**TITLE**: ANA2 – BAER Testing in Mice Treated With Anatoxin-a

**QAPP** L-PHITD-0033913-QP-1-3 “Anatoxin Toxicity in Mice”

**PURPOSE**: This experiment will examine BAERs recorded in CD-1 mice exposed to anatoxin-a (a nicotinic agonist at central α4β2 and α4 and peripheral muscle nicotinic acetylcholine receptors. Animals will be implanted with screw electrodes to increase the signal-to-noise ratio, allow non-anesthetized testing. In ANA1, we saw a possible increase in BAERs 1 week after dosing and a possible decrease in BAERs 3 weeks after testing. Anatoxin will be delivered for 5 days at 6 mg/kg (po). Surgery for electrode implantation will occur one week after dosing, and animals will be tested two weeks after treatment.

**PERSONNEL**:

David Herr: PI, Experimental Design, Data Collection, Data Analysis, Data Interpretation

Garyn Jung: Animal Health, Calibration, Neurophysiology, Data Analysis

Donna Hill: Experimental Design, Dosing, Data Collection, Data Interpretation

**STUDY START DATE**: 04/14/2025

**STUDY TERMINATION DATE**: 08/20/2025 (not including data analysis)

**ANIMALS**: Adult, CD-1 male mice (Charles River, Raleigh, NC) will arrive at EPA at 10-12 (Squad1 with #167 and #168 at 13-15 weeks) or 8-10 weeks of age (Squad 2). All animals will be treated in accordance with federal regulations for animal care, housing, and welfare. This facility follows National Institutes of Health guidelines for animal care and is accredited by the American Association for Accreditation of Laboratory Animal Care International. All protocols were approved by the Office of Resource Management, Office of Research and Development Institutional Animal Care and Use Committee. A licensed veterinarian is maintained on-site, who regularly inspects the animal facilities.

A. Animal Environment

1. Temperature and Humidity: 22 ± 2⁰C, 40 ± 20% relative humidity

2. Light-Dark Cycle: 12 hr light-dark cycle, lights on 06:00 am EDT, off at 6:00 pm.

3. Water: Tap water is available ad libitum from water bottles.

4. Food: LabDiet 5001 is available ad libitum. Food stocks will be stored at room temperature in sealed bags or covered containers. Feed will be used within 4 months of the milling date. Hanging feeders will be refilled two times a week.

5. Bedding: Hardwood chips, Enviro dry nesting material

6. Cages, Lids, Feeders, and Racks: Animals will be single housed in hanging polycarbonate cages. Cages are equipped with hanging feeders and access to a water bottle with sipper tube with a double ball bearing. Cages are placed on 5 tier stainless steel racks and identified with an external hanging card. Dams are singly housed.

7. Animal Room Air Changes: 10-15 changes per hour of 100% filtered fresh air.

8. Sanitization Schedule: Cages are sanitized and receive fresh bedding as well as water bottles are changed twice a week. Cage racks and feeders are changed every two weeks.

**TEST SUBSTANCES:**

Name: Anatoxin-a - 1-(9-azabicyclo[4.2.1]non-2-en-2-yl)-ethanone

CAS #: 64285-06-9

Source: Cuspidothrix issatschenkoi, New Zealand culture at Cawthron Institute

Purity: 96.6% by LC-MS

 M.W.: 165.24 g/mol

Formula: C10H15NO

Density: N/A

Water Solubility: 28.13 mg/mL

Storage: Store at -20 in amber glass, in dark with pH of 4-5

Boiling Point: N/A

Volatility: N/A

Octanol/Water Partition (logP): 0.8

Vehicle: MilliQ Ultrapure Water

Mixing: Stir/Vortex

Route of Administration: Oral gavage

Frequency of Dosing: 5 Days

Protective Clothing: Gloves, Mask, Lab Coat, Goggles

**TREATMENTS:**

0 mg/kg

6 mg/kg

**PREPARATION:** Dosing solutions will be prepared and administered by Dr. Donna Hill

**EQUIPMENT CALIBRATION**: Prior to testing, all amplifiers and collection systems will be calibrated using the Clarke-Hess calibrator for the EP System using L-PHITD-NETB-SOP-2929. Correction factors will be calculated and used to automatically adjust the acquired data. Auditory stimuli will be calibrated using L-PHITD-NETB-SOP-2927. All calibration data will be recorded in the log book.

A. List of Calibration Tables:

1. Table 1. Amplifier Calibration Factors

2. Table 3. Auditory Stimulus Calibration

**EXPERIMENTAL DESIGN**:

Dosing & Testing Procedures

1. Subjects will be coded as ANA2-XXX.

2. Animals will be implanted with screw electrodes one week after dosing.

Electrode Pin # Function A-P Bregma Distance (mm) M-L Bregma Distance (mm)

1 GND +1 1.5 LL

4 Ref +1 1.5 LR

3 Active -5.3 0

3. The electrode impedance will be measured at the conclusion of surgery (Grass Electrode Impedance Meter, Grass Instrument Div., Astro-Med, Inc., West Warwick, RI; kOhm at 30 Hz).

4. Animals will be tested about 2 weeks after dosing.

5. Animals will be tested in boxes 1/3 or 5/7. This will use both DACs to minimize the A/D conversion on a single DAC (boxes 1 & 5 are on DAC1 and boxes 3 & 7 are on DAC2).

6. On testing day, the mouse will be restrained, placed in the test chamber, and connected to the recording wire.

7. A mouse temperature probe connected to a thermometer located outside the chamber, will be inserted 2 cm rectally and colonic temperature will be recorded after collection of each BAER waveform.

8. Auditory stimuli, such as tones or clicks, are presented from overhead speakers in the test chamber. Stimuli will be rarefaction clicks and tone pips centered at 4, 16, and 64 kHz (all at 75 and 100 dB peak SPL, re: 20 µPa). The stimulus duration will vary depending on the frequency: 50.0 µs (click), 2.54 ms (4 kHz), 634 µs (16 kHz), and 159 µs (64 kHz). Each test condition will require about 4 min for collection (total of about 35 min), and the stimuli will be presented in a random order between animals.

8. Stimuli order and colonic temperature will be recorded in Table 4.

9. After testing is complete, the mice will be removed from the decapicone and returned to their home cage.

10. Names of computer programs used to collect data:

a. BAERs: BAER\_Needle + BAER\_Needle VSA

11. Chambers will be cleaned between rats.

TIME REQUIREMENTS

1. Neurophysiological Testing = 1-2 days/time point

2. Data analysis = 4 weeks

**DEPENDANT VARIABLES**:

A. Body Weights: Body weights

B. Evoked Potentials:

1. EPs will be recorded on the EP System

2. Evoked Potentials will be collected using the test procedures indicated in the attached **Table 5**

D. Colonic Temperature: Colonic temperature will be collected during BAER testing using a Physitemp Thermometer Model TH-8) and inserting a probe (Ret-3, Physitemp) approximately 2 cm rectally, and allowing the temperature reading to stabilize.

**RECORDS TO BE MAINTAINED**: Evoked potential waveforms will be maintained on the EP System database. Printouts (including amplitude, latency, study protocol, subject files, experimental notes, statistical results, and graphs) will be stored in 3 ring binders.

**QUALITY CONTROL:** Amplifier and auditory calibration results will be compared to historical values for that particular instrument. If the data varies by more than 10%, the equipment will be examined for malfunction, and replaced if necessary. All experiments will be performed with control animals, and the results from these subjects should be similar to historical control data for this laboratory. Group average waveforms will be created and compared with previously published data from this laboratory.

**STATISTICAL METHODS**: Dependent variables will be examined for outliers using PROC UNIVARIATE. Data may be considered an outlier if it lies greater than 3 interquartile distances (distance between the 25th and 75th sample percentages) above or below the 25th or 75th sample percentages. If the data point is judged to be related to an improper scoring of the waveform, the data may be rescored.

Neurophysiological measures will be examined for a normal distribution. If non-normal, the dependent measure may be transformed to achieve normality, analyzed using GLMMIX, or non-parametric analysis. Appropriate step-down analysis will be used to examine appropriate effects. Post hoc comparisons will be performed using Tukey-Kramer or non-parametric DSCF tests. All analysis will be performed using SAS. Group average waveforms will be made as statistical analysis deems appropriate. Graphs will be made using SigmaPlot, based on statistical results.

Between-Subjects Within-Subjects

Dose (2) kHz (4)

dB (2)

**SPECIFIC STATISTICAL TESTS:** The individual mouse will be the unit of statistical analysis. The influence of tail temperature on neurophysiological endpoints may be analyzed using ANCOVA, with rectal temperature as the covariant.

**DATA STORAGE AND RETRIEVAL**: Data will be backed up and archived on network drives and burned to CD at the completion of the study. Hard copies of all data and analysis will be stored in EPA room A256. After publication, data will be archived as per EPA protocols.

**APPROVAL**: David Herr

**DATE**: 04/15/2025

**ANA2: Experimental Notes**

**Doses Original n-Size Final n-Size**

0 14 10

6 14 11

**Deleted Animals** **TRT**

172 6

170 0

177 0

181 0

182 6

187 6

188 0

**04/30/2025: Squad 1**

1. **Mouse #172 (TRT6)** died on day 3 of dosing. No data collected.

2. **Mouse #170 (TRT 0)** died during restraint. No data collected.

**05/13/2025: Squad 2**

1. **Mouse #177 (TRT 0)** was euthanized.

**05/17/2025**

1. **Mouse #181 (TRT 0)** was euthanized

**05/22/2025**

1. **Mouse #182 (TRT 6)** died during restraint. No data collected.

2. Mouse #179 (TRT 0) had mostly poor BAER responses. Retested later in the day, and still got poor BAER responses. Deleted 2nd run and kept the data from the 1st run (consistent with other mice).

**08/07/2025: Squad 3**

1. **Mouse #187 (TRT6)** died after dosing. No data collected.

**08/15/2025**

1. Mouse #191 (TRT6) has GND electrode in Pin #2. The impedance is only 0.2 kΩ.

**08/18/2025**

1. Due to a failure of the L-Pad gating box for chambers #6-10, squad 3 animals were testing in boxes #1 & 3. This is different from squad #1 & 2 animals, which were tested in boxes #5 & 7.

**08/20/2025**

1. **Mouse #188 (TRT 0)** died during restraint. No data collected.

2. Run Time parameters were blanked by default (cause unknown). DWH manually re-created Run Time parameters post-acquisition form mice #185 (TRT 0) and #192 (TRT 6).

3. Compute froze during VSA1 (8th in sequence) for mice #185 & 192. Rebooted and reacquired VSA1.

4. Compute froze during VSA5 (4th in sequence) for mice #194 & 193. Rebooted and reacquired missing VSA sets.