# Steroid Standard Operating Procedures

Blood sampling for mice

Animals are sacrificed and blood is collected from the trunk into red top blood collection tubes with no additives. Tubes are kept on ice until centrifugation; they are then centrifuged at 3000 rpm for 15 minutes at 4ºC and plasma is aliquoted and frozen at –80ºC until assayed for neuroactive steroids.

Brain sampling for mice

Animals are sacrificed, the brain is removed and chilled in ice-cold saline for 1-2 minutes; the brain regions of interest are rapidly dissected over ice, immediately frozen on dry ice and stored at –80ºC until assayed for neuroactive steroids.

Blood sampling for non-human primates

Monkeys were trained to comply with awake venipuncture to collect blood for the steroid assays. Each step in the behavioral training was considered complete when the animals performed the behavior readily and with minimal observable distress. Twice a day each monkey was trained with positive reinforcement to move to the front of the cage and present its leg through an opening in the cage (10 x 10 cm). Blood draws from the femoral vein were performed multiple times per week continuing throughout the experiment. To administer the pharmacological challenges to the monkeys and collect the necessary blood samples following these challenges under non-stressful conditions, the monkeys were trained to sit in a primate restraining chair. For each challenge test, the order in which the animals were handled was randomly assigned although each animal was assigned a specific chair for the entire endocrine profile to ensure proper fit. As the animals became comfortable sitting in the chairs, blood draws were obtained via the femoral vein to simulate the blood sampling during the pharmacological challenges. Each week during endocrine profiling, a blood sample was obtained for a hematocrit value to monitor for anemia.

Femoral blood samples are obtained with a 22g x 1 inch Vacutainer needle and a 3 ml Vacutainer hematology tube (Becton Dickinson, Franklin Lakes, NJ, USA). All blood samples are stored on ice until centrifuged (approximately 5 minutes). Samples are spun at 3000 rpm for 15 minutes at 4ºC in a Beckman Coulter refrigerated centrifuge (Model Allegra 21R, Beckman Coulter, Fullerton, CA, USA). The plasma is pipetted into 2 ml microtubes in 100 µl aliquots. Plasma samples for neuroactive steroid analysis are frozen at -80ºC and stored until processing.

Brain sampling for non-human primates

Monkeys underwent ketamine anesthesia immediately before sacrifice. The different brain regions of interest are rapidly dissected over ice, immediately frozen on dry ice and stored at –80ºC until assayed for neuroactive steroids.

Shipping procedures

Samples to be shipped are packed in Styrofoam boxes with dry ice to ensure proper storage of samples at –80ºC. Shipment will take place early in the week to avoid any delivery problems over the weekend.

Corticosterone assay in mouse samples

The corticosterone assay is performed according to company's instructions using the kit # 07-120102 from MP Biomedicals (Orangeburg, NY, USA).

3α,5α-THP assay in mouse samples

3α,5α-THP in the brain regions of interest is determined as follows. Samples are weighed, digested in 0.3 N NaOH by a sonic dismembrator, spiked with 2000 cpm of [3H]3α,5α-THP for recovery determination and extracted three times in 3 ml aliquots of 10% (v/v) ethyl acetate in heptane. The aliquots are combined and diluted with 4 ml of heptane. The extracts are applied to solid phase silica columns (Burdick & Jackson, Muskegon, MI, USA), washed with pentane, and steroids of similar polarity to 3α,5α-THP are eluted off of the column by the addition of 25% (v/v) acetone in pentane. The eluant is then dried under $N_2$ and steroids are redissolved in 20% (v/ v) isopropanol/50% RIA buffer (0.1 M $NaH_2PO_4$, 0.9 M NaCl, 0.1% w/v BSA). Extraction efficiency is determined in 50 µl of the redissolved extract by liquid scintillation spectroscopy. Extraction efficiency ranges between 95 and 100%. Samples (75 µl) are assayed in duplicate for 3α,5α-THP by radioimmunoassay. Reconstituted sample extracts (75 µl) and 3α,5α-THP standards (5–40,000 pg in 6.25% v/v ethanol, 31% v/v isopropyl alcohol in RIA buffer) are assayed by the addition of 725 µl of RIA buffer, 100 µl of [3H]3α,5α-THP (20,000 dpm), and 100 µl of anti-3α,5α-THP antibody (1:500). Total binding is determined in the absence of unlabeled 3α,5α-THP, and nonspecific binding is determined in the absence of antibody. The antibody-binding reaction is allowed to equilibrate for 120 min at room temperature and was terminated by cooling the mixture to 4ºC. Bound 3α,5α-THP is separated from unbound 3α,5α-THP by incubation with 300 µl of cold dextran-coated charcoal (DCC; 0.04% dextran, 0.4% powdered charcoal in double-distilled $H_2O$) for 20 min. DCC is removed by centrifugation at 2000 x g, 8ºC for 20 min. Bound radioactivity in the supernatant is determined by liquid scintillation spectroscopy. Sample values were compared to a concurrently run 3α,5α-THP standard curve produced using a one-site competition model (GraphPad Prism 4.0; GraphPad, Software, San Diego, CA, USA). Sample values are adjusted to account for the previously determined extraction efficiency. The sensitivity of the assay is 15–20 pg/tube, and the interassay coefficient of variation is 7-9%.