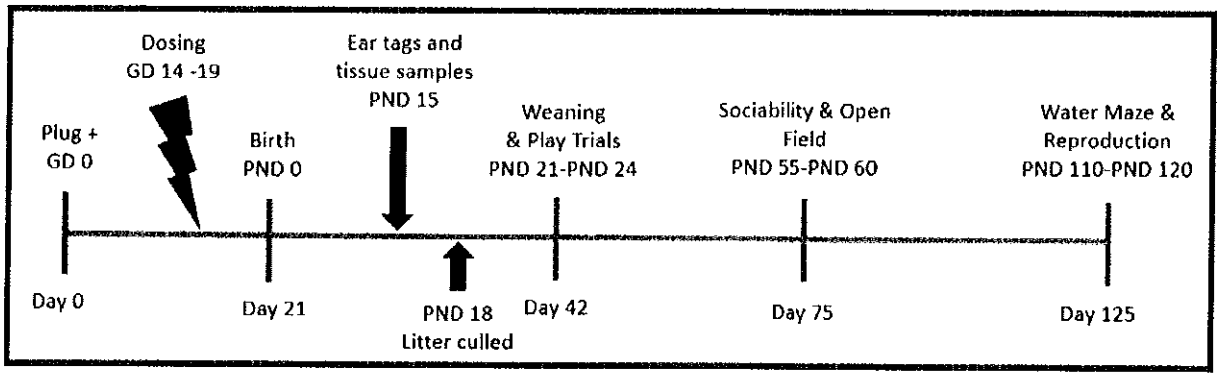

Figure 1. Experiment timeline

On PND 21, pups will be weaned and all pups will be housed in same sex groups. Details of behavioral studies are outlined in the appropriate sections below. Briefly, same sex pairs will be habituated to a readily sanitizable (glass) play arena/box over a three-day period and then will be housed singly for 24 hours. After this 24-hour separation play-pairs will be reunited in the play arena and behavior will be recorded in order to assess social play.

On PND 55-60, sociability and open field behavioral tests will be run (detailed below). After these trials, rats will be returned to their same sex sibling cages until the next behavioral tests. On PND 110-120, the Morris water maze and reproductive behavior testing will be conducted. After these behaviors are recorded individuals will be deeply anesthetized and blood will be collected via cardiac puncture. Then animals will be euthanized and perfused with fixative (see below).

In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

Please refer to DCM and IACUC websites for relevant guidelines and SOPs.

B. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:

Adequate records describing anesthetic monitoring and recovery must be maintained for all species.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:

Click here to enter text.

1. Describe the pre-procedural preparation of the animals: N/A

a. Food restricted for Click here to enter text. **hours**

b. Food restriction is not recommended for rodents and rabbits and must be justified:

Click here to enter text.

c. Water restricted for Click here to enter text. **hours**

d. Water restriction is not recommended in any species for routine pre-op prep and must be justified:

Click here to enter text.

2. Anesthesia/Analgesia *for Procedures Other than Surgery*

	Agent	Concentration	Dose (mg/kg)	Max Volume	Route	Frequency	Number of days administered

Pre-procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Reason for administering agent(s):
Click here to enter text.

4. For which procedure(s):
Click here to enter text.

5. Methods for monitoring anesthetic depth:
Click here to enter text.

6. Methods of physiologic support during anesthesia and recovery:
Click here to enter text.

7. Duration of recovery:
Click here to enter text.

8. Frequency of recovering monitoring:
Click here to enter text.

9. Specifically what will be monitored?
Click here to enter text.

10. When will animals be returned to their home environment?
Click here to enter text.

11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:
Click here to enter text.

C. Use of Paralytics

1. Will paralyzing drugs be used? Choose an item

2. For what purpose:

Click here to enter text.

3. Please provide scientific justification for paralytic use:

Click here to enter text.

4. Paralytic drug:

Click here to enter text.

5. Dose:

Click here to enter text.

6. Method of ensuring appropriate analgesia during paralysis:

Click here to enter text.

D. Blood or Body Fluid Collection

1. Please fill out appropriate sections of the chart below:

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	cardiac	21gauge	Max 15mL	one	Terminal at sacing
Body Fluid Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

E. Injections, Gavage, & Other Substance Administration

1. Please fill out appropriate sections of the chart below:

	Compound	Location & Route of admin	Needle/ catheter /gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
Injection/ Infusion	Estradiol, dihydrotestosterone progesterone	Subcutaneous injection into the scruff (the loose skin over the interscapular area)	22 G X ½ in.	1.0 ml	Once per day	Max of 5 days	500 ug/kg, 5mg/kg, 200 ug/kg
Gavage	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. **Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:**

a. **Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.**
b. **Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).**

All hormones injected will be pharmaceutical grade.

F. Prolonged restraint with mechanical devices

Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks. Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.

1. For what procedure(s):
Click here to enter text.

2. Explain why non-restraint alternatives cannot be utilized:
Click here to enter text.

3. Restraint device(s):
Click here to enter text.

4. Duration of restraint:
Click here to enter text.

5. Frequency of observations during restraint/person responsible:
Click here to enter text.

6. Frequency and total number of restraints:
Click here to enter text.

7. Conditioning procedures:
Click here to enter text.

8. Steps to assure comfort and well-being:
Click here to enter text.

9. Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.

1. Describe methodology:
Subcutaneous administrations are made into the loose skin over the interscapular or inguinal area. Subcutaneous injections will be delivered by doing the following:

1. The animal will be weighed and the appropriate dosing volume will be determined.
2. The animal will be restrained. To restrain the rat we will: scruff the rat, grasping the skin over the shoulders with the thumb and forefinger. The animal will then be placed on a clean towel or sterile surface.
3. The needle will be inserted under the skin of the interscapular area tented by the thumb and forefinger and the injection will be delivered.
4. Return the animal to the cage and provide moist chow.

2. Expected model and/or clinical/pathological manifestations:
Offspring of our treated dams are expected to develop some genital abnormalities (females in the DHT treatment will have masculinized genitalia). Offspring of treated dams are

expected to develop some behavior abnormalities (reduced sociability, reduced spatial learning, altered reproductive behavior). The exposed dams are not expected to show any adverse effects.

3. Signs of pain/discomfort:

The signs of pain and discomfort include: depression, anorexia, labored respiration, increased aggression, periocular/nasal discharge, abnormal posture and immobility. If dams are injected correctly there should not be any signs of pain and discomfort from any of the exposure treatments. All animals, will be provided moist chow after each injection.

4. Frequency of observations:

Daily

5. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

If any of the above signs of distress are seen in individuals a veterinarian will be consulted. If the animal needs to be euthanized, isoflurane will be used with decapitation to insure death.

H. Treadmills/Swimming/Forced Exercise

1. Describe aversive stimulus (if used):

Click here to enter text.

2. Conditioning:

Animals will be trained to learn where the hidden platform is for the Morris water maze so that spatial learning can be assessed. No aversive stimuli will be used.

3. Safeguards to protect animal:

Animals will be gently guided to the platform by the observer if unable to find it. Animals will be removed from the water if swimming for more than 90 seconds.

4. Duration:

Animals will not be required to swim for more than 90 seconds.

5. Frequency:

Each animal will complete the Morris water maze one time. One water maze trial consists of 15 sub-trials. Including, three training trials (60 second each), and three experimental sub-trials (max 90 seconds) from each of the four cardinal directions (north, east, south, and west = 12).

6. Total number of sessions:

For the Morris water maze each animal will undergo 15 sub-trials with sufficient rest time in between each trial.

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

The animals may experience temporary stress due to the forced swimming task, but adverse effects are typically not expected during a water maze test. If an animal shows signs of excessive distress, they will be immediately removed from the water by the observer and will not be required to complete the test.

I. Projects Involving Food and Water Regulation or Dietary Manipulation

(Routine pre-surgical fasting not relevant for this section)

1. Food Regulation

a. Amount regulated and rationale:

Click here to enter text.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):

Click here to enter text.

c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

2. Fluid Regulation

a. Amount regulated and rationale:

Click here to enter text.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):

Click here to enter text.

c. Frequency of observation/parameters documented (body weight, hydration status, etc.):

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

3. **Dietary Manipulations**

a. **Compound supplemented/deleted and amount:**
Click here to enter text.

b. **Frequency and duration (hours for short term/week or month for long term):**
Click here to enter text.

c. **Frequency of observation/parameters documented:**
Click here to enter text.

d. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
Click here to enter text.

J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging

1. **Describe animal methodology:**
Click here to enter text.

2. **Duration of procedure:**
Click here to enter text.

3. **Frequency of observations during procedure:**
Click here to enter text.

4. **Frequency/total number of procedures:**
Click here to enter text.

5. **Method of transport to/from procedure area:**
Click here to enter text.

6. **Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
Click here to enter text.

7. **Please provide or attach appropriate permissions/procedures for animal use on human equipment:**
Click here to enter text.

K. Polyclonal Antibody Production

1. **Antigen/adjuvant used and justification for adjuvant choice:**
Click here to enter text.

2. **Needle size:**
   Click here to enter text.
3. **Route of injection:**
   Click here to enter text.
4. **Site of injection:**
   Click here to enter text.
5. **Volume of injection:**
   Click here to enter text.
6. **Total number of injection sites:**
   Click here to enter text.
7. **Frequency and total number of boosts:**
   Click here to enter text.
8. **What will be done to minimize pain/distress:**
   Click here to enter text.
9. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
   Click here to enter text.

L. Monoclonal Antibody Production

1. Describe methodology:
Click here to enter text.

2. Is pristane used: Choose an item.

Volume of pristane:
Click here to enter text.

3. Will ascites be generated: Choose an item.

i. **Criteria/signs that will dictate ascites harvest:**
   Click here to enter text.
ii. **Size of needle for taps:**
   Click here to enter text.
iii. **Total number of taps:**
   Click here to enter text.
iv. **How will animals be monitored/cared for following taps:**
   Click here to enter text.

4. What will be done to minimize pain/distress:
Click here to enter text.

5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

M. Temperature/Light/Environmental Manipulations

1. Describe manipulation(s):
Click here to enter text.

2. Duration:
Click here to enter text.

3. Intensity:
Click here to enter text.

4. Frequency:
Click here to enter text.

5. Frequency of observations/parameters documented:
Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

N. Behavioral Studies

1. Describe methodology/test(s) used:
Social Play. Social play will be tested using methods described by Himmler et. al (2013). A controlled environment that is designed to maximize the occurrence of play behavior will be used. Play behavior will be recorded in a "play box"—a large arena (36"L x 18"W x 16"H) with neutral bedding which will create a natural setting for playing. Pups will be weaned on PND 21 and separated into sex-specific cages with their sibling. Starting on PND 21, habituation to the play box will begin. On PND 21, play pairs will be placed in the play box for 30 minutes to allow habituation to occur. After 30 minutes of habituation, play pairs will be returned to their home cages. The play box will be emptied and sanitized using Quatricide PV-15. The next play pair will be placed inside and their first 30 minute habituation will begin. This process will be repeated again on PND 22 and PND 23. After the final habituation session on PND 23, play pairs will be housed singly for 24 hours. This isolation is a standard procedure, does not induce excessive stress, and is known to increase motivation for play behavior upon reuniting the play pair. On PND 24, each play partner will be marked on their dorsal side with a "+" or a "0" so that the trained observer can distinguish between individuals when reviewing the recording. Play pairs will be reunited in the play box on PND 24 and their behavior will be recorded for 30 minutes under red light. After the 30 minutes of recording are finished, play pairs will be returned together to a new cage and housed together until the next behavioral trial. The observer will use Jwatcher behavioral analysis program to code behaviors performed by each focal animal including non-aggressive play fighting, approach, chasing, sniffing and pouncing.

Open field test-The open field test will measure anxiety and hyperactivity, which will provide information regarding whether or not autism-like behavioral phenotypes are expressed. Open field behavior will be tested on PND 55-60. Each rat will be placed in the periphery of the open field and allowed to explore the apparatus for 20 minutes, with the experimenter out of the rat's sight. The behavior will be video recorded and analyzed using Jwatcher software. The distance travelled, the number of rears, and time spent in the central and peripheral regions will be recorded and measured per test session. The number of entries into the center, and resting time in each zone of the arena will also be recorded.

Sociability and preference for social novelty-At the same time (PND 55-60) but using different animals than used above in the open field test, sociability and preference for social novelty tests will be conducted to assess the presence or absence of autism-like behavioral phenotypes in the animals. A social behavior apparatus (36"L x 18"W x 16"H) with three sections will be used (illustrated by Figure 3; Moy et al. 2007). Procedures for the sociability and preference for social novelty will follow those described by Moy et al. (2007). The test rat will be placed in the middle chamber and allowed 10 minutes for exploration and habituation. The doors to the neighboring chambers will then be opened and each chamber will contain an empty wire cage. After 10 minutes, the test rat will be enclosed in the center compartment and an unfamiliar rat of the same sex (stranger 1) will be placed in the sectioned off side compartment of the testing apparatus. The location of the unfamiliar rat will be randomized to the left or right chamber per testing session. Once the unfamiliar rat is placed, the doors to the side compartments will be opened and the test rat will be

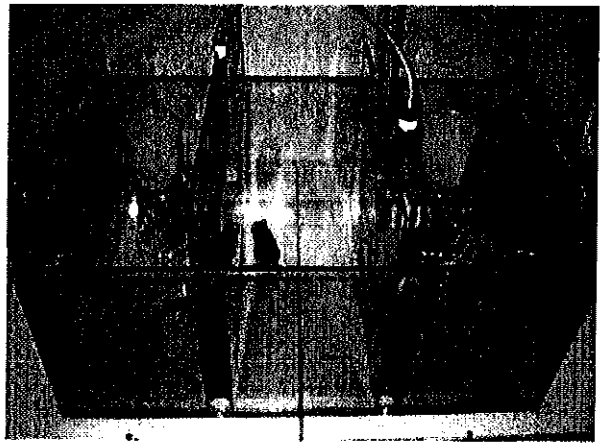

Figure 4. Social approach and sociability apparatus (Moy et al. 2007)

observed for another 10 minutes. All observations will be video recorded and analyzed to assess the amount of time the test rat spends with the unfamiliar rat or the empty chamber, as well as the number of entries into each side chamber. After the first 10 minutes pass, the test rat will again be temporarily isolated to the center chamber while a second unfamiliar of the same sex (stranger 2) rat is placed in the empty wire cage. The test rat will again be permitted to explore all areas of the testing apparatus. The test rat will be video recorded for another 10 minutes to assess how much time is spent investigating the now

familiar rat (stranger 1) versus the novel unfamiliar rat (stranger 2). The apparatus will be sanitized with Quatricide PV-15 after every trial.

Home cage behavior-In order to measure the extent to which autism-like behavioral phenotypes are expressed in the animals, home cage behavior will be monitored. Home cage behavior observations will be modeled after the methods described by Moy et al. (2007). Observations will take place at PND 61-64. Observations of same-treatment, same-sex siblings in their home cages will be made for 20 minutes. Nest shredding, nest building, sleeping in huddles, activity, non-aggressive play fighting, social approach and any aberrant behaviors will be measured. Female mounting behavior will be recorded as evidence for masculinized reproductive behavior.

Water maze test-The Morris water maze will be used to assess spatial learning abilities in the rat (Beatty 1984). Tests will be conducted between PND 110-120. A standard Morris water maze protocol will be followed to test the rats (Nunez 2008). The Morris water maze is a warmed pool of water that has a hidden, slightly submerged platform that the animals must navigate to using four visual cues that are located at each cardinal direction (north,

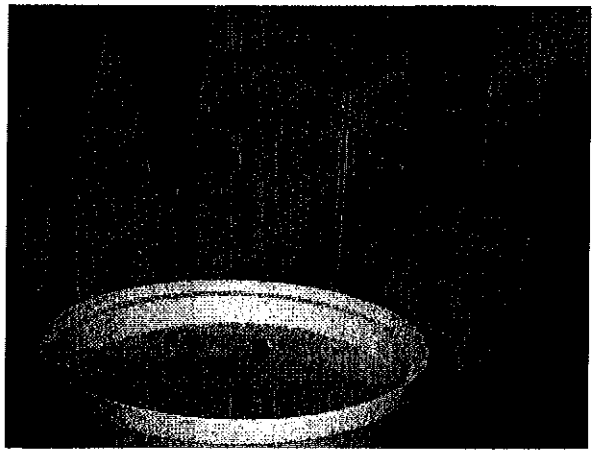

Figure 5. The Morris water maze (Moy et al 2007)

south, east and west). The animals will be pre-trained to learn where the hidden platform in the pool of water is. Each animal will undergo three 60 second training trials. If the animal is unable to find the platform, the observer will gently guide the animal to the platform using their hand. This will ensure that the animal learns where the platform is. After the learning trials are conducted for each animal, the testing trials will begin. Twelve testing trials will be completed per animal. This is to provide three data sets per starting point per individual. Each trial will consist of no longer than 90 seconds of swimming while the animal's trajectory is recorded by the video software. Animals will have opportunity to rest and dry off in between the 12 trials inside individual test boxes. Once the 12 trials are completed, animals will be dried off and returned to their cage.
The water in the maze is maintained at 25-27°C via a temperature-regulated pond heater. The water will be changed and the pool will be sanitized according to the number of days it

is used. If behavioral tests are run for 5 days, the pool will be cleaned after day 3. If behavioral tests are run for 10 days, the pool will be cleaned after day 5. After the pool is drained on the day of cleaning, it will dried and be sanitized with Quatricide PV-15, allowed to air dry again, rinsed with tap water then re-filled with tap water.
The test boxes in which the rats will be housed in between water maze trials will be sanitized after each rat completes all trials. The test boxes will be emptied of feces and sanitized with Quatricide PV-15 and then dried.

Sexual reproduction-Masculine sexual reproduction behaviors are programmed prenatally by the actions of estrogens and ER (K. McCoy, unpublished data). Measuring masculine reproductive behavior will indicate the extent to which ER programmed the brain prenatally. Reproductive behavior will be assessed between PND 110-120. Male and female rats will be paired with a sexually receptive (hormonally primed) female in his home cage. Behavior will be recorded for 20 minutes and a trained observer will score and quantify sexual behavior (e.g., mount latency, number of mounts, total time spend mounting).

Hormonal priming of non-exposed females will involve three injections. Two days before the female is paired with the male she will be injected with estradiol benzoate (10ug/kg). The following day she will be injected with another dose of estradiol benzoate (5ug/kg). The day of reproductive trials (four hours before the female is paired with male) she will be injected with progesterone (~10ug/kg).

2. Will conditioning occur? If so, describe:

Conditioning will occur for the Morris water maze prior to testing trials. The animals will be pre-trained to learn where the hidden platform in the pool of water is by being introduced to the hidden platform by the observer. Each animal will undergo three 60 second training trials. If the animal is unable to find the platform, the observer will gently guide the animal to the platform using their hand. This will ensure that the animal learns where the platform is.

3. If aversive stimulus used, frequency, intensity and duration:

N/A

4. Length of time in test apparatus/test situation: *(i.e., each test is ~10 mins)*

Play trials: 30 minutes
Open field: 20 minutes
Water maze: 15 trials lasting no longer than 90 seconds each
Reproductive trials: 20 minutes

5. Frequency of testing and duration of study: *(i.e., 5 tests/week for 6 months)*

Play trials: One test over one day
Open field: One test over one day
Water maze: One test consisting of fifteen trials over one day
Reproductive trials: One test over one day

6. Frequency of observation/monitoring during test:

Play trials: Continuous monitoring
Open field: Continuous monitoring
Water maze: Continuous monitoring
Reproductive trials: Continuous monitoring

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Play trials: There is no evidence that juveniles should be aggressive, however if aggression is observed the animals will be immediately separated by the observer.

Open field: Animals may experience mild stress associated with being exposed in the open field, but this stress should not have adverse effects.

Water maze: Animals will be monitored during all trials and removed from the water by the observer if they show signs of distress or struggling. There is a risk for hypothermia, but this risk is low as animals will not be placed in the water maze unless the water is within the range of 25-27°C .

Reproductive trials: Potential for aggressive interactions. If aggression is observed, animals will be separated by the observer. Aggression has never been observed by K. McCoy during similar mating trials.

O. Capture with Mechanical Devices/Traps/Nets

1. Description of capture device/method:

Click here to enter text.

2. Maximum time animal will be in capture device:

Click here to enter text.

3. Frequency of checking capture device:

Click here to enter text.

4. Methods to ensure well-being of animals in capture device:

Click here to enter text.

5. Methods to avoid non-target species capture:

Click here to enter text.

6. Method of transport to laboratory/field station/processing site and duration of transport:

Click here to enter text.

7. Methods to ensure animal well-being during transport:

Click here to enter text.

8. Expected mortality rates:

Click here to enter text.

9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

P. Manipulation of Wild-Caught Animals in the Field or Laboratory

1. Parameters to be measured/collected:
Click here to enter text.
2. Approximate time required for data collection per animal:
Click here to enter text.
3. Method of restraint for data collection:
Click here to enter text.
4. Methods to ensure animal well-being during processing:
Click here to enter text.
5. Disposition of animals post-processing:
Click here to enter text.
6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

Q. Wildlife Telemetry/Other Marking Methods

1. Describe methodology (including description of device):
Click here to enter text.
2. Will telemetry device/tags/etc. be removed? Choose an item. **If so, describe:**
Click here to enter text.
3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

R. Other Animal Manipulations

1. Describe methodology:
Click here to enter text.
2. Describe methods to ensure animal comfort and well-being:

Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

S. Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species.

1. Location of Surgery (Building & Room #):

Click here to enter text.

2. Type of Surgery (check all that are appropriate):

Click here to enter text.

☐ **Non-survival surgery (animals euthanized without regaining consciousness)**

☐ **Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**

☐ **Minor survival surgery**

☐ **Multiple survival surgery**

If yes, provide scientific justification for multiple survival surgical procedures:

Click here to enter text.

3. Describe the pre-op preparation of the animals:

a. **Food restricted for** Click here to enter text. **hours**
b. **Food restricted is not recommended for rodents and rabbits and must be justified:**

Click here to enter text.

c. **Water restricted for** Click here to enter text. **hours**

d. Water restriction is not recommended in any species for routine pre-op prep and be justified:

Click here to enter text.

4. Minimal sterile techniques will include (check all that apply):

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

☐ **Sterile instruments**

How will instruments be sterilized?

Click here to enter text.

If serial surgeries are done, how will instruments be sterilized between surgeries:

Click here to enter text.

☐ **Sterile gloves**
☐ **Mask**
☐ **Cap**
☐ **Sterile gown**
☐ **Sanitized operating area**
☐ **Clipping or plucking of hair or feathers**
☐ **Skin preparation with a sterilant such as betadine**
☐ **Practices to maintain sterility of instruments during surgery**
☐ **Non-survival (clean gloves, clean instruments, etc.)**

5. Describe all surgical procedures:

a. Skin incision size and site on the animal:

Click here to enter text.

b. Describe surgery in detail (include size of implant if applicable):

Click here to enter text.

c. Method of wound closure:

Click here to enter text.

i. Number of layers

Click here to enter text.

ii. Type of wound closure and suture pattern:

Click here to enter text.

iii. Suture type/size/wound clips/tissue glue:

Click here to enter text.

iv. Plan for removing of skin sutures/wound clip/etc:

Click here to enter text.

6. Anesthetic Protocol:

a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:

Click here to enter text.

b. Anesthesia/Analgesia For Surgical Procedures

	Agent	Dose (mg/kg or %)	Volume	Route	Frequency	Number of days administered
Pre-operative analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post-operative Analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):

Click here to enter text.

d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):

Click here to enter text.

e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:

Click here to enter text.

f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):

Click here to enter text.

g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:
Click here to enter text.

7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)
Click here to enter text.

a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:
Click here to enter text.

b. How frequently (times per day) will animals be monitored:
Click here to enter text.

c. How long post-operatively (days) will animals be monitored:
Click here to enter text.

8. Surgical Manipulations Affecting Animals

a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:
Click here to enter text.

b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):
Click here to enter text.

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

V. Euthanasia

Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.

A. Euthanasia Procedure. ***All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.***

1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:

Click here to enter text.

2. Inhalant Method

(if other, describe the agent and delivery method)

Click here to enter text.

3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)- Please provide the following:

a. Agent:

Rodent euthanasia mix or pentobarbital overdose solution

b. Dose or concentration:

390mg/ml, 100mg/kg IP

c. Route:

Rats will receive a left cardiac ventricular perfusion under a fume hood at Ragsdale Hall Room 9 (maintained by EH&S) within 10 days after water maze and sexual behavior assays. They will be euthanized with rodent euthanasia mix or pentobarbital overdose solution (390mg/ml, 100mg/kg IP) obtained from DCM.

B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):

Death (absence of reflexes, including corneal and palpebral reflexes, and glazing of eyes) will be confirmed. Bilateral incisions will be made to reveal the chest cavity and a left ventricular blood collection will be performed. Remaining blood will be flushed initially with saline (normally 0.9%), followed by brain tissue fixation with 4% paraformaldehyde.

C. Describe disposition of carcass following euthanasia:

Carcasses will be dissected, tissues preserved, and carcasses will be frozen and stored at -20C until they can be properly discarded.

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: e-mail **Date:** 1/31/14

Veterinarian: _____ **Date:** 2/19/14

IACUC Chair: _____ **Date:** 2/19/14

APPENDIX 1-HAZARDOUS AGENTS			
Principal Investigator: Krista McCoy	Campus Phone: 737-2730		Home Phone: 252-565-5780 Cell: 571-315-2884
IACUC Protocol Number: PENDING	Department: Biology		E-Mail: mccoyk@ecu.edu
Secondary Contact: Bevin Blake Department: Biology	Campus Phone: 440-465-7246	Home Phone:	E-Mail: Blakeb13@students.ecu.edu
Chemical Agents used: Estradiol, 5-alpha dihydrotestosterone		Radioisotopes used: None	
Biohazardous Agents used: None	Animal Biosafety Level: N/A	Infectious to humans? No	
PERSONAL PROTECTIVE EQUIPMENT REQUIRED:			
Route of Excretion: Urine and feces			
Precautions for Handling Live or Dead Animals: Nitrile gloves			
Animal Disposal: Nitrile gloves			
Bedding/Waste Disposal: Nitrile gloves, lab coat, eye protection			
Cage Decontamination: Nitrile gloves, lab coat, eye protection			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment:			
Initial Approval Safety/Subject Matter Expert Signature & Date _____			

Davenport, Janine

From: Bagley, Alana
Sent: Wednesday, February 19, 2014 10:30 AM
To: Davenport, Janine
Cc: McCoy, Krista Ann-Marie
Subject: AUP D300 Approval K. McCoy
Attachments: AUP D300 approval-02192014112503.pdf

Importance: High

Good Morning,

Attached are the approved safety documents for AUP D300. If you have any questions or concerns do not hesitate to contact me.

Thanks,

Alana B.

Alana E. Bagley, AOEE
EH&S Specialist
252-328-6166 (office)
252-737-1458 (fax)
www.ecu.edu/oehs

Laboratory Safety Plan for Progesterone injections

Alana Bagley 2-19-14

*Process	Progesterone injections
*Hazardous Chemical/ Chemical Class	Progesterone Hazards: Reproductive hazard, suspected carcinogen Target Organs: Reproductive (Male and Female), Liver, Nervous system
*Hazardous Equipment	Injection: Needle/Syringe
*Potential Hazards	Harmful by ingestion, inhalation and skin absorption. Considered a suspected carcinogen. May cause respiratory tract irritation. Possible reproductive hazard for men and women. Consult physician if pregnant or possibility of pregnancy. Chronic disorders may cause reproductive disorders.
*Personal Protective Equipment	Safety glasses/goggles; closed front or back lab coat, compatible gloves (nitrile), long pants and closed toed shoes.
*Engineering and Ventilation Controls	All handling and preparation should be completed in a certified chemical fume hood (Howell S112). Administration of injections will be in the biosafety cabinet in Ragsdale Annex. All extraneous equipment should be removed from the hood before work begins. All equipment required for dilutions will be placed in the hood prior to beginning work. All chemical containers can only be removed from fume hood if tightly capped and the exterior wet wiped. Before relocating to Ragsdale Annex ensure the chemical containers are securely closed and placed in an absorbent lined secondary container, preferably with a lid. When transporting to Ragsdale take the most direct route without interruptions. All sharps should be placed in a red biohazard sharps container. Do not recap sharps.
Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins	Prepare the stock solutions in the laboratory and store away from other chemical storage, preferably in a locked cabinet.
Special Use Procedures	Hands will be washed with a disinfectant soap after handling any chemical containers.
Special Handling and Storage Requirements	Keep container tightly closed in a dry, well-ventilated space. Store stock solutions separately from other chemicals, in a secure area. Store away from oxidizers. Keep away from ignition sources. Substance should be stored away from light. Avoid creating and breathing dust/aerosols from substance.
*Spill and Accident Procedures	Clean spills only if proper materials are available and if researcher is properly trained to do so. All other spills should be reported to EH&S for clean-up. Needed: absorbent materials, plastic bags, sealable container to hold contaminated cleanup materials, protective clothing, gloves, and safety glasses. For minor spills ventilate area. If spill occurs outside hood, cover liquid with absorbent material; and place in plastic bag. Spill materials should be picked up by EH&S Hazardous Waste Management.
*Waste Minimization Plan	Order only the amount needed.
*Hazardous Waste Disposal	Maintain all hazardous waste in closed containers with hazardous waste tag and contact EH&S for disposal. All sharps should be placed in a closed red sharps disposal container. Contact Prospective Health for pickups of biohazards and sharps.
Decontamination Procedures	Hood will be decontaminated, if necessary, by removing the paper liner and wiping the hood interior surfaces from top to bottom then back to front. (PPE as listed above must be worn during decontamination.)
Animal Care Precautions	Animal care workers should wear PPE including double gloves, lab coats and closed -toed shoes animal and waste removal.
*Chemical Procurement	Inventories kept by PI or laboratory manager. Minimum quantities necessary for the procedures confirmed by current funding.
*Revision Date	2-13-2014

APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator: Krista McCoy	Campus Phone: 737-2730		Home Phone: 252-565-5780
IACUC Protocol Number: D300	Department: Biology		EMail: mccoyk@ecu.edu
Secondary Contact: Bevin Blake Department: Biology	Campus Phone:	Home Phone: 440-465-7246	EMail: blakeb13@students.ecu.edu
Chemical Agents Used: Progesterone		Radioisotopes Used: N/A	
Biohazardous Agents Used:		Animal Biosafety Level:	Infectious to humans?
PERSONAL PROTECTIVE EQUIPMENT REQUIRED: STANDARD PERSONAL PROTECTIVE EQUIPMENT FOR DCM ANIMAL LAB TECHNICIANS.			
Route of Excretion: Urine			
Precautions for Handling Live or Dead Animals: Use standard precautions when handling alive or dead animals. Always don personal protective equipment when handling animals (dead or alive) and bedding.			
Animal Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Bedding / Waste Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Cage Decontamination: Normal cage washing.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: Always don personal protective equipment when handling animals (dead or alive) and bedding. Use provided biosafety cabinet in Ragsdale Annex when handling alive or dead animals, bedding and waste.			
Initial Approval Safety/Subject Matter Expert Signature & Date Alana Bagley 2-19-14			

Laboratory Safety Plan for Testosterone injections (Controlled Substance)

Alana Bagley 2-19-14

*Process	Testosterone injections
*Hazardous Chemical/ Chemical Class	Testosterone – Controlled Substance Hazards: Reproductive hazard, suspected carcinogen Target Organs: Reproductive (Male and Female), Liver, Nervous system
*Hazardous Equipment	Injection: Needle/Syringe
*Potential Hazards	Considered a carcinogen. Harmful by ingestion, inhalation and skin absorption. May cause respiratory tract irritation. Possible reproductive hazard for men and women. Consult physician if pregnant or possibility of pregnancy. Chronic disorders may cause reproductive disorders.
*Personal Protective Equipment	Safety glasses/goggles; closed front or back lab coat, compatible gloves (nitrile), long pants and closed toed shoes.
*Engineering and Ventilation Controls	All handling and preparation should be completed in a certified chemical fume hood (Howell S112). Administration of injections will be in the biosafety cabinet in Ragsdale Annex. All extraneous equipment should be removed from the hood before work begins. All equipment required for dilutions will be placed in the hood prior to beginning work. All chemical containers can only be removed from fume hood if tightly capped and the exterior wet wiped. Before relocating to Ragsdale Annex ensure the chemical containers are securely closed and placed in an absorbent lined secondary container, preferably with a lid. When transporting to Ragsdale take the most direct route without interruptions. All sharps should be placed in a red biohazard sharps container. Do not recap sharps.
Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins	Prepare the stock solutions in the laboratory and store away from other chemical storage, in a locked cabinet according to federal regulations for controlled substances.
Special Use Procedures	Hands will be washed with a disinfectant soap after handling any chemical containers.
Special Handling and Storage Requirements	Keep container tightly closed in a dry, well-ventilated space. Store stock solutions separately from other chemicals, in a locked and secure area according to federal regulations for controlled substances. Store away from oxidizers. Keep away from ignition sources. Substance should be stored away from light. Avoid creating and breathing dust/aerosols from substance.
*Spill and Accident Procedures	Clean spills only if proper materials are available and if researcher is properly trained to do so. All other spills should be reported to EH&S for clean-up. Needed: absorbent materials, plastic bags, sealable container to hold contaminated cleanup materials, protective clothing, gloves, and safety glasses. For minor spills ventilate area. If spill occurs outside hood, cover liquid with absorbent material; and place in plastic bag. Spill materials should be picked up by EH&S Hazardous Waste Management.
*Waste Minimization Plan	Order only the amount needed.
*Hazardous Waste Disposal	Maintain all hazardous waste in closed containers with hazardous waste tag and contact EH&S for disposal. All sharps should be placed in a closed red sharps disposal container. Contact Prospective Health for pickups of biohazards and sharps.
Decontamination Procedures	Hood will be decontaminated, if necessary, by removing the paper liner and wiping the hood interior surfaces from top to bottom then back to front. (PPE as listed above must be worn during decontamination.)
Animal Care Precautions	Animal care workers should wear PPE including double gloves, lab coats and closed -toed shoes animal and waste removal.
*Chemical Procurement	Inventories kept by PI or laboratory manager. Minimum quantities necessary for the procedures confirmed by current funding.
*Revision Date	2-13-2014

APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator: Krista McCoy	Campus Phone: 737-2730		Home Phone: 252-565-5780
IACUC Protocol Number: D300	Department: Biology		EMail: mccoyk@ecu.edu
Secondary Contact: Bevin Blake Department: Biology	Campus Phone:	Home Phone: 440-465-7246	EMail: blakeb13@students.ecu.edu
Chemical Agents Used: Testosterone	Radioisotopes Used: N/A		
Biohazardous Agents Used:	Animal Biosafety Level:	Infectious to humans?	
PERSONAL PROTECTIVE EQUIPMENT REQUIRED: STANDARD PERSONAL PROTECTIVE EQUIPMENT FOR DCM ANIMAL LAB TECHNICIANS.			
Route of Excretion: Testosterone metabolites are excreted in feces and urine			
Precautions for Handling Live or Dead Animals: Use standard precautions when handling alive or dead animals. Always don personal protective equipment when handling animals (dead or alive) and bedding.			
Animal Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Bedding / Waste Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Cage Decontamination: Normal cage washing.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: Always don personal protective equipment when handling animals (dead or alive) and bedding. Use provided biosafety cabinet in Ragsdale Annex when handling alive or dead animals, bedding and waste.			
Initial Approval Safety/Subject Matter Expert Signature & Date Alana Bagley 2-19-14			

Laboratory Safety Plan for Estradiol injections

Alana Bagley 2-19-14

*Process	Estradiol injections
*Hazardous Chemical/ Chemical Class	Estradiol Hazards: Reproductive hazard, suspected carcinogen Target Organs: Reproductive (Male and Female), Liver, Nervous system
*Hazardous Equipment	Injection: Needle/Syringe
*Potential Hazards	Harmful by ingestion, inhalation and skin absorption. Considered a suspected carcinogen. May cause respiratory tract irritation. Possible reproductive hazard for men and women. Consult physician if pregnant or possibility of pregnancy. Chronic disorders may cause reproductive disorders.
*Personal Protective Equipment	Safety glasses/goggles; closed front or back lab coat, compatible gloves (nitrile), long pants and closed toed shoes.
*Engineering and Ventilation Controls	All handling and preparation should be completed in a certified chemical fume hood (Howell S112). Administration of injections will be in the biosafety cabinet in Ragsdale Annex. All extraneous equipment should be removed from the hood before work begins. All equipment required for dilutions will be placed in the hood prior to beginning work. All chemical containers can only be removed from fume hood if tightly capped and the exterior wet wiped. Before relocating to Ragsdale Annex ensure the chemical containers are securely closed and placed in an absorbent lined secondary container, preferably with a lid. When transporting to Ragsdale take the most direct route without interruptions. All sharps should be placed in a red biohazard sharps container. Do not recap sharps.
Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins	Prepare the stock solutions in the laboratory and store away from other chemical storage, in a secure area.
Special Use Procedures	Hands will be washed with a disinfectant soap after handling any chemical containers.
Special Handling and Storage Requirements	Keep container tightly closed in a dry, well-ventilated space. Store stock solutions separately from other chemicals, in a secure area. Store away from oxidizers. Keep away from ignition sources. Substance should be stored away from light. Avoid creating and breathing dust/aerosols from substance.
*Spill and Accident Procedures	Clean spills only if proper materials are available and if researcher is properly trained to do so. All other spills should be reported to EH&S for clean-up. Needed: absorbent materials, plastic bags, sealable container to hold contaminated cleanup materials, protective clothing, gloves, and safety glasses. For minor spills ventilate area. If spill occurs outside hood, cover liquid with absorbent material; and place in plastic bag. Spill materials should be picked up by EH&S Hazardous Waste Management.
*Waste Minimization Plan	Order only the amount needed.
*Hazardous Waste Disposal	Maintain all hazardous waste in closed containers with hazardous waste tag and contact EH&S for disposal. All sharps should be placed in a closed red sharps disposal container. Contact Prospective Health for pickups of biohazards and sharps.
Decontamination Procedures	Hood will be decontaminated, if necessary, by removing the paper liner and wiping the hood interior surfaces from top to bottom then back to front. (PPE as listed above must be worn during decontamination.)
Animal Care Precautions	Animal care workers should wear PPE including double gloves, lab coats and closed -toed shoes animal and waste removal.
*Chemical Procurement	Inventories kept by PI or laboratory manager. Minimum quantities necessary for the procedures confirmed by current funding.
*Revision Date	2-13-2014

APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator: Krista McCoy	Campus Phone: 737-2730		Home Phone: 252-565-5780
IACUC Protocol Number: D300	Department: Biology		EMail: mccoyk@ecu.edu
Secondary Contact: Bevin Blake Department: Biology	Campus Phone:	Home Phone: 440-465-7246	EMail: blakeb13@students.ecu.edu
Chemical Agents Used: Estradiol	Radioisotopes Used: N/A		
Biohazardous Agents Used:	Animal Biosafety Level:	Infectious to humans?	
PERSONAL PROTECTIVE EQUIPMENT REQUIRED: STANDARD PERSONAL PROTECTIVE EQUIPMENT FOR DCM ANIMAL LAB TECHNICIANS.			
Route of Excretion: Urine			
Precautions for Handling Live or Dead Animals: Use standard precautions when handling alive or dead animals. Always don personal protective equipment when handling animals (dead or alive) and bedding.			
Animal Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Bedding / Waste Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Cage Decontamination: Normal cage washing.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: Always don personal protective equipment when handling animals (dead or alive) and bedding. Use provided biosafety cabinet in Ragsdale Annex when handling alive or dead animals, bedding and waste.			
Initial Approval Safety/Subject Matter Expert Signature & Date Diana Bagley 2-19-14			