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| IACUC Use Only | |
| Date: 5/7/2011 | IACUC Protocol No.: 11-1194R |

**ANIMAL USE PROTOCOL**

**ARIZONA STATE UNIVERSITY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE**

**(revised March 2010)**

Please read “Instructions for Completing the Animal Use Protocol” before completing. **Upon approval, this protocol will become a public record so please follow instructions carefully.**

**PROJECT/PROGRAM TITLE**: Sex-specific Programming of the HPA Axis by Glucocorticoids

**SPECIES REQUESTED**: Rat (*Rattus norvegicus*)

**I.** **PERSONNEL INFORMATION**

A. A single member of the university faculty and/or Principal Investigator (PI) is considered the responsible individual.

|  |  |  |  |
| --- | --- | --- | --- |
| NAME: | Jane Doe, Ph.D. | TITLE: | Professor |
| AFFILIATION: | School of Life Sciences | Office Phone # | 480-555-1234 |
| Cell Phone #: | 602-555-2468 | Dept. Phone #: | 480-555-4321 |
| Fax # | 480-555-1111 | E-Mail: | Jane.Doe@asu.edu |

B. Additional contact, if any, for IACUC business

|  |  |  |  |
| --- | --- | --- | --- |
| NAME: | John Buck | TITLE: | Research A**s**sociate |
| AFFILIATION: | School of Life Sciences | Office Phone # | 480-555-3579 |
| Cell Phone #: | 602-555-8642 | Dept. Phone #: | 480-555-4321 |
| Fax # | 480-555-1111 | E-Mail: | John.Buck@asu.edu |

C. Protocol Type

Non-funded research

Grant / Contract (Also submit grant proposal with this protocol)

|  |  |  |  |
| --- | --- | --- | --- |
| Granting Agency: | NIH | Deadline: | none |
| Proposal Title: | Sex Differences in Depression: A Translational Approach | | |
| Proposal Number: | P01 MM1111111 | | |
| Co-Investigator(s): | none | | |

Teaching

Course Title, Schedule:

1. Protocol Status:

New

Renewal—Previous Protocol #:

Revision—Previous Protocol #:

1. List all persons involved in this protocol. The first person listed should be the PI.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Name | Title | Role in Protocol (What procedures will each person be doing?) | Species with which individual will have direct contact (“all” or list species)\* | IACUC  USE ONLY Training  (mm/yy) |
| Jane Doe | Professor | PI, all procedures: surgery, injections, blood collection, ear-tagging, behaviors, euthanasia | rat |  |
| John Buck | Research Associate | all procedures: surgery, injections, blood collection, ear-tagging, behaviors, euthanasia | rat |  |
| Susan Elk | Postdoctoral Fellow | all procedures: surgery, injections, blood collection, ear-tagging, behaviors, euthanasia | rat |  |
| Faye Fawn | Undergrad student (SOLUR) | injections, behaviors | rat |  |
| Billy Moose | Lab. Assistant | injections, behaviors | rat |  |
| Mark Deer | Lab. Assistant | injections, behaviors | rat |  |

For each individual, describe the individual’s training and years experience with all listed species and procedures:

Jane Doe, Ph.D.: PI, over 25 years of experience working with rats and mice: small animal surgery (gonadectomy, cannulation), injections, euthanasia, breeding, and behavior testing.

John Buck, Ph.D.: Research Associate: Over 10 years of experience working with rats and mice: small animal surgery (gonadectomy, cannulation) injections, euthanasia, breeding, and behavior testing.

Susan Elk, Ph.D.: Postdoctoral Fellow, Over 5 years of experience working with mice: gonadectomy, cannulation, injections, euthanasia; 1 year experience: behavior testing.

Faye Fawn: Laboratory Assistant (SOLUR program), 1 year of experience working with mice: injections, euthanasia, and behavior testing.

Billy Moose: Laboratory Assistant (undergraduate), 2 years of experience working with mice: gonadectomy, injections, euthanasia, and behavior testing.

Mark Deer: Laboratory Assistant (undergraduate), 1 year of experience working with rats: injections, euthanasia, and behavior testing

\* The answer provided in this column dictates which Level II species-specific IACUC training modules are required for each individual. An individual only needs to complete Level II certification for those species with which he or she will directly work.

**Note: ASU requires that all personnel engaged in animal research or teaching be qualified through training or experience in order to conduct the work humanely. The IACUC requires the successful completion and renewal of Level I – The Humane Care and Use of Laboratory Animals as well as Level II species-specific training at least once every 3 years. A link to the individual training modules is available on the IACUC ASU homepage at:** [**http://hazel.forest.net/latanet/client/asu/introduction.htm**](http://hazel.forest.net/latanet/client/asu/introduction.htm)**.**

1. Have all personnel on this protocol completed the required IACUC Level I and Species-Specific Level II Training Modules as well as the Occupational Health and Safety Program Health Surveillance Questionnaire? The Training Modules and the Health Surveillance Questionnaire (HSQ) can be found at <http://researchintegrity.asu.edu/iacuc/training/exams.htm>

Yes. Proceed to section B.

No. List the individuals who are not in compliance and identify their deficiencies with an “X” in the appropriate columns:

|  |  |  |  |
| --- | --- | --- | --- |
| **Name** | **Training Modules** | | **HSQ**  **Clearance** |
| **Level I** | **Level II** |
|  |  |  |  |
|  |  |  |  |
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|  |  |  |  |

1. Describe any non-routine measures such as special vaccines or personal protective equipment that is required for animal and/ or human safety:

None.

H. Do you plan to use Department of Animal Care & Technologies (DACT) personnel and resources? If yes, for what facilities and procedures? (If this use is new or an expansion of previous use, please contact the DACT well in advance of need). Yes, DACT personnel will provide husbandry and perform daily checks of the animals.

**II. Project Description and Program Requirements**

The University Animal Care and Use Committee (IACUC) is composed of both active animal users and lay persons. Regardless of background, each member has one vote, and it is therefore particularly important that the language of the application be understood by all. This applies to all sections of the application, but it is especially important that the goals and justifications of the proposed research be spelled out in the clearest possible terms. NOTE: Upon approval, this protocol will become a public record, so please do not disclose proprietary information such as home telephone number and address.

1. Please provide a brief (300 words or less) synopsis in **laymen terms** of proposed research.

This project will use a rodent model to test a series of hypotheses concerning sexual differentiation in the functioning of organs involved in the stress response. Thes results, if extrapolated to humans, may underlie the presence of sex differences in the prevalence of major depressive disorders. Glucocorticoid hormones from the adrenal gland are a major component of the stress response. Exposure to these hormones near the time of birth in rodents can increase the number of dying cells within a part of the brain’s hypothalamus in female, but not male, newborns. This treatment also increases the expression of some genes involved in programmed cell death in the newborn brain. This raises the possibility that exposure to glucocorticoids near the time of birth can permanently change the hormonal response to stress in adult animals by altering the numbers of specific neuron populations in specific brain areas. Alternatively, there may be programming of gene expression as a result of newborn glucocorticoid exposure that is due to altered methylation of DNA. Studies in this project will test two hypotheses regarding the fetal programming of adult stress responsiveness: 1) Fetal or newborn exposure to stress causes permanent changes in the function of the adult hormonal response to stress by either increasing the incidence of death in a particular type of neuron in the brain or by causing permanent changes in gene expression in the brain’s hypothalamus through DNA methylation, and 2) There are sex-specific affects of fetal/newborn glucocorticoid exposure on the developing hypothalamus as a result of molecular interactions between glucocorticoid and estrogen receptors. These studies are important in determining the fetal insults and their underlying mechanisms that make some individuals more susceptible to stressful life events during adulthood.

B. PLANNED USE OF ANIMALS. Begin with a clear statement of purpose and briefly provide background information and references to previous work. Include a clear description of the experimental design for all animal experiments planned and explain why the experiments must be performed. **It is critical that for each procedure you provide a detailed sequence of events that effectively describes what happens to the animals.** Flow charts, diagrams or tables are strongly recommended for complicated experimental designs. Please state how the research is expected to benefit the human community, the animal community, and/or society as a whole. (Details regarding surgical procedures and drug treatments are not necessary, as they will be addressed later.)

This project examines the effects of perinatal glucocorticoid exposure and interaction with gonadal steroid hormones on cell death mechanisms and epigenetic influences on gene expression in brain regions important for the control of the hypothalamo-pituitary axis (HPA), which regulated the stress repsonse. The overarching goal of this project is to identify some of the long-term consequences of fetal/neonatal glucocorticoid hormone exposure on the developing HPA axis and determine how these changes occur in a sex specific fashion to impact hormonal and behavioral stress reactivity in adulthood. Using rodent models, it is established that prenatal stress affects the developing brain, to the extent that these animals are hyper-responsive to stress and show enhanced anxiety and depressive-like behaviors during adulthood. Of interest for this project is the fact that females are more susceptible to these insults than are males. A causal factor appears to be the exposure of the developing brain to glucocorticoids. This indicates that glucocorticoid hormones initiate an abnormal developmental program of the neural components regulating the HPA axis resulting in aberrant responses during adulthood.

In this project, we will determine some of the cellular mechanisms that are responsible for programming the memory of early glucocorticoid exposure to adult stress reactivity. Prenatal stress or perinatal glucocorticoid exposure will increase the number of dying cells in the paraventricular nucleus (PVN) of female but not male offspring (preliminary data). This time period is analogous to mid gestation in humans. Thus, developmental exposure to glucocorticoids may cause permanent changes in adult HPA axis reactivity by altering neurotrophic support for neurons in brain areas involved in regulating stress reactivity and stress related behaviors.

The studies described in this project will test two hypotheses in a rat model. 1) Developmental exposure to glucocorticoids causes permanent changes in the function of the HPA axis by increasing the incidence of cell death in a select neuronal phenotype in brain areas controlling hormonal and behavioral responses to stress or by causing permanent alterations in gene expression in neurons in these same brain areas through DNA methylation events. 2) There are sex-specific affects of perinatal glucocorticoid exposure on the developing HPA axis as a result of the perinatal steroid hormone millieu.

In some studies, we will utilize behavior tests to estimate the degree of anxiety in adult offspring of glucocorticoid treated dams. These tests are outlined below:

Test #1: Open field test. This is a non-invasive behavioral test. This test will consist of a 100 cm x 100 cm square black plexiglass box with 40 cm walls. Lines are painted on the floor to demarcate 25 20-cm squares. The box is placed in an isolated room and elevated approximately 30 cm. from the floor. It is lit from above by a 25 watt lightbulb located approximately 1 meter above the surface. The animal is placed into the center of the box and its behavior is monitored remotely by a videocamera. Measurements will be taken of the amount of total activity, time spent in certain regions of the grid, time spent with novel objects placed into the arena, time spent sniffing the air above, and time spent grooming. Animals will be monitored for 20-minutes and then replaced into their home cage. After each animal is tested, the box is cleaned with a 70% ethanol/acetic acid mixture.

Test #2: Light:Dark Transition. This test uses a Plexiglas box with 2 compartments separated by a black Plexiglas wall with a 7 cm by 10 cm wide opening to allow transit between the compartments. One side of the compartment is made of the same black Plexiglas and is 38 cm, 38 cm and 30 cm long and enclosed at the top. The opposite side is made of clear Plexiglas and has the same dimensions except it is 45 cm long and is open at the top. The animals will be placed in the center of the clear compartment, facing away from the door to the dark compartment. Behavior will be monitored remotely for 15 minutes by a videocamera. Measurements are time before entering the closed compartment, number of compartment entries, and total time spent in each compartment.

Test #3: Elevated plus maze. This test uses a maze consisting of 4 Plexiglas arms each beign 60 cm long and 14 cm wide radiating out from a center platform that is 30 cm square. The center platform and 2 arms that are opposite each other will be made from Plexiglas and have no walls. The other 2 arms will be made of black Plexiglas and have walls that measure 13 cm high. Animals will be placed at the center of the middle platform and be observed for 10 minutes and the amount of time in each arm, number of entries into each arm, and amount of time spent in the center platform will all be measured.

Test 4: Forced Swim Test: The forced swim test is an assessment of immobility level, experimentally analogous to depressed mood, a primary symptom of human depression. Clinically active anti-depressants have been found to delay the onset of the first phase of immobility and to reduce the total time of relative immobility in this animal model (Borsini et al, 1988; Kirby and Lucki, 1997; Porsolt, et al., 2000). Animals are placed in glass cylinders (height = 40 cm, dia = 18 cm) containing tap water maintained at 23 C. Animals are initially trained for the Forced Swim Test by placing them in the water for 10 min. Animals are tested the following day by placing them in the water for 5 minutes. All tests are videotaped to prevent observer interference, however, in all instances the investigator is monitoring the animal’s behavior from the adjacent room and can immediately enter if a problem arises (e.g., the animal sinks without an effort to swim). In the Forced Swim Test, the animals can exhibit a number of behaviors, all of which have been characterized in the literature as being a reuslt of different neural components. These behaviors can be grouped into three main types: struggling, swimming, and floating. In the case of struggling, because the swimming chamber is made of glass or clear Plexiglas, the animal can see out and will sometimes try to climb the sides of the chamber - this is classified as climbing. In other cases, the animal will dive toward the bottom in an attempt to identify a way out. Both of these behaviors are classified as "active coping" as the animal is actively trying to find a way out of the stressful situation. In contrast, floating is considered a "learned helplessness" response - the animal has given up, and this is considered a despair or depressive-like behavior. Thus, we will record the time climbing, struggling, diving, active swimming, or immobile over the course of 5 min. Pharmacologically, antidepressant drugs work to increase time struggling and swimming and decrease floating time in the Forced Swim Test. After each test, the animal is removed and dried thoroughly before being returned to its home cage. A heating pad is placed under ½ of the cage until the animal is completely dry.

Test 5: Tail Suspension Test: A complement to the Forced Swim Test is the Tail Suspension Test (TST). The TST was developed as an alternative to the forced swim test, and “behavioral despair” is imposed by hanging the animal from its tail for a period of 5 minutes (Porsolt 1987). The animal is suspended by taping the tail to a polyethylene tube.The tail suspension test is similar in theory to the Forced Swim Test in that animals subjected to the short-term, inescapable stress of being suspended by their tail will develop an immobile posture (absence of initiated movements, including passive swaying) as a "learned helplessness" response. The durations of immobility and struggling are the parameters measured. Antidepressant drugs will act to increase struggling and decrease immobility. The animals are suspended about 10 cm from the table top, so that they can see the "floor" and actively attempt to reach it. If a rat tries to climb its tail, or spins , it is prevented from doing so by the investigator, and if it persists, it is removed from the test. Each rat is given only one trial lasting for 5 minutes. In the unlikely event that an animal would tear the tape (we have never had one do this), it would only fall a very short distance (~10 cm). An investigator is always monitoring these animals during the test. The number of struggling events and time to achieve immobility (helplessness) is determined. The main advantage of this system is its simplicity. Although the TST and Forced Swim Test are similar, they do not always correlate. For example, Chan et al (2006), when examining knockout of brain-derived neurotrophic factor (BDNF) targeted to the fetal period, showed depression like phenotype in the TST, but not the forced swim test. Thus, performing both tests adds experimental rigor to our design.

**Experiment 1** Can sex differences in glucocorticoid-mediated cell death be reversed by changing the gonadal steroid hormone environment

Our studies thus far have demonstrated that perinatal glucocorticoid exposure increases cell death in the hippocampus in a sex specific fashion (females > males). We have further characterized gene expression changes that may correlate (for example: BDNF and its receptor, TrkB). For these studies, timed pregnant dams (n = 5 / treatment) will be injected with dexamethasone (DEX, 0.2 mg/kg BW SQ) and/or testosterone propionate (TP, 2 mg/kg BW; SQ) once daily (in the a.m.) for four consecutive days, at gestational day (GD)-17,18 ,19, and 20. Safflower oil will be used as the vehicle for the drugs as well as serve as the control injection for those animals not receiving one or both drugs. Thus, there will be four treatment groups: DEX/oil, DEX/TP, oil/TP and oil/oil. This perinatal TP treatment regimen was chosen because it has been previously used to masculinize the serotonin and tyrosine hydroxylase fiber distributions and sexually dimorphic cytoarchitectonic features of the medial preoptic nucleus (Simerly et al., 1985a, Simerly et al 1985b). Male and female neonates from each treatment group (n=10 neonates/sex/group) will be sacrificed on the day of birth because our preliminary data show that this time point yields the greatest sex difference in activated Caspase-3 immunoreactivity (a marker for apoptotic cells). Two animals of each sex per litter will be selected for each treatment to prevent inter-litter variation from biasing the data. Brain tissue will be harvested from neonates after they have been intracardially perfused with saline followed by 4% neutral buffered paraformaldehyde.

Summary for Experiment 1:

DEX Veh. TP DEX + TP

P0 males 10 10 10 10

P0 females 10 10 10 10

Two offspring of each sex from each of 20 dams (5 dams/treatment) will be euthanized on the day of birth for this experiment. Thus there will be 80 experimental neonates (2 offspring/sex X 2 sexes X 5 dams/treatment X 4 treatments). The 20 litters (5 dams/treatment X 4 treatments) from which these offspring will originate will likely average 12 pups/litter = 240 total neonates. These 240 offspring will be split between Experiment 1 (80 animals) and Experiment 2 (80 animals, see below). The excess 80 offspring will be euthanized at birth. As with all experiments, the dams are euthanized after pups are harvested. All euthanized Category B animals are made available to other PIs for research subjects or food for rodent-consuming animals (e.g., snakes).

**Experiment. 2.:** Are perinatal glucocorticoid-dependent alterations in gene expression sex-specific, and can they be altered by the gonadal steroid hormone environement?

Our preliminary studies have identified a number of genes that are altered in the hypothalamus of prenatal DEX-exposed male and female rats. Therefore, this experiment will be performed to determine if those genes in which we have detected sex differences in the response to DEX treatment are responsive to testosterone treatment. The approach taken will be to determine if concomitant treatment with testosterone can prevent the effect of glucocorticoids that has been noted in female offspring. Neonates to be used in this experiment will come from the littlers produced for Experiment 1. These offspring will be euthanized on the day of birth. Animals will be intracardially perfused with RNAse-free saline followed by buffered 4% paraformaldehyde.

Summary for Experiment 2:

DEX Veh. TP DEX + TP

P0 males 10 10 10 10

P0 females 10 10 10 10

This experiment will use the same 20 dams and their litters from Experiment 1. Two offspring of each sex from each dam will be euthanized on the day of birth for this experiment (2 offspring/sex X 2 sexes X 4 treatment = 80 offspring). Thus, of the estimated 240 offspring produced in these liters, 160 will be used in Experiments 1 and 2, and approximately 80 will not be used in an experiment.

**Total for Experiments 1 and 2: 20 timed-pregnant females + 240 offspring (160 to be used for experiments and 80 to be culled) = 260 total (80 Category B, 180 Category C)**

**Experiment. 3** Does prenatal testosterone treatment prevent the actions of prenatal glucocorticoid exposure on the adult female HPA reactivity and depressive-like behaviors?

Timed pregnant dams will be treated as described in Experiment 1 (i.e., four overall treatment groups: DEX/oil, DEX/TP, oil/TP and oil/oil once dailyfor four consecutive days, beginning at GD-17). Males and females from each of the four groups will be allowed to grow to adulthood, prior to being tested for HPA response to restraint stress and depressive-like behaviors. Offspring will be maintained in mixed-sex litters of 5 females and 5 males. All animals are weaned at 23 days of age, separated by sex, and group housed 2/cage according to treatment group.

At 60 days of age, the 128 experimental offspirng (see below) will be gonadectomized (see Appendix 2) to remove circulating gonadal hormone levels. We have chosen to gonadectomize adult animals in these studies in an attempt to remove variance and allow us to best interpret the results of the tests. For example, treatment of neonatal females with even small amounts of testosterone will defeminize the reproductive neuroendocrine axis and as a result they will not ovulate in adulthood, whereas those treated with oil will show regular 4 day estrous cycles (Handa et al., 1985a,b). Since fluctuations in estrogen can influence the HPA response to stress (Burgess and Handa, 1992, Handa et al., 1994) as well as depressive-like behaviors (Estrada-Camarena et al., 2003; Walf et al, 2004), it stands to reason that this confounding factor should be removed. Additionally, prenatal stress has been reported to reduce circulating testosterone levels in adult males (Gerardin et al. 2005), and androgens have also been shown to influence behaviors in the Forced Swim Test (Frye and Wawrzycki, 2003). Thus for accurate comparisons across sex and to truly determine the effects of neonatal testosterone on adult stress responses, the behavioral evaluations should take place in adult gonadectomized animals.

One week following gonadectomy, animals will be subjected to a battery of tests to examine depressive like behaviors. These include the forced swim test, the tail suspension test, the light:dark test, the open field test and the elevated plus maze (see descriptions of behavior tests above). All tests are non-invasive. Individual animals will be tested in all five tests, but in random order so that test order does not influence performance in any particular test. Two days will be allotted between each test to minimize any carryover. Three days after the final test, animals will be subjected to a restraint stress as previously described (Lund et al., 2006). Animals will be placed in a Plexiglas restraint tube for 15 min. At the end of this time, animals will be rapidly decapitated and trunk blood will be collected for the determination of plasma levels of corticosterone and ACTH using radioimmunoassay. Unstressed controls will be sacrificed immediately upon removal from their home cage. For this study, we will include 16 animals in each group for behavior testing. This will allow sufficient numbers of animals for the final restraint stress test (8 stressed, 8 unstressed controls per experimental group). As above, to control for any litter effects. We will use only two males and two females from each litter. Thus, we will need 8 dams per treatment group (32 dams total) to produce 16 males and 16 females experimental offspring in each of the four treatments. Data from behavior tests will be analyzed by multi-way ANOVA.

As above, litter size will likely average 12/litter, but extra offspring above 10/litter will be euthanized on the day of birth. Litters are maintained at 10 pups (mixed sex) per litter until weaning in order to control for suckling competition and accommodate any potential early mortality. At weaning, 2 males and 2 females will be randomly selected from each litter to continue on in the study (2 individuals/sex X 2 sexes X 8 females/treatment X 4 treatments = 128 adult offspring). Any unused weanlings will be euthanized. All euthanized animals are made available to other PIs for research subjects or food for rodent-consuming animals (e.g., snakes).

Summary for Expeirment 3:

DEX Veh. TP DEX + TP

P60 males 16 16 16 16

P60 females 16 16 16 16

**Total for Experiment 3: 32 timed pregnant dams + 384 offspring (128 to be used for the experiment and 256 to be culled at birth or weaning) = 416 total (256 Category B, 32 Category C, 128 Category D)**

Summary of animals to be used under this protocol:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Experiment | Catgory B | Category C | Category D | Total |
| Experiment 1 | 80 | 100 |  | 180 |
| Experiment 2 |  | 80 |  | 80 |
| Experiment 3 | 256 | 32 | 128 | 416 |
| Total | 336 | 212 | 128 | 676 |

1. RATIONALE FOR INVOLVING ANIMALS AND THE APPROPRIATENESS OF THE **SPECIES AND NUMBER** USED. Keeping in mind the principles of the “3 R’s” (Refinement, Reduction, and Replacement answer the following:
   1. Why must live vertebrates be used in this study?

These studies examine the consequences of prenatal exposure to glucocorticoids on the development of brain structure and function. Currently there is not enough information known about neural development to accurately model the intricacies of any brain region, let alone the influence of environmental stress or hormones. Consequently, these studies can only be performed *in vivo* in a species that has a typical vertebrate brain structure.

* 1. Why are you using the requested species rather than other species?

The rat has been used in all of our previous studies on this topic which form the background for our studies. This species is particularly well suited for our endocrine and behavioral manipulations since there is extensive literature characterizing the response to physiological and psychological stressors.

* 1. What is the rationale supporting the numbers of animals proposed? Typically, a power analysis should be performed to support the proposed sample sizes. A table depicting the number of animals to be used is required.

We have performed power analysis to identify the minimum number of animals required in each of these studies. We have utilized SigmaStat 6.1 software. Tables for each experiment are shown in section B above.

Expt. 1: Analysis by ANOVA: a = 0.05, no. of groups = 8, minimal detectable = 1.0, SD = 0.6, group size = 10, power = 0.74

Expt. 2: Analysis by ANOVA: a = 0.05, no. of groups = 8, minimal detectable = 1.0, SD = 0.6, group size = 10, power = 0.74

Expt. 3: Analysis by ANOVA: a= 0.05, no of groups = 8, minimal detctable = 1.0, SD = 0.5 group size = 8, power = 0.8 [Power analysis was used to describe the response to stress, which uses half of each group in stress / no stress conditions].

* 1. What refinements, if any, have been made to reduce the number of animals used and the potential detrimental effects on the study animals?

We have carefully examined the literature in the design of these studies. Furthermore, over the course of many years we have been able to reduce the variance in our assays and surgical procedures thereby reducing the number of animals that are required in order to have sufficient power to see differences. An examination of our previous studies, and those of other laboratories have determined that the numbers of animals proposed are minimal amounts to determine significant effects given the variance in the assays in question. To further reduce the numbers of animals, we will conduct Experiments 1 and 2 using the same litters. In Experiment 3, where animals will be gonadectomized, we are using a combination of anesthetics and analgesics that should minimize pain and distress.

* 1. If the procedures will cause pain or distress to the animals, provide a written narrative of the methods used to determine whether or not alternatives exist to these procedures.

Based on our literature searches (see below) and conversations with colleagues, the only alternative to our surgical gonadectomies is to use chemical approaches. However, such chemicals are less effective and can have more unintended side effects, making them less preferred for both the research and animal well-being.

**III. CONTROLLED SUBSTANCES**

1. Does this protocol involve the use of DEA-listed control substances (e.g., ketamine, pentobarbital, androgens, diazepam, buprenorphine)?

No. Proceed to section **IV**.

Yes. List all controlled substances:

testosterone

buprenorphine

1. **EMERGENCY CONTACT**
2. Who should be contacted in case of an animal emergency? **Note: This information will be redacted if this protocol is requested as a public document.**

Name: Jane Doe

Home Phone # 480-555-4343

Office Phone # 480-555-1234

Cell Phone #: 602-555-2468

**V. DUPLICATION AND ALTERNATIVES**

The Animal Welfare Act requires that you document your justifications with data from **two** or more sources. One source **must** be a set of searches of a relevant database: name the database searched, the terms searched, when it was searched and the frequency of searches. The second source can be a set of searches of a second relevant database, or consultation with a laboratory animal science veterinarian, or courses/meetings/consultations with qualified personnel. Sufficient documentation, such as the consultant's name and qualifications and the date and content of the consult, should be provided to the IACUC to demonstrate the expert's knowledge of the availability of alternatives in the specific field of study. Consultation with the university attending veterinarian **must** be performed if the animals are expected to experience pain or distress. Examples include PUBMED, Web of Science. **[**Note:USDA Animal Welfare Information Center provides an in depth listing of database resources on their website at <http://awic.nal.usda.gov/nal_display/index.php?info_center=3&tax_level=1&tax_subject=184>.**]**

1. Provide the following details for the most recent literature search used to explore for duplicative research. (The literature search documents that the research will not unnecessarily duplicate previous research). **Teaching protocols do not need to conduct this search.**

Date that search was conducted (Must be within 60 days of the IACUC review date): 7/14/2011

Database used: PubMed

Publication years covered by the search: all years (1966 - 2011)

Keywords used: various combinations of testosterone, development\*, hypothalam\*, sex difference\*,

dexamethasone, prenatal, brain develop\*, stress, HPA axis, glucocorticoid\*

1. Provide the following details for the most recent literature search used to explore for alternatives to animal use and alternatives to painful procedures. Alternatives should be considered for any aspect of the protocol that may cause more than momentary or slight pain or distress to the animal. Alternatives to be considered include those that would: 1) refine the procedure to minimize discomfort that the animal(s) may experience; 2) reduce the number of animals used overall; or 3) replace animals with non-animal alternatives (e.g., computer models or tissue culture). **All protocols (both research and teaching) MUST conduct this search.**

Date that search was conducted (Must be within 60 days of the IACUC review date): 7/14/2011

Database used: AltWeb

Publication years covered by the search: 1970-2011

Keywords used: various combinations of rat, euthanasia, gonadectom\*, castrat\*, ovariectom\*, restraint, immobilization, elevated plus maze, light dark box, open field test, tail suspension test, forced swim test

1. Results of search for alternatives: Please comment on the application(s) of any identified alternatives, including how these alternatives may be or may not be incorporated to modify a procedure to either lessen or eliminate potential pain and distress. You must include sufficient information for the IACUC to determine that a reasonable, good faith effort was made to determine the availability of alternatives. If the search identified any alternative methods (ones that could be used to accomplish the goals of the animal use proposal), you must clearly explain and justify why this alternative cannot be used.

We found no procedures available that will meet our scientific objectives that are less painful or distressful than the ones proposed in this protocol. Animals receive multi-modal analgesia following surgical procedures to minimize any post-operative pain. The animals are continually monitored during behavioral testing by trained laboratory personnel.

1. Describe any other procedures (e.g., participation in meetings, review of journals) that are used to explore and evaluate alternatives: PI and laboratory personnel attend the annual meeting of the Society for Neuroscience and the Endocrine Society where we look for alternative methodologies and approaches. In addition, frequent Pubmed searches are made with alternatives in mind. To date, there have been no effective alternative approaches to remove circulating gonadal steroid hormone other than gonadectomy.
2. Does this research replicate previous work?

No. Proceed to section **VI.**

Yes. Explain why the replication is necessary:

Not applicable. This is a teaching protocol.

**VI**. **CATEGORY OF PAIN OR DISTRESS**

The USDA Regulations define a “painful or distressful procedure” as “any procedure that would reasonably be expected to cause more than slight or momentary pain or distress in a human being to which that procedure was applied; that is, pain in excess of that caused by injections or other minor procedures.” Using the table below, list all species of live vertebrate animals to be used in the proposed study and indicate the number of animals to be used under the appropriate USDA category. For an animal undergoing multiple procedures, list the animal under the highest level of pain expected for that animal.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Species | Number per USDA Category\* | | | | Total number of animals requested |
| B | C | D | E |
| *Rattus norvegicus* | 336 | 212 | 128 |  | 676 |
|  |  |  |  |  |  |
|  |  |  |  |  |  |

\*USDA PAIN CATEGORIES: (see <http://researchintegrity.asu.edu/iacuc/apply/USDApaincategories-examples.doc> for a more complete description of the below categories)

Classification B: Includes animals that are used solely for breeding (e.g., to produce experimental animals or to maintain experimental lines).

Classification C: Includes the use of animals in procedures involving no pain or distress (e.g., non-invasive parenteral drug delivery, peripheral blood collection, euthanasia, short-term manual or chemical restraint, and tumor propagation or toe-clipping performed according to ASU guidelines).

Classification D: Alleviated pain. Animals used in procedures that could cause pain or distress but appropriate anesthetic, analgesic, or tranquilizing drugs are used (e.g., surgery, periorbital blood collection, perfusion, or administration of irritating chemicals.

Classification E: Unalleviated pain. Includes the use of animals in procedures that involve pain or distress but the use of appropriate anesthetic or analgesic would have an adverse effect (e.g., negative conditioning, unrelieved post-surgical pain, death without euthanasia).

**VII. ASSURANCE**:

The information contained herein is accurate to the best of my knowledge. I have carefully compared the proposed work with the current state of knowledge in this field by reviewing the literature and it is my professional opinion that the proposed work meets high standards of scientific merit. If the study involves pain and distress to the animal, whether or not it is relieved by anesthetics or analgesics, I have (1) reviewed the literature related to this work and have found no significant studies which could make this protocol unnecessarily duplicative, and (2) considered alternatives to animal use and found none available, as described above. Procedures involving animals will be carried out humanely and all procedures will be performed by or under the direction of trained or experienced persons. Any revisions to animal care and use in this project will be promptly forwarded to the Institutional Animal Care and Use Committee for review. Revised protocols will not be used until Committee clearance is received. The use of alternatives to animal models has been considered and found to be unacceptable at this time.

The principal investigator, by signing below, and the IACUC recognize that other medications may be given to the animals for veterinary care purposes (including humane euthanasia of animals in pain that cannot be controlled, as determined by the University Veterinarian or an euthanasia-certified principal investigator).

Jane Doe 7/14/11

Principal Investigator – Please Print Date

Principal Investigator Signature Date

\*\*\*Department Chair Date

\*\*\*College Dean Date

**\*\*\*ASU Polytechnic requires these signatures.**

NOTE: Principal investigators are requested to attach a two-page biosketch reflecting their most recent pertinent

experience. **Also include a current curriculum vitae for all senior participants.**

## **DETAILED USE OF ANIMALS**

## **This section must be completed for each species used.**

## [Link](http://researchintegrity.asu.edu/iacuc/apply/documents/Upated%20Forms%204-08/Final%20forms%204-2008/Detailed%20Use%20of%20Animals%20Form.doc) to additional Detailed use of Animals form:

**Common Name:** RAT

**Scientific Name:** *Rattus norvegicus*

1. **ANIMAL INFORMATION**
2. Is this a threatened or endangered species?

No. Proceed to section I. B.

Yes. Describe why this work must be done on this species and why the project will not have a significant

negative impact on the species:

B. Maximum # of animals to be used

|  |  |  |  |
| --- | --- | --- | --- |
| Per Year: | 416 | Entire three years of protocol: | 676 |

C. Sex: M and F Age or Weight Range: variable – neonates through adult (~300g)

1. Source (e.g., commercial versus other, donated, captured from wild): commercial
2. Please LIST all labs and/or rooms **outside of the ASU centralized vivaria** where youintend to keep or use live animals in connection with the animal use covered under this protocol.  This list is for IACUC information to assure each location is inspected semi-annually.  **Listing rooms here does not assure approval of this space for use**.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Building | Room # | Max Length of Stay | Method of Transport | Purpose |
|  |  |  |  |  |

**II.** **MAJOR CATEGORIES OF USE**

1. Will animals be immunized for antibody production?

No. Proceed to section II. B.

Yes. Complete the following table.

Injection:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Volume of injectate | Adjuvant | Route | Min. Frequency | Max. # of injections |
|  |  |  |  |  |

Collection: If terminal, check here  Otherwise complete the following.

|  |  |  |  |
| --- | --- | --- | --- |
| Route | Max. Volume | Min. Frequency | Max. # of collections |
|  |  |  |  |

1. Will tissues or blood be harvested (other than for antibody production)?

No. Proceed to section II. C.

Yes. Will tissues be collected post-mortem only?

Yes. Proceed to section II.C.

No. Complete Appendix 1.

1. Will animals be food restricted (calorically or specific constituents)?

No. Proceed to section II. D.

Yes.

What are the restriction parameters? Provide scientific justification

How will you monitor for negative effects of food restriction (include information on how you will account for animal growth)?

1. Will animals be water restricted?

No. Proceed to section II. E.

Yes.

What are the restriction parameters? Provide scientific justification

How will you monitor for negative effects of water restriction (include information on how you will account for animal growth)?

1. Will pharmacologic or toxicologic materials be used apart from surgical use, including but not limited to tranquilizers, sedatives, analgesics, and anesthetics?

No. Proceed to section II. F.

Yes. Complete the following for each material.

|  |  |  |  |
| --- | --- | --- | --- |
| Agent | Dose | Route | Purpose |
| dexamethasone  testosterone  propionate  safflower oil  isoflurane | 0.2 mg/kg/day  to pregnant dams at  gestation days 17-20  2.0 mg/kg/day  to pregnant dams at  gestation days 17-20  0.05ml/injection  2% | subcutaneous  subcutaneous  subcutaneous  inhalation via  mask | glucocorticoid  androgen  vehicle for other agents  light anesthesia for ear-tagging |

Provide scientific justification:

Dexamethasone is a synthetic glucocorticoid that we are using to activate glucocorticoid receptors in the late gestation fetus. We are using it to mimic glucocorticoid changes that might occur as a result of prenatal stress and monitor outcomes.

Testosterone propionate is a conjugated androgen that we are using in an attempt to determine if the presence of androgen prenatally (in male fetuses) is responsible for the sex differences that we have observed in neuronal cell death in hippocampus and hypothalamus. The testosterone propionate and dexamethasone are both pharmaceutical grade compounds.

Sterile safflower oil will be used as the delivery vehicle, since both of these steroids are highly lipophilic.

Isoflurane is the standard gas anesthetic used in rats. It’s advantage is that it is very safe and has a rapid induction and recovery.

F. Will irradiation or radioisotopes be used?

No. Proceed to section II. G.

Yes. List here and attach ASU Radioisotope Approval Form.

|  |  |  |  |
| --- | --- | --- | --- |
| Agent | Dose | Route | Purpose |
|  |  |  |  |

Provide scientific justification

G. Will toxic chemicals, carcinogens, recombinant DNA, or infectious agents be used in conjunction with animal use?

No. Proceed to section II. H.

Yes. List the agent, dose, route, and purpose in the table below \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_.

|  |  |  |  |
| --- | --- | --- | --- |
| Agent | Dose | Route | Purpose |
|  |  |  |  |

Provide the Institutional Biosafety Committee (IBC) approval #:

Provide scientific justification for the need to use these agents:

H. Will animals be exposed to trauma, injury, burning, freezing, or electric shock?

No. Proceed to section II. I.

Yes. List and justify each exposure.

Provide scientific justification:

1. Will animals be exposed to environmental stress (e.g., temperature, physical restraint, forced exercise)?

No. Proceed to section II. J.

Yes. List and scientifically justify each exposure.

Adult offspring will be tested for stress reactivity by being placed into a Plexiglas restraint tube for 15 min. The Plexiglas restraint tube allows for some movement. Ventilation holes have been made in the top and bottom and a nose-poke hole is present at the front end. The restraint tube is considered to be a modest psychological stressor rather than a physical stressor. Hormonal responses to restraint have been well characterized and have been used in my laboratory for over 20 years.

J. Will animals undergo surgery?

No. Proceed to section II. K.

Yes. Attach Appendix 2.

1. Will any animals have a device (e.g., thermocouple, cannula, electrode) that extends chronically through the skin?

No. Proceed to section II. L.

Yes. Describe wound management measures to minimize chances of infection around the device where it penetrates the skin:

1. Will animals need any special husbandry considerations?

No. Proceed to section II. M.

Yes. Describe special procedures and provide scientific justification:

1. Will any animals need to be individually identified?

No. Proceed to section III.

Yes. Describe the marking technique to be used, why that technique was chosen, how it will be performed, and on what age range of animals?

Animals used in Experiment 3 will undergo serial behavioral testing and will be pair-housed. Therefore, o assure known identity of each animal throughout the trials, we will ear tag the experimental offspring in Experiment 3 at weaning We will use small, sterile, self-piercing and self-locking ear tags. To reduce pain and discomfort, the animal will be lightly anesthetized with isoflurane before performing the procedure.

**III. DETRIMENTAL SEQUELAE**

1. Will animals possibly experience clinical signs intentionally or as a possible side effect of the study?

No. Proceed to section IV.

Yes. Complete the following.

|  |  |  |
| --- | --- | --- |
| Possible Clinical Effect | Probability of Occurrence | Treatment |
|  |  |  |

1. **END POINT CRITERIA**
2. What clinical signs will be used as a basis for removal of an animal from the study?

We expect no adverse reactions to these experimental manipulations. However, if an animal appears lethargic, is immobile, fails to groom, or shows any other sign of illness, the animal will be treated or euthanized following consultation between the DACT veterinary staff and the PI.

**V**. **EUTHANASIA**

A. Chemical/Gas Methods, if any:

|  |  |  |
| --- | --- | --- |
| Agent | Dose | Route |
| CO2 (for Category B animals) | 100% | Inhalation |

If using a chemical method for euthanasia, what secondary physical means (e.g., thoracotomy) will be used to assure euthanasia? Bilateral thoracotomy

B. Physical Methods, if any:

Cervical dislocation (mice, immature rats)\*

Decapitation\* (for experimental animals)

Exanguination under anesthesia

For methods that are marked with an \*, provide a scientific justification here for the need to use this method (provide references if possible):

As we are attempting to determine plasma concentrations of stress hormone as well as gene expression changes (in adult animals), anesthesia prior to decapitation will confound the results. For adults, the animal will be placed into a Decapicone and decapitated with a standard rodent guillotine. The guillotine will be monitored weekly for sharpness by feel (fingernail test) and will be sharpened and conditioned every 6 months or whenever it appears dull. In neonates, we are also examining glucocorticoid sensitive endpoints, but neonates are in a stress hyporesponsive period. Therefore, we will anesthetize them by hypothermia prior to decapitation. Animals are placed on wet ice and decapitated once they do not respond to touch. For adults, animals will be rapidly decapitated within 30 seconds following removal from their home cage or the restraint tube. For neonates, animals will be decapitated following cryoanesthesia, usually about 1 min. after removal from their mother.

C. Name(s) and qualifications of person(s) performing euthanasia:

|  |  |
| --- | --- |
| Name | Qualification |
| Jane Doe | 25 years of experience with CO2 and decapitation (+/-  hypothermia) |
| John Buck | 10 years of experience with CO2 and decapitation (+/-  hypothermia) |
| Susan Elk | 5 years of experience with CO2 and decapitation (+/-  hypothermia) |

**APPENDIX 1: ANTEMORTEM SPECIMEN COLLECTION**

1. **BLOOD COLLECTION**
2. Will blood be collected?

No. Proceed to section II.

Yes. Complete the following.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Site | Volume (ml) | % BW | Max. # of collections | Min. Interval |
| Lateral saphenous vein | 0.5ml | <1% | 1 | N/A |

B. Will anesthetics, sedatives, or other drugs be used during blood collection?

No. Proceed to section I. C.

Yes. Complete the following.

|  |  |  |  |
| --- | --- | --- | --- |
| Drug | Dose | Route | Purpose |
|  |  |  |  |

C. Describe the methods used to draw the blood including physical restraint, if any.

The rat will be placed in a commercial rodent restrainer so the hind limb can be extended and stabilized. A sterile 1ml X 27g syringe will be used to collect blood from the lateral saphenous vein. Pressure will be applied to the site of collection using a gauze sponge for 30 seconds to assure no residual bleeding occurs.

D. Provide scientific justification for blood collection and justification for the frequency of it.

In Experiment 3, three days after gonadectomy we will collect a blood sample to assay for sex steroids and confirm the effectiveness of the surgical procedure.

E. Who will draw the blood?

Name: Jane Doe, John Buck, and Susan Elk

Qualifications: 25, 10, and 5 years of experience collecting blood samples from rats, respectively.

1. **OTHER TISSUE/BODY FLUID COLLECTION**
2. Will other tissues or body fluids be collected prior to death?

No. Appendix 1 is completed.

Yes. Complete the following. Surgical procedures should be described more fully in Appendix 2.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Tissue/Fluid | Site and Method | Amt | # of collections | Min Interval |
|  |  |  |  |  |

B. Will anesthetics, sedatives, or other drugs be used during tissue/body fluid collection?

No. Proceed to section II. C.

Yes. Complete the following.

|  |  |  |  |
| --- | --- | --- | --- |
| Drug | Dose | Route | Purpose |
|  |  |  |  |

C. Describe the methods used to collect the samples, including physical restraint, if any.

D. Provide scientific justification for the sample collection(s) and justification for the frequency of it

E. Provide name and qualifications of individual who will collect tissues or body fluids.

Name:

Qualifications:

**APPENDIX 2: SURGICAL PROCEDURES**

1. **GENERAL INFORMATION**
2. Species

*Rattus norvegicus*

1. Surgical Procedure(s)

Gonadectomy (ovariectomy, orchidectomy)

1. Room/location of surgery

ISTB1 vivarium procedure room

1. Name(s) and qualifications of person(s) performing surgery

Jane Doe – 25 years performing gonadectomy

John Buck – 8 years performing gonadectomy

Susan Elk – 5 years performing gonadectomy

1. **SURGICAL PROCEDURE**:

Survival  Nonsurvival

1. Describe and justify scientifically each surgical procedure (e.g., approach, tissue manipulation, closure):

Ovariectomy: The animals will be anesthetized with isoflurane. The fur over the lateral abdominal wall is shaved, and the area is cleaned with dilute chlorhexidine and alcohol. An incision is made through the skin of the lateral abdominal wall, midway between the ribs and the pelvic crest. An incision is then made through the abdominal musculature. The ovary in located in a fat pad within the lateral abdominal cavity and exteriorized. The oviduct and uterine horn are identified and clamped with a hemostat. The oviduct and uterine horn are cut and the ovary is removed. The distal uterine horn is tied off with 4-0 absorbable suture, the tissue is placed back into the abdominal cavity and the muscle wall is closed with 4-0 absorbable suture. The skin wound is closed with 2-3 staples, which are removed approximately 7 days later. The procedure is repeated on the other side. The animal is returned to its home cage, half of which is placed on a warming pad. The animal is monitored until it is awake and mobile.

Orchidectomy: The animal will be anesthetized with isoflurane, and the scrotum will be shaved and swabbed with dilute chlorhexidine and alcohol. An incision is made through the scrotal skin and the underlying connective tissues. The testis is identified and the spermatic cord is clamped. The spermatic cord is then tied off with 4-0 absorbable suture and the spermatic cord is cut and the testis is removed. The spermatic cord is gently replaced into the scrotum. The process is repeated on the other side to remove the other testis. The connective tissue and skin are then closed with 5-0 absorbable suture. The animal is returned to its home cage, half of which is placed on a warming pad. The animal is monitored until it is awake and mobile.

1. Will pre-anesthetic drugs be used?

No. Proceed to section II. C.

Yes. Complete the following.

|  |  |  |  |
| --- | --- | --- | --- |
| Drug & concentration (e.g.,  mg/ml) | Dose (e.g., mg/kg) | Route | Purpose |
| buprenorphine 0.3mg/ml | 0.01– 0.05 mg/kg  once prior to surgery | SQ | analgesia |
| carprofen 5% | 5 mg/kg prior to the  onset of surgery and  every 24 hrs for 3 days | SQ | analgesia |
| bupivacaine 0.25% | 2.5 mg/kg | SC infiltration of the  incision line prior to  cutting | local anesthesia |

1. Anesthetic regimen:

|  |  |  |
| --- | --- | --- |
| Drug and concentration | Dose | Route |
| isoflurane | 1-5% | Inhalation |

Describe measures used to indicate surgical plane of anesthesia to keep animals from getting both too light and/or too deep: Animals will be tested by examining the hind limb pedal withdrawal response to toe-pinch. Anesthesia will be continued until they are non-responsive. To determine whether animals are too deeply anesthetized, ventilatory rate and mucous membrane color will be monitored.

1. Additional pharmacological agents used during surgery (include analgesics, supportive medications, and research drugs):

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Drug and concentration | Dose | Route | Frequency | Purpose |
|  |  |  |  |  |

E. Who will administer the drugs?

Surgeon.

1. Describe the steps taken to maintain an aseptic surgery:

The surgeon will wear a mask, shoe covers, a clean laboratory coat, and sterile gloves. Sterile instrumentation will be used (autoclaved). If multiple surgeries are to be performed on the same day, instruments will be sterilized between animals using a glass bead sterilizer. A sterile drape will be used to cover the majority of the animal, the animal’s fur will be shaved, and the surgical site will be cleaned with dilute chlorhexidine followed by a 70% ethanol swab.

1. What is the maximum duration of surgery?

15 min

1. Will any animals recover from surgery?

No. This involves terminal, or non-survival, procedures; Appendix 2 is complete.

Yes. Complete Section III.

**III.**  **POST-SURGICAL CARE**

1. Is post-operative pain or distress anticipated?

No. Proceed to section C.

Yes.

1. Will analgesics be used?

(For analgesic use in rodents see The Guidelines for Rodent Analgesia: <http://researchadmin.asu.edu/compliance/iacuc/sop/rodentanalgesia.htm>)

No. Provide a scientific justification:

Yes. Complete the following.

|  |  |  |  |
| --- | --- | --- | --- |
| Drug | Dose | Frequency | Route |
| carprofen 5% | 5 mg/kg | every 24 hrs after  surgery for 3 days | SC |

Who will administer these drugs?

All investigators

C. Post-operative routine care:

What drugs will be administered, if any (e.g., antibiotics, fluids)?

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Drug and concentration | Dose | Route | Frequency | Purpose |
|  |  |  |  |  |

What other post-operative support and monitoring will be provided, how often, for how long, and by whom?

Lab personnel will check the surgical sites and the animal’s general health daily until the staples are removed.

D. Is post-operative intensive care required?

No. Proceed to section E.

Yes.

What special care is required?

Who will provide special care and what are their qualifications?

For how long will special care be needed?

E. Will animals undergo multiple survival surgical procedures?

No. Appendix 2 is complete.

Yes. Describe which surgeries, the sequence (timeline), and frequency. Provide scientific justification: