Behavioral Standard Operating Procedures

BEHAVIORAL SOPS – GOLDOWITZ/COOK/HAMRE

1. Stress-related tasks and neuroendocrine measures
a. Tail suspension: The tail suspension apparatus is manufactured by MedAssociates, St. Albans, VT. Each unit consists of a linear load cell and load cell amplifier and filter which are connected to a transducer to which the animals' tail will be taped. Each unit is enclosed in a cubicle. Each mouse will be weighed at least one hour prior to testing. Weight we be recorded to the nearest 1/10th of a gram and entered into the tail suspension software program. The software uses the animals' weight to determine a threshold for struggling versus immobility. Mice will be suspended by the tail with tape attaching them to the transducer. During the 6 minute test, force of movement or lack thereof will be recorded. Time spent below threshold reflects the time the animal spent immobile during the test and this is our primary variable of interest. Data over the 6 minute test will be collected in 30 second bins which will allow us to also evaluate bouts of struggling versus immobility over the course of the test session. Immediately following the tail suspension test, animals will be returned briefly to their home cages (to allow for transport away from the testing equipment) decapitated and trunk blood collected in heparinized collection tubes. Blood will be spun down to collect serum and the serum will be frozen at -80° C until the time of assay for corticosteroid levels (corticosterone).

b. Unavoidable footshock: Unavoidable footshock will be used as our other acute stressor. Footshock has been one of the stressors that has been somewhat consistently linked to the reinstatement of alcohol drinking behavior in mice and rats (Le et al., 1998; Liu & Weiss, 2002; Martin-Dardon et al., 2000; for review see Le & Shaham, 2002). In addition to alcohol, footshock stress will also reliably reinstate cocaine seeking (Erb et al., 1996; McFarland et al., 2004), nicotine seeking (Buczek et al., 1999) and heroin seeking (Shaham et al., 1997; Shalev et al., 2001) behavior in rodents. The shock chambers are manufactured by Med Associates, St. Albans, VT. Determination of vocalization threshold: The Med Associates, Inc Shock Titration Package for Mice will be used in our determination of vocalization thresholds. In small groups of animals from each mutant line, we will establish the shock threshold for vocalization (if the threshold has not been previously determined for any particular mutant line). We will start with 0.05 mA and progressive upwards in increments of 0.05 mA until the animal vocalizes. The shock level at which the animal vocalizes will be recorded. Mean values for each mutant line will be evaluated to determine a shock threshold level. Unavoidable footshock as an Acute stressor: Once the shock threshold (respective of mutant line) has been established, all animals will undergo a 15 minute session consisting of intermittent footshocks, 0.5 seconds in duration, separated by 10 to 40 seconds intervals (mean off time will be 40 seconds) as developed by Le et al., 1998. Of course, in the test portion of this assay, there will a cohort of animals that are placed into the shock chambers, but do not receive any footshock. At the end of the 15 minute session, animals will be returned briefly to their home cages (to allow for transport away from the testing equipment) decapitated and trunk blood collected in heparinized collection tubes. Blood will be spun down to collect serum and the serum will be frozen at -80° C until the time of corticosterone determination via assay. We will, of course, note differences in vocalization thresholds between mutant lines, but our primary variable of interest here is corticosteroid response to the footshock stressor.

For both the tail suspension and unavoidable footshock tests, we will use three separate cohorts of mutant mice (one control and two experimental). Each cohort will consist of three (3) male and three (3) female mice from each (mutant pedigree or consomic strain). The control cohort will be used to obtain baseline data on corticosterone levels. Animals will not be tested in the tail suspension test; but simply sacrificed and trunk blood collected for corticosteroid analysis. Another control cohort will be placed into the shock chambers but

not receive shock. They will be sacrificed and have trunk blood collected for corticosteroid analysis. One experimental group will be tested in the tail suspension assay and the other in the unavoidable footshock paradigm.
Measurement of corticosterone levels will be assessed using RadioImmunoAssays kits. RIA assays will be carried out at UTHSC in accordance with established protocols within the INIA-East consortium.

2. Ethanol-related tasks
Ethanol preference, most commonly measured via the various two bottle choice paradigms is one of the hallmarks in ethanol drinking behavior in rodents. We will use this assay to evaluate ethanol preference in our mutant lines. We will also use handling-induced convulsions to evaluate ethanol withdrawal severity. Handling-induced convulsion scores may provide insight into the mechanisms involved in ethanol dependence and withdrawal as well as insight into mechanisms involved in ethanol-seeking and ethanol drinking behaviors related to withdrawal experience.

a. Self-administration of alcohol in two bottle choice: Ethanol self-administration will be determined using the method of Whatley et al., (1999). Briefly, mice receive one bottle of fluid on days 1 and 2 that contain 10% ethanol before receiving two bottles for the next 10 days (days 3 – 12). On days 3 – 12 one bottle is filled with tap water while the other bottle is filled with 10% ethanol. The amount of fluid consumed is measured each day in mL and the bottles refilled with fresh solution and alternated (left/right) to prevent a positional bias. All bottle changes are done between 2 and 3 p.m. Lights in the colony are turned off at 3 p.m. Animals are never food deprived. This method has produced great consomic strain differences and also yields the typical sex difference. For the final ethanol solution (15% ethanol/ 0% sucrose) only and in only a subset of the animals, a blood sample will be taken from the retro-orbital sinus 1 hr after the start of the access period. Heparinized capillary tubes will be used to collect the blood sample. Samples will be spun down and plasma collected. Plasma will be subjected to a standard spectrophotomeric assay to quantify blood ethanol levels.

b. Acute EtOH withdrawal and handling-induced convulsions. We will administer a single high dose of ethanol by intraperitoneal injection to evaluate acute ethanol withdrawal. Briefly, animals will be given an i.p. injection of 4.0 g/kg ethanol and placed in a trough. (Possible addition to also get a measure of sensitivity of CNS to ETOH: Using a stopwatch/time, we will measure the latency to loss of righting response (LORR), or when place on its back in the trough the animal is no longer able to right itself. At LORR, we will also take a blood sample from the retro-orbital sinus as described above.) At four, six, and seven hours post ethanol injection, we will assess acute withdrawal using handling-induced convulsions as the phenotypic endpoint. Each mouse will be picked up by the tail, held in place and then rotated in a 360° arc. We will use the following modified criteria (Crabbe et al., 1985, Kosobud and Crabbe, 1990) to rate handling-induced convulsions:
Handling-Induced Convulsions (HIC) Scoring Scale

Score Description of Behavior
0 No activity on tail lift or after gentle 360°spin
1 Facial grimace after 360°spin
2 Tonic convulsion after 360°spin
3 Tonic/clonic convulsion after 360°spin
4 Tonic convulsion after tail lift
5 Tonic/clonic convulsion on tail lift/delayed onset
6 Tonic/clonic convulsion; no delay
7 Severe tonic/clonic convulsion prior to tail lift

As previously mentioned, HIC scores will be obtained four, six, and seven hours post-ethanol injection. HIC scores at each time point will be determined by removing baseline HIC scores (noting a negative HIC score, ie, baseline HIC greater than post ethanol HIC which will be scored as 0).

3. Anxiety-related tasks
In our screening, we will also assess several anxiety-related measures in separate cohorts of mutant mice. High-throughput, informative tests will be used to assess anxiety-related behaviors. Elevated Zero-maze, open-field and light/dark tests will be used. Because all of our tests will be performed in cohorts of genetically identical animals, information from these tests may allow us to make some hypotheses about the relationship

between baseline anxiety levels, stress reactivity, and alcohol-related behaviors (although the elevated zero-maze, open field and light/dark tests could be considered as psychogenic stressors themselves).
The elevated zero-maze is an alternative to the elevated plus-maze and is thought to be more sensitive in measuring anxiety-related behaviors (Shephard et al.,1994). The zero-maze lacks any central platform and therefore eliminates the ambiguity associated with time spent and behavior on the central area of the plus-maze. The open-field test is perhaps among the oldest tasks used to assess mouse behaviors, including reactivity, exploration, and anxiety among others. The light/ dark box is also widely used to assess anxiety-related behaviors. Each of these tests is based on the natural tendency of rodents to avoid brightly light, open, or elevated areas.
Because animals will not be housed in the same room as testing equipment, for all behavioral tests, animals will be moved to the testing rooms and allowed to acclimate to the testing room for a minimum of 30 minutes before any testing begins.

a. Elevated Zero-Maze: We will use automated elevated zero-mazes manufactured by AccuScan Instruments. Briefly, each maze is a continuous, elevated circular platform with open and closed quadrants used to assess anxiety-related behaviors. Five-centimeters in width, the black platform is elevated 108.9 cm off the floor. The walls of the closed areas are constructed of clear Plexiglas and are 28.5 cm high. The maze diameter is approximately 40 cm, with the inner diameter being about 30 cm. Three mazes are separated by solid office dividers. To start the test, an animal will be placed into a closed quadrant of each respective maze (a beam break in this area triggers the start of the test). Variables of interest will be latency to enter an open quadrant, time spent in open and closed quadrants, and activity in the closed areas of the maze (only closed quadrants are equipped with photobeams).

b. Open Field : We will use the VersaMax system manufactured by AccuScan Instruments. The boxes measure 42cm L x 42cm W x 30cm H. Boxes are equipped with an 8X4 horizontal photobeam array which detects movements of the animal. Specifically, the pattern of beam breaks is transmitted to an analyzer that converts the patterns and sequence of beam breaks into total distance traveled, rest time, center time, and margin time. Boxes are also equipped with a vertical photobeam array that transmits information on vertical movements of the animals (or rears, thought to represent exploratory behavior). We will use a short test session, 10 minutes in duration. Data will be collected in 2-five minute bins. To begin the test animals will be placed into the center of the open field. Our primary variables of interest will be total distance traveled, rears, and center time. We will also evaluate intrasession habituation by looking at the difference in distance traveled between the two-five minute bins. Many of the variables collected in open field testing are also thought to reflect reactivity in rodents (ref).

c. Light/Dark. For the light/dark test, we will also use the VersMax system, but with a light/dark insert option. The insert is made of black plexiglass and is 21cm L x 42cm W, effectively dividing the open field into two equal parts. The front of the insert is 30 cm in height; however the remainder of the insert is covered and approximately 20 cm in height. The front of the insert has a semi-circular opening in the middle that allows the animals to pass between the light and dark halves of the open field. A white sheet of poster board (21cm L x 42 cmW) is placed under the clear Plexiglas floor of the light side of the box. A gooseneck desk lamp with a 15W bulb is also placed above the light half of the open field, but such that the light does not disrupt photo beam communications. The insert also has holes drilled at the level of both the horizontal and vertical sensors so that communication between photobeams is not disrupted. Animals will be placed in the light half of the box to start the 10 minute test. Data will be collected in 2-five minute bins. The VersaMax software allows us to instruct the system to divide the field into two halves, one designated light, and the other dark. Variables obtained from this test include time spent in the light vs. dark side, distance traveled in each side, rest time in each side, and rears. We can also have the data reduced such that we can determine the latency of the animal to transition from the light to dark portion of the field.

4. Corticosterone (CORT) measurements. The protocol used is that of Roberts et al. (1995). All mice tested will be 60-80 days of age. At least 8 potential mutant mice will be analyzed from each pedigree: four mice will be injected with saline to determine the baseline stress response and four will be tested for the ethanol-induced response to stress. Mice will be injected with 4 g/kg of ethanol (20% v/v in saline). Corticosterone measurements will be taken one hour and six hours after the injection. Blood will be obtained from the tail vein of each mouse and collected into capillary tubes. Blood is then centrifuged to separate the plasma. Plasma

CORT will be measured by radioimmune assay (RIA) using a kit (ImmunChemTM Double antibody corticosterone 125I RIA kit for rats and mice) from ICN Biomedicals Inc. (Diagnostics), Costa Mesa, CA. A gamma scintillation counter (Beckman) will measure 125-Iodine at 90% efficiency and counts per minute (c.p.m.) will be obtained from the samples. A set of seven standards in the range (0-2000ng/ml) will be used to construct a standard or calibrator curve. In addition, blank tubes for the determination of non-specific binding (NSB) and tubes containing the total radioactivity aliquot (total tubes) will be measured. All standard, sample, NSB and total radioactivity tubes will be run in triplicate to obtain an accurate mean c.p.m. value. Plasma CORT concentrations in the samples will be calculated with respect to the standard curve and expressed as ng/ml.