

VERTEBRATE ANIMALS - PROJECT 2 - PRECLINICAL MODEL FOR ANTIEPILEPTOGENIC THERAPY SCREENING IN POST-TRAUMATIC EPILEPSY

PROPOSED USE

Animals: Adult male 12 wk old Sprague-Dawley rats (300-350 g) will be purchased from a local vendor. Exclusion criteria: >15% weight loss during first post-TBI weeks, loss of electrode headset more than once, poor well-being assessed using standard Animal Center protocol. Rats will be housed in AAALAC-accredited animal facilities, supervised by the investigator's staff and 3 available veterinarians. 12h:12h light:dark cycle will be used and approved humidity and room temperature conditions. All methods have been approved by the local Institutional Animal Care and Use Committees (IACUC). The ARRIVE guidelines and the recommendations of the AES/ILAE (American Epilepsy Society, International League Against Epilepsy) Translational Research Task Force of the ILAE will be followed. We estimate that we will need 213 rats/site (Einstein, Melbourne) in Year 1, 95 rats per site per year (Einstein, Melbourne, UCLA) in Year 2 and in Year 3, and 92 rats per site (Einstein, Melbourne, UCLA, UEF) in Year 4 per site (Einstein, Melbourne, UCLA, UEF).

SUMMARY: In this project, we plan to utilize a rat model of posttraumatic epilepsy (PTE), induced by lateral fluid percussion injury (LFPI) to identify therapies that prevent PTE. All methods are described below. Adult rats will be subjected to LFPI or SHAM injury and will be randomized to the following cohorts:

- 1) rats will be euthanized for histology (perfusion) or brain tissue collection to evaluate the expression of certain molecular targets (histology, qRT-PCR, Western blots) at various timepoints (2d, 1wk, 2wk, 4wk, 8wk).
- 2) LFPI-induced rats will be randomized to be implanted with subcutaneous osmotic pumps (vehicle vs drug) for 1wk and will be followed for 1wk off treatment; rats will be sacrificed at specific timepoints (2d, 1wk, 2wk) to test the effect of drug on the targets (histology, qRT-PCR, Western blots). Blood samples will be collected (under isoflurane anesthesia) at 2d, 7d, 8d, 14d) to determine drug levels during and off treatment.
- 3) LFPI-induced rats will be implanted with EEG electrodes after LFPI and minipumps (drug vs vehicle) (1 wk ON, 1wk OFF treatment) to monitor the effect of the treatment on EEG activity (biomarkers, seizures). Blood will also be collected under isoflurane anesthesia, through the tail vein, to determine the effects on plasma biomarkers.
- 4) LFPI-induced rats will be implanted with minipumps (drug vs vehicle) for 8wk (2 minipumps per rat will be placed sequentially), blood for drug levels will be collected at each minipump removal, and EEG electrodes will be implanted at 6months. Video-EEG monitoring will be done for 1 month (7th month) and a second 1month-monitoring will be done at 12 months to detect seizures. MRI will be done at 2 timepoints (1st and 5th month) and blood collection for plasma biomarkers (target proteins, miRNA) or drug levels will be done at 2 timepoints, under anesthesia (isoflurane).

We expect to determine whether specific novel treatments have antiepileptogenic effect on PTE in the LFPI model and if the effect of treatments on early stage biomarkers (plasma, EEG, imaging) predicts the antiepileptogenic effect.

JUSTIFICATION: Traumatic brain injury (TBI) carries significant risk for post-traumatic epilepsy (PTE) (~50%) and is associated with significant comorbidities and seizures can often be intractable to medical treatment. The need to identify better therapies that can prevent PTE and improve long-term outcomes has been recognized as a priority. With this project we aim to utilize a standardized model of PTE, the lateral fluid percussion model of PTE in rats, in the preclinical screening for the identification of new antiepileptogenic therapies. There are no *in vitro* or computer models of PTE that could be used for such a purpose. The use of phylogenetically lower species is not possible as they are more distant to the biology, brain structure and function of the humans.

NO EXPERIMENTAL PROCEDURE WITHOUT PAINLESS DEATH.

Euthanasia: Pentobarbital (100mg/kg i.p.) followed by decapitation will be used for euthanasia and rats will be tested by stimulation to confirm they do not respond to pain. A body conditioning score of 2, signs of infection, moribund appearance, prolonged seizures (convulsive seizures more than 30min), over 15% weight loss, reduced grooming, signs of reduced well-being beyond what is expected to be TBI-related will be considered as humane endpoint for euthanasia.

RODENT SURGERY AND SURVIVAL

Blood collection: Blood (1.2 ml) will be collected from tail vein (<http://www.nc3rs.org.uk/bloodsamplingmicrosite/page.asp?id=420>) of light isoflurane sedated rats. Blood cells and plasma will be separated (half for plasma protein extraction – clotting to be done at room temperature, half to microRNA extraction – clotting to be done at +4°C), and both will be stored at -70°C at each study site. Blood samples for drug levels will be collected in sterile EDTA-tubes (0.3ml minimum/rat), centrifuged at 3,000g (4°C) and plasma will be transferred to a tube for storage at -25°C.

LFPI, subcutaneous osmotic minipump placement: 1 wk after arrival, rats are anaesthetized with isoflurane, placed in a Kopf stereotactic frame, skull is exposed, and periosteum extracted. A circular craniotomy (diameter 5 mm) is performed over the left posterior convexity (center AP-4.5, L2.5; Figure 9), leaving the dura intact. The edges of craniotomy are sealed with a modified Luer-Lock cap filled with saline. Calvaria is covered with dental acrylate. Severe LFPI will be induced 90 min after anaesthesia induction by connecting the rat to a fluid-percussion device. The mean severity of the impact (≈ 2.8 -3.0 atm) is adjusted to produce severe TBI ($\approx 30\%$ mortality). SHAM animals receive anesthesia and all surgical procedures without LFPI. Wound edges are covered with antibiotic cream and rats are treated with analgesics. Sham-operated controls will undergo all procedures except the impact. Minipumps (ALZET 2004 or 2006) will be placed subcutaneously immediately after LFPI, under isoflurane anesthesia.

Neuroscore: Neuroscore will be done 1d prior, and at 2d, 7d, 14d post-LFPI⁹⁵. A score from 0 (severely impaired) to 4 (normal) is given for each of the following 7 indices: (i and ii) left and right (2 indices) forelimb flexion during suspension by the tail, (iii and iv) left and right (2 indices) hindlimb flexion when the forelimbs remain on hard surfaces and the hindlimbs are lifted up and back by the tail, (v and vi) ability to resist a lateral propulsion toward the left and right (2 indices), and (vii) angle board. A composite neuroscore (0—28 points) was generated by combining the scores for each of these seven tests.

EEG electrode and microelectrode placement and long-term video-EEG monitoring: Immediately after LFPI (SA 1C) or at 6 months (SA 2), rats will be anesthetized with 2.0% isoflurane on a heating pad and fixed in the stereotaxic frame. After cleaning with Betadine/70% alcohol, 0.5 mm holes will be drilled to secure the bipolar electrodes around the perilesional cortex and at the sites of the screw electrodes (see Figure 9) and. Reference and ground electrodes are positioned above the cerebellum. After fixation of the connector head cap, wound edges are infiltrated with 0.5 % Marcaine, and closed with a wound clip. Rats will be treated with analgesics. For the 1st 2 post-operative days rats receive 0.9% NaCl to prevent dehydration. If any inflammation appears, neosporin ointment will be applied to wound edges. We will regularly monitor (a) general well-being of animals using the standardized form provided by Animal Center, (b) weight (once per week), (c) rectal temperature (once per week). At night, monitoring is done under infrared light.

vEEG to diagnose epilepsy. Experiments will be carried out under freely moving conditions in the animal's own cage. Wide band EEG recording and analysis that will be recorded on days 1-3 and 28-30 has been described in SA1. Standard video-EEG (vEEG) (0.1-300 Hz; sampling rate 1-2 kHz/channel) will be monitored continuously. The recording montage will consist of bipolar intracortical and intrahippocampal electrodes and epidural electrode (stainless steel jewelers screws, 0.5 mm OD) positioned bilaterally as shown in Figure 9). The likelihood for correctly diagnosing epilepsy, if it exists, was calculated assuming that seizure appearance has exponential distribution, and expected frequency based on our previous published experience is 0.2 seizures/d. The likelihood that we will detect seizures during the 4-wk monitoring, if they exist, is 99.75%. Even if the seizure frequency will be 0.1seizures/d, the likelihood would still be 95.02%. Digitized EEG recordings will be screened on computer screen by an experienced technician, blinded to the experimental group, and seizures and epileptiform discharged identified. An electrographic seizure is defined as high-amplitude rhythmic discharges that represents a new pattern of activity (repetitive spikes, spike-and-wave discharges, and slow waves) that last ≥ 10 s. Epileptic events occurring with an interval < 5 s without the EEG returning to baseline are defined as belonging to the same seizure. Visual screening of EEGs will be stopped after the 1st electrographic seizure is detected, hallmarking epilepsy diagnosis. The behavioral severity of seizures will be checked from time-locked video (to confirm that the EEG pattern was not artifact related, e.g., due to grooming). For complete analysis, EEG data files will be sent to the IAC to be assessed using unsupervised analysis tools.

MRI studies: During MRI experiment rats will be anesthetized with isoflurane (1.5-2%) inhalation anesthesia in 30%/70% O₂/N₂ carrier gas mixture. For monitoring of level of anesthesia and physiology, MRI compatible rectal temperature probe, breathing sensor pad and pulse oximetry sensor (for heart rate and blood oxygenation) will be used. **Ex vivo MRI and Histology.** Animals will be intracardially perfused with 0.9% NaCl (2 min) and 4% paraformaldehyde (30 min), and brains will be postfixed in the same fixative. For ex vivo MRI

brains will be placed in the plastic sample tube filled with perfluoronated liquid (Fomblin). *Ex vivo* MRI will be carried out using the same protocols as in vivo. After that brains will be cryoprotected in 20% ethyleneglycol in 0.02 M KPBS, frozen in dry ice, and stored at -70°C.

Transcardiac Perfusions (terminal procedure): Rats will be injected with a euthanasia-level dose of pentobarbital (100 mg/kg i.p.) to effect deep anesthesia. When the rats do not respond to noxious stimuli, an incision will be done under the diaphragmatic area, the heart will be exposed with separating the diaphragm from the thoracic wall and two dissections along the thorax will be done to lift the thoracic wall anteriorly and expose the heart. Sterile, cold saline will be infused with a pump through a needle placed at the apex of the heart and connected to an infusion pump, and a small incision will be done at the right atrium for the blood to escape. Perfusion with formalin will then be done. Brains will be collected and fixed 2hr to overnight (depending on protocol and intended use) in 4% paraformaldehyde (4°C) and then transferred to 30% sucrose till sunken and then they will be frozen in dry ice / isomethylbutane and stored to a -80°C freezer.

DESCRIPTION OF PROCEDURES FOR MINIMIZING DISCOMFORT, DISTRESS, PAIN, AND INJURY.

Ambulatory rats will be returned to the home cage in the Animal Institute. In the procedures described under subheading "*Procedure causes insignificant pain or distress*" no further minimization of discomfort or pain is possible either because it would directly interfere with the test results (seizures, behavioral tests and therapies) or the procedure for minimization of pain or discomfort would cause at least similar or larger discomfort than the experimental procedure itself (injections of analgesics). Regardless, all personnel handling these animals and performing these procedures will be trained to adopt standards of practice compatible with the recommendation for Animal Handling that have been approved by our IACUC, the NIH Guide on Animal handling and the ARRIVE/NC3R guidelines. All surgical procedures are conducted under deep anesthesia (e.g., induction of craniotomies, collection of blood via tail vein). After completion of surgery for induction of brain injury, bupivacaine (0.25%) is locally infiltrated into all wound margins and topical antibiotic ointment is applied.

VETERINARY CARE

Full-time veterinarians participate in our AAALAC-accredited program of care and use. Veterinary care includes a program for prevention of disease, daily observation and surveillance for assessment of animal health; appropriate methods of disease control, diagnosis, and treatment; guidance of animal users in appropriate methods of handling, restraint, anesthesia, analgesia, and euthanasia; and monitoring of surgical programs and postsurgical care.

The animals will be housed in an approved animal facility, which is maintained under supervision of the vivarium staff. Veterinary expertise is continuously available. Animals are single-housed together in a clear plastic bin (20X10X10 inches) that is lined with bedding material. Food and water are available *ad libitum*. Cages are cleaned twice weekly and food and water are checked daily. Daily monitoring of rats includes (a) general well-being of animals using the standardized form provided by Animal Center, (b) weight (once per week), (c) rectal temperature (once per week). Each animal is checked daily for loss of stereospecific behavior and coat color – any wound incisions/sutures are also examined daily. If any major health problems are noticed (over 15% weight loss, reduced grooming, signs of reduced well-being beyond what is expected to be TBI-related), animal will be euthanized, and excluded from the study.

ASSURANCE

No significant changes or additions to the above procedures will be implemented until reviewed and approved by our animal care and use committee. These procedures are not unnecessarily duplicative of previous experiments. Discomfort and injury will be limited to that which is unavoidable in the conduct of scientifically valuable research. Analgesic, anesthetic and tranquilizing drugs will be used where indicated and appropriate to minimize discomfort and pain to animals. The animals will be housed in our AAALAC-accredited animal facility at all times, where they are observed daily and are not deprived of water or food at any time nor subjected to prolonged physical restraint. Animals that become moribund will be killed painlessly with pentobarbital anesthesia overdose.

We agree to abide by the provisions of the PHS Animal Welfare Policy, and we give permission for veterinary care of animals showing evidence of pain or illness unrelated to the intent of the experiment.

EUTHANASIA

When experiments end, animals are killed painlessly by methods consistent with the recommendations of the AVMA Panel on Euthanasia. Our attending veterinarians and IACUC have approved the specific methods, described above. A body conditioning score of 2, signs of infection, moribund appearance, prolonged seizures (convulsive seizures more than 30min), over 15% weight loss, reduced grooming, signs of reduced well-being beyond what is expected to be TBI-related will be considered as humane endpoint for euthanasia. At Einstein, euthanasia will be done by pentobarbital (100mg/kg i.p.) followed by decapitation, once no reaction to pain (toe, tail response) is noted. This method has been approved by the Institutional Animal care and Use Committee (IACUC) of the Albert Einstein College of Medicine.

At the other 3 sites, euthanasia of anesthetized rats will be performed by: i) terminal intravenous or intraperitoneal injection of an overdose of sodium pentobarbital (75 mg/kg, i.v.). This method has been selected because it is painless and rapid; the method is consistent with the recommendations of

- (a) UEF: The Committee for the Welfare of Laboratory animals of the University of Kuopio and by the Provincial Government of Kuopio (ESAVI/5146/04.10.07/2014) and European Community Council Directive 2010/63/EU.
- (b) UM: The University of Melbourne/Florey Neurosciences Institute Animal Ethics Committee and the Australian and New Zealand Council for the Care of Animals in Research and Teaching.
- (c) UCLA: The University of California, Los Angeles, (UCLA) Chancellor's Animal Research Committee and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

HAZARD CONTROL

The protocols used for handling and administrative have taken into consideration the existing literature and have been approved by our Environmental, Health & Safety Committee. Toxic substances (i.e. paraformaldehyde during the perfusion) will be handled within a fume hood and will be disposed in special containers that will be collected by the Environmental Health & Safety Office. Isoflurane use will be done using charcoal traps for isoflurane, which will be checked by eight after each use and will be discarded if they exceed 50g from starting weight.