# research strategy – project 2 – PRECLINICAL MODEL FOR ANTIEPILEPTOGENIC THERAPY SCREENING IN POST-TRAUMATIC EPILEPSY

# 1. SIGNIFICANCE

There is currently no AEG treatment available in clinical practice for acquired epilepsies. While several animal studies reported drugs with AEG effects[77](#_ENREF_77), these have not reached the clinics in at least part because of a lack of rigorous pre-clinical validation. The need to develop strategies that “prevent epilepsy and its progression” is Benchmark II of the 2014 NINDS Benchmarks for epilepsy research[1](#_ENREF_1) and the top priority among the 50 international investigators participating at the 1st Joint American Epilepsy Society (AES) / International League Against Epilepsy (ILAE) Translational Epilepsy Workshop in 2012[37](#_ENREF_37), [81](#_ENREF_81). In this Project 2, we propose to (1) *implement a preclinical multi-step rigorous model to screen and validate AEG treatments for PTE*, using a standardized PTE model, the LFPI model in adult rats[51](#_ENREF_51),[54-57](#_ENREF_54),[80](#_ENREF_80), and (2) *deliver at least one novel AEG treatment for PTE*, in a manner that will (3) *prepare for testing in future clinical AEG trials for PTE*. Efforts to identify and develop AEG treatments in animal models of epilepsies and transition them to clinical trials for testing in human cohorts face significant challenges[11](#_ENREF_11),[81](#_ENREF_81): high demands in monitoring time, sample sizes, and effort resulting in prohibitive costs; uncertainty about whether the treatment targets are also relevant for epileptogenesis in humans, and reproducibility issues[11](#_ENREF_11),[35](#_ENREF_35),[36](#_ENREF_36),[58](#_ENREF_58),[63](#_ENREF_63),[81](#_ENREF_81). To de-risk the process of AEG therapy development, there is an intense interest to develop strategies that (a) reduce the “*number needed to treat*” (NNT), among those at risk, so that the treatment prevents one person from developing epilepsy[20](#_ENREF_20) and (b) accelerate the time to confirm an AEG effect. One such strategy is the development of reliable biomarkers of epileptogenesis and its progression (validated across species)[11](#_ENREF_11),[27](#_ENREF_27),[28](#_ENREF_28),[35](#_ENREF_35),[81](#_ENREF_81), and their use in preclinical and clinical studies to reduce the NNT to those only who are likely to develop epilepsy and respond to AEG treatment. *Project 1* aims to identify EEG, MRI, and plasma biomarkers of PTEgenesis in the standardized model of LFPI which will then be cross-validated in humans, in *Project 3*. In Project 2, we will first determine which biomarkers of PTEgenesis can be modified by the tested treatments early after TBI in the LFPI model and if these biomarkers can predict “AEG treatment response,” i.e., prevention of epilepsy in the model. Validation of biomarkers of AEG treatment response will provide a uniquely powerful tool to make AEG trials most effective and efficient by reducing both the NNT and the *time to document AEG effect.*

We propose to conduct the first *multicenter “Phase II” preclinical AEG trial*[72](#_ENREF_72),[94](#_ENREF_94), using a blinded, randomized, and vehicle-controlled study design, using clinically relevant treatment paradigms in a consortium of four preclinical epilepsy research centers. We plan to develop and adopt harmonized protocols and CDEs for data collection and analysis to improve the rigor and reproducibility of our study[107](#_ENREF_107). This approach has been advocated by the AES/ILAE Translational Research Task Force of the ILAE (several of our PIs are members) and their multi-PART collaborators (<http://www.dcn.ed.ac.uk/multipart/about.html>). If successful, Project 2 will provide a new testing paradigm and platform for rigorous preclinical screening of new therapies for their AEG potential. Our close interactions with both a preclinical *DSMB* and the *Public Engagement Core* will ensure that our strategies and GO/NO GO decisions will be considering clinical relevance, rigor standards, and sensitivity to the needs of the consumer parties, i.e., individuals affected by TBI. We include expert consultants on pHFOs (Dr. Bragin, Dr. Mody), and the tested drugs (Dr. Bush, Dr. Hovens) to advise us. We will create a *Rodent Biospecimen Repository (BioBank)*to also provide opportunities for future collaborations with investigators from the wider research community to apply advanced methods for the identification of new biomarkers or targets of PTEgenesis or treatment response.

**2. INNOVATION**

Project 2 offers the following innovation elements:

(1) The design and validation of a preclinical trial protocol to parallel human clinical trials: This *blinded* *multicenter “Phase II” preclinical AEG trial* adopts rigorous standards of practice and harmonized methods of data collection and analysis across 4 epilepsy research centers, mimicking procedures that are now accepted as standard in clinical trials.

(2) Rigorous validation of at least one novel AEG treatment for PTE, in the standardized animal model of PTE using drugs that prevent tau hyperphosphorylation, iron deposits in the brain, block T channels, or inhibit the IL-1 signaling inflammatory pathway.

(3) Validation of a panel of biomarkers of PTEgenesis predicting treatment response.

(4) Development of a pipeline for the screening of candidate AEG treatments for PTEgenesis, combining innovative strategies to ensure rigor and transparency (rigorous study design, preclinical DSMB), clinical relevance, as well as preserving the sensitivity to the needs of consumer parties (individuals affected by TBI) during the decisions affecting drug development and selection of the lead treatment.

# 3. APPROACH

3.1 Background and Preliminary Data: We propose to test five treatment options which (a) target mechanisms implicated in PTE and (b) have already been tested in early phase trials in humans for other indications, with a demonstrated favorable safety and tolerability profile. Sodium selenate has undergone Phase I testing in adults with prostate cancer[21](#_ENREF_21), a Phase II study for Alzheimer’s Disease (ACTRN12611001200976), and in infants as supplementation (NCT02066610), deferiprone has been approved by the Food and Drug Administration (FDA) for the treatment of iron excess[2](#_ENREF_2), Kineret (rrIL-1ra) has been FDA approved for rheumatoid arthritis[3](#_ENREF_3), VX-765 has undergone phase 2 clinical testing in patients with refractory epilepsy (NCT01048255, NCT01501383), and Z944 has completed phase 1 and 2 testing in healthy volunteers and people with pain disorders[4](#_ENREF_4). Levetiracetam is currently used for the acute management of early post-TBI seizures; we will test here if it has an effect on early stage biomarkers[13](#_ENREF_13),[16](#_ENREF_16),[31](#_ENREF_31),[46](#_ENREF_46),[60](#_ENREF_60),[85](#_ENREF_85),[89](#_ENREF_89),[97](#_ENREF_97),[100](#_ENREF_100),[112](#_ENREF_112),[116](#_ENREF_116). The rationale in selecting these drugs for testing as potential AEG treatments in the LFPI model is as follows.

Targeting Protein Phosphatase 2A (PP2A), h-tau: Sodium Selenate: The tau protein is a microtubule-associated protein that stabilizes microtubules and controls axonal transport[41](#_ENREF_41),[109](#_ENREF_109). H-tau has been implicated in neurofibrillary tangles, a characteristic pathology of Alzheimer’s dementia[41](#_ENREF_41) but also in TBI and in individuals with epilepsy[8](#_ENREF_8),[41](#_ENREF_41),[83](#_ENREF_83),[98](#_ENREF_98),[99](#_ENREF_99),[115](#_ENREF_115). Protein phosphatase 2A (PP2A) accounts for almost 70% of the phosphatase activity that regulates tau phosphorylation in the brain[47](#_ENREF_47). PP2A consists of 3 subunits: a structural, a catalytic and a regulatory subunit, like PR55, which is the principle regulatory subunit in the brain[47](#_ENREF_47),[66](#_ENREF_66). In the rodent models of TBI, increased phosphorylation of tau (p-tau) has been shown (reviewed in[73](#_ENREF_73)) but with model, time, or method-specific differences. In the LFPI model of TBI used here, the Shultz and O’Brien group showed early reduction in PR55 expression and PP2A activity, within 2hr post-TBI, an increase in h-tau, at 72hr post-TBI (**Figure 1**) and persisting chronically for at least 12 weeks post-TBI[93](#_ENREF_93). Similar findings are reported in humans who died following a TBI[93](#_ENREF_93). Sodium selenate, activates the regulatory subunit of PP2A, thus PP2A de-phosphorylates h-tau and prevents the resultant neurodegenerative pathologies (**Figure 2**)[22](#_ENREF_22),[93](#_ENREF_93),[102](#_ENREF_102). The group of Jones, Shultz, and O’Brien has shown that sodium selenate is AEG in the amygdala kindling, post-kainic acid (KA) status epilepticus (SE) and post-LFPI (Liu et al., submitted[49](#_ENREF_49)).

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| **Figure 1.** Time course of changes in the expression of p-tau and PP2A subacutely post-TBI in the LFPI model, based on Western blots from cortex. **A-B.** Increased p-tau at Ser 198 and Ser 262 is seen at 72hr post-LFPI. Results are expressed as % of sham control of the ratio p-tau / tau-5. **C-E.** LFPI reduces the expression of PR55 regulatory subunit of PP2A between 2–72hr (**C**), followed by reduction in PP2A activity (**D**). There are no significant differences in the expression of the catalytic subunit of PP2A (PP2Ac) (**E**). Results are expressed as % of sham control in the ratio of PR55 /GAPDH. N=5 rats/group. \*: P<0.05 vs sham control. These show that *the dysregulation of the PP2A/p-tau pathway occurs within 2hr from TBI*. |

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|  | **Figure 2.** Sodium selenate activates PP2A which de-phosphorylates tau and may prevent or restore the pathologies associated with h-tau[50](#_ENREF_50). |

In adult male rats, sodium selenate administration starting immediately after LFPI and continuing for 12 weeks (1 mg/kg/day via osmotic minipump), reduced the levels of p-tau phosphorylation at Ser 198 and/or Ser 262 to sham control levels (**Figure 3** F-G)[93](#_ENREF_93), increased PR55 expression and PP2A activity, reduced the extent of the often progressive injury seen in MRI and improved behavioral outcomes[93](#_ENREF_93). A subsequent study by Jones, Shultz, and O’Brien found that the same dose of sodium selenate administered for 14 weeks after LFPI significantly reduced the number and duration of spontaneous seizures at 12-14 weeks. There were also fewer seizures after washout (16-18 weeks after LFPI), suggesting an AEG effect (**Figure 4**) (Liu et al., manuscript submitted). A similar AEG effect of sodium selenate was seen after 10 weeks of treatment following KA-induced SE and after 4 weeks of treatment during amygdala kindling (Liu et al., manuscript submitted). Based on these promising data, we have selected *sodium selenate* to test in our standardized LFPI model for its effect on EEG and plasma biomarkers of PTEgenesis (Aim 1) and, if selected for testing in Aim 2, for a sustained AEG effect in the LFPI model, using a rigorous multicenter preclinical trial.

**Targeting iron deposits: Deferiprone:**The risk for PTE is significantly increased in patients with intracranial

hemorrhages[23](#_ENREF_23),[29](#_ENREF_29),[44](#_ENREF_44),[82](#_ENREF_82). Intracranial hemorrhages may increase iron deposits in the cerebral cortex, which may

trigger epileptogenesis[92](#_ENREF_92),[108](#_ENREF_108),[110](#_ENREF_110). Hemorrhages are also observed in the LFPI model, detected by hematoxylin & eosin (H&E) stain and T2\* MRI within 24hr[69](#_ENREF_69),[114](#_ENREF_114). The iron chelator deferoxamine attenuated the acute hydrocephalus in LFPI rats, 24hr post-LFPI[114](#_ENREF_114). In this project, we will study the effect of deferiprone, currently in use for treatment of iron overload in thalassaemia major, to test whether deferiprone doses that prevent iron accumulation in the perilesional region of rats subjected to LFPI can prevent PTE. To monitor the response to deferiprone, we will monitor the number and extent of intracerebral hemorrhages by MRI, but also the levels of ceruloplasmin, a ferroxidase that oxidizes ferrous iron (Fe2+) to ferric iron (Fe3+), facilitating its transport in the plasma via transferrin. TBI patients with reduced ceruloplasmin levels, within the first 24hr, developed increased intracranial pressure[24](#_ENREF_24). However, at three days (3d) post-TBI ceruloplasmin was elevated in the serum[24](#_ENREF_24). Ceruloplasmin was also increased in the serum of animals 2hr-1month post-TBI[5](#_ENREF_5), possibly due to compensatory changes to the increased iron load.

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| **Figure 3.** Sodium selenate (SS) administration (1mg/kg/day in SC osmotic minipumps) for 12 weeks, starting immediately after LFPI in adult male rats ameliorated the progressive injury and reduced h-tau in the cortex. **A.** T2-weighted MRI images show the progressive injury in LFPI rats, which is greater in vehicle (VEH) than sodium selenate (SS) treated LFPI rats. **B-E.** Volumetric analysis shows significant reduction in injury of LFPII+SS rats compared to LFPI+VEH rats in the ipsilateral cortex (**B**) and corpus callosum (**C**), size of ipsilateral ventricle (**E**) but has no effect on ipsilateral hippocampus (**D**). \*\*: P<0.05 vs both SHAM groups,\*\*\*: P<0.05 vs all other groups. **F-G.**SS normalized p-tau immunoreactivity IR) (ptau at Ser 198 (**D**) or Ser 262 (**E**)) ratios over total tau (Tau-5). \*\*\*: P<0.05 vs all other groups. Data are from[93](#_ENREF_93). N=12-13 rats /group. |

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| **Figure 4.** Sodium selenate attenuates epileptogenesis after LFPI in adult rats. **A.** Adult male rats received sodium selenate (1mg/kg/day) for 14 weeks in SC osmotic minipumps implanted immediately after the LFPI. **B-C.** The number of spontaneous seizures per day (**B**) and their duration (**C**) decreased in selenate-treated rats. The frequency of seizures was low 4 weeks after treatment but there was no difference in seizure duration. N= 14-16 rats/group pre-washout and 7 rats/group during post-washout. Data are from Jones Shultz & O’Brien (manuscript submitted). \*: P<0.05 vs bar-linked group. |

Targeting T channels: Z944**:**Low threshold T-type calcium channels underpin the neuronal burst firing, a key component of epileptic oscillatory neuronal network firing in both genetic and acquired epilepsies[90](#_ENREF_90),[111](#_ENREF_111). T-Type channels play a critical role in acquired epileptogenesis, in particular temporal lobe epilepsy. In the pilocarpine-induced SE model, upregulation of the T-type Ca2+ channel expression and T-type Ca2+ channel alters the intrinsic bursting firing properties of the hippocampal CA1 pyramidal cells that may be responsible for the initiation of epileptiform events[90](#_ENREF_90). In the same model, a selective and transient increase in Cav3.2 mRNA expression in CA1 pyramidal neurons was found coupled with an up-regulation of T-type calcium currents[12](#_ENREF_12),[90](#_ENREF_90),[96](#_ENREF_96),[111](#_ENREF_111). Critically knocking out the T-type channel gene, Cav3.2, prevented the development of epilepsy and associated neuropathology following pilocarpine induced SE[12](#_ENREF_12). In a model of penetrating ballistic-like TBI in male rats, ethosuximide, a relatively low-potency and non-selective T-channel blocker, reduced the incidence, frequency and duration of early nonconvulsive seizures[70](#_ENREF_70), but the authors did not study convulsive seizures. Z944 is a new high affinity selective pan-T type Calcium channel antagonist that inhibits absence seizures by 85-90% in the GAERS model[101](#_ENREF_101). Z944 delays progression of seizures in the amygdala kindling model of acquired TLE[17](#_ENREF_17), in the absence of any overt antiseizure effects on kindled seizures[17](#_ENREF_17), O’Brien’s group has further demonstrated that Z944, given 4hr after KA-induced SE for 4 weeks, can prevent spontaneous seizures 8-10 weeks post-SE (**Figure 5**) and reduced expression of Cav3.1 and Cav3.2, channels in the hippocampus of Z944-treated post-SE rats lasting beyond its washout period (**Figure 6**) (unpublished data).

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|  | **Figure 5.** Z944, a T channel blocker, has AEG effects in adult rats subjected to KA- SE. **A.** Study design. Rats implanted with EEG electrodes underwent KA induced SE. After 4h of KA induced SE, rats were assigned to 5 different cohort groups. Rats were implanted with SC osmotic pumps containing either vehicle, Z944 (30mg/kg/day), or LEV (200mg/kg/day) that delivered the treatment continuously for 4 weeks. |
| Immediately after the pump implantation, SE was stopped with diazepam and the rats underwent 4 weeks of continuous video-EEG. After 4 weeks of washout, EEG monitoring continued for 2 weeks. **B-D.** Z944, but not levetiracetam, treated rats for 4 weeks after SE, have significantly less spontaneous seizures (**B**), but of the same duration (**C**), or seizure class (Racine scale) (**D**). Shams had no seizures. Data show means ± SEM. \*\*\*: P<0.0001 between linked groups. (Casillas-Espinosa et al., Manuscript in Preparation). | |

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|  | **Figure 6.** Z944 treatment after SE reduced mRNA expression of T-type calcium channels in the hippocampus 9 weeks after the treatment ended (i.e., time of video-EEG in Figure 5. The relative mRNA expression to shams was used to assess the different groups. **A.** CaV 3.1 mRNA expression was significantly reduced in post-SE + Z944 treated animals when compared to post-SE + vehicle and post-SE+ levetiracetam (LEV) (\* *p*<0.05). **B.** Similarly, Z944 treatment significantly reduced CaV 3.2 mRNA when compared to post-SE + vehicle (\*\**p<*0.01) and post-SE + levetiracetam (\* *p*<0.05). mRNA CaV3.2 expression was also reduced compared to shams (\* *p*<0.05). |
| Relative mRNA expression is compared to shams. Sham groups were combined because there were no expression differences across the CaV isoforms. Data shows mean + S.E.M., n=7-8. (Casillas-Espinosa et al., Manuscript in Preparation). | |

These findings raise the possibility that Z944 may have AEG effects against acquired epilepsies. We will use here the LFPI model of PTE, which demonstrates epilepsy with both convulsive and nonconvulsive seizures to determine whether Z944 modifies early stage candidate EEG/plasma biomarkers of PTEgenesis (Aim 1C-D) and if it has AEG effects on both convulsive and nonconvulsive seizures post-TBI (Aim 2). We will also use Cav3.1, Cav3.2, and Cav3.3 mRNA levels as targets to determine the target relevance and optimal treatment window in the LFP model (Aims 1A, 1B). If selected for Aim 2 (AEG trial), the effects of Z944 will also be correlated with the MRI data [i.e., apparent diffusion coefficient (Dav)] in the thalamus and cortex), which implicated the thalamocortical network in the ictogenic network of post-TBI seizures[45](#_ENREF_45).

Targeting IL-1Kineret, VX-765*:*Small cohorts show increased susceptibility to either hemorrhagic injury or PTE in individuals with polymorphisms of the IL-1 receptor antagonist (IL-1ra) gene[42](#_ENREF_42) or of the IL-1 gene respectively[25](#_ENREF_25). Inflammatory responses post-TBI with increase in IL-1 have also been shown in rat LFPI[59](#_ENREF_59) during the first day of TBI (peak at 3hr). Mild LFPI in rats increased IL-1 and microgliosis in the hippocampus and cortex within 3-6hr[74](#_ENREF_74), whereas a single injection of Kineret (IL-1ra, 75µg i.p.) 30min after LFPI reduces microgliosis, prevents loss of myelin basic protein (MBP) and neuronal loss and improves locomotor behavior in the foot fault test at 18d[75](#_ENREF_75). Kineret immediately after TBI in rats also reduced inflammation and injury[43](#_ENREF_43). There is evidence for proepileptogenic effects of IL-1[86-88](#_ENREF_86),[103-105](#_ENREF_103), which raises the question of whether IL-1 receptor inhibition might prevent PTE. VX-765 is a caspase-1 inhibitor, which blocks synthesis of IL-1 and has shown antiseizure effects[87](#_ENREF_87), [103](#_ENREF_103). Because Kineret is not stable at room temperature, we will combine administration of Kineret (i.p. bolus) with VX-765 (minipump SC) to test if they normalize the early stage biomarkers of PTEgenesis (Aim 1C-D) and have AEG effects in the LFPI model (Aim 2).

**Rationale for studying levetiracetam*:*** Levetiracetam binds to synaptic vesicle protein SV2A, which is

involved in vesicle exocytosis[67](#_ENREF_67), and is currently used for the acute management of early seizures in patients

with TBI[13](#_ENREF_13),[16](#_ENREF_16),[31](#_ENREF_31),[46](#_ENREF_46),[60](#_ENREF_60),[85](#_ENREF_85),[89](#_ENREF_89),[97](#_ENREF_97),[100](#_ENREF_100),[112](#_ENREF_112),[116](#_ENREF_116). Levetiracetam has shown better cognitive outcomes in the Operation Brain Trauma Therapy (OBTT) study in 3 rat TBI models[62](#_ENREF_62). However, no differences in long-term functional outcomes or PTE development have been seen in human TBI patients treated with levetiracetam[31](#_ENREF_31). We will test here whether levetiracetam has an effect on early stage EEG and plasma biomarkers. This will be important for the design of the future clinical AEG study since TBI patients will have to be on levetiracetam, with the new drug being added on. It will also be important for the interpretation of the biomarker data (*Projects 1, 3*), since all of the TBI patients in *Project 3* receive levetiracetam for 7 days post-TBI.

pHFOs and rHFOSs: biomarkers of PTEgenesis***:*** Recent published work from UCLA[15](#_ENREF_15) (see **Appendix 2**) found pHFOs (**Figure 7**) in cortical areas within or adjacent to the injury site of 58% of TBI rats (n=12), but none in controls (n=14). All pHFOs were detected between 2 and 7 d after TBI. In these same areas within 1-3 d after the appearance of pHFOs, paroxysmal activity was found consisting of repetitive pHFOs and EEG spikes (rHFOSs) in a 10-16 Hz arcuate-shaped pattern. The EEG spike component of rHFOSs had a mean duration of 25±11 ms and corresponded with an increase in neuronal spike firing. However, four rats that had pHFOs and rHFOSs within the first week developed late seizures (follow up 31-168 days post-TBI), whereas none of the rats without these events developed late seizures. In *Project 2*, we will determine the effects of tested treatments on early stage (week 1) pHFOs and rHFOSs (Aim 1C), and will test whether these can be used as biomarkers predicting AEG effect in Aim 2.

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|  | **Figure 7.** pHFOs in the LFPI rat model recorded with wide band EEG. **A.** pHFOs associated with local slow waves in a rat 3d after LFPI. **B.** Local generation of pHFO next to the TBI site (ATBI). Black lines are recorded at a 0.1Hz-10kHz frequency band. Red lines went through 100-600Hz band pass. **C.** Electrode placement. Please see also Bragin et al[15](#_ENREF_15) in **Appendix 2** for more details. |

**3.2 Experimental Design**

3.2.1 Specific Aim 1:To apply a multi-modality early stage post-TBI screening protocol for selecting early onset candidate AEG treatments in the adult rat LFPI model, using the following criteria:

**Specific Aim 1A:** define target relevance and treatment window for the tested drugs;

**Specific Aim 1B***:*evidence for modification of early stage candidate epileptogenic targets by the tested drugs;

**Specific Aim 1C:** prevention of early post-TBI seizures and EEG biomarkers of PTEgenesis (pHFOs, rHFOSs, spikes), as a function of dose and treatment exposure;

**Specific Aim 1D:** normalization of early plasma biomarkers of PTEgenesis, as a function treatment exposure.

**Hypothesis 1**: A multi-modality early stage post-TBI screening platform for target relevance and persisting modification of early stage post-TBI seizures and EEG/plasma biomarkers beyond treatment exposure will help select optimal treatment protocols for candidate AEG treatments for PTE.

Deliverables:(1) Validate a rapid multi-modality screening platform for optimization and selection of lead AEG treatments for PTE. (2) Identify early stage MRI/EEG/plasma biomarkers predicting AEG treatment response.

Rationale: The scientific premise of Aim 1 is that the optimal treatment protocol to be tested for AEG potential in the standardized animal model of PTE will need to demonstrate: (1) evidence for target relevance during the treatment window (Aim 1A) and target engagement (Aim 1B) and (2) normalization of early stage PTE epileptogenesis biomarkers that predict treatment response (Aim 1C-D).

These criteria would eventually help select and optimize rational treatments and implement them only in subjects at risk (PTEgenesis-biomarker positive) and likely to benefit from the specific treatment (PTEgenesis biomarkers for treatment response), reducing therefore the NNT and follow up period in a future AEG trial. There are no validated biomarkers of PTEgenesis. Thus, in this Aim we will use the panel of general and mechanistic candidate biomarkers of PTEgenesis, while these are assessed and validated in

*Project 1.* In Year 3, the combined data from *Projects 1 and 2* will be cross-correlated to identify biomarkers of PTEgenesis that predict treatment response to be used in the preclinical AEG trial of Aim 2.

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|  | **Figure 8.** Study design of Aims 1A, 1B. *Aim 1A* will determine the target relevance and relevant treatment windows for each target, in rats subjected to LFPI comparing them to SHAM rats. Target investigation will be done at 2days (2d), 1 week, 2weeks, 4weeks, and 8 weeks, at which timepoints brains will be collected for either perfusion and histology or for |
| qRT-PCR or Western blots[17](#_ENREF_17), [18](#_ENREF_18), [32](#_ENREF_32), [48](#_ENREF_48), [93](#_ENREF_93). *Aim 1B* will determine the effects of treatments on the respective targets. After LFPI, rats will be injected with a bolus dose of the tested drug, 30min post-LFPI, and osmotic minipumps filled with either drug (either of 2 drug doses) or vehicle will be implanted SC. Minipumps will be removed 1week post-LFPI and rats will be sacrificed for histology or brain tissue collection for target investigation at 2weeks. Blood will be collected for drug levels at baseline, 30min post bolus drug i.p., 24hr, 72hr, 7d, and at 30min, 2hr, 4hr after minipump removal, to confirm rapid elevation to target levels, steady levels during treatment (Rx ON) and washout by the end of 2nd week (Rx OFF). See section 3.3, General Methods. We will use 5 rats/group. | |

## Aims 1A-1B: Experimental design and specific methods:

*Animals:* Adult Sprague-Dawley male rats (300-350g) will be used to minimize sample size and avoid potential sex differences in outcomes[6](#_ENREF_6),[33](#_ENREF_33),[34](#_ENREF_34),[38](#_ENREF_38),[76](#_ENREF_76),[78](#_ENREF_78) (See General Methods, section 3.3).

*Study design (Figure 8):* Einstein and Melbourne sites will be involved. A blinded, randomized, vehicle

controlled study design will be followed[63](#_ENREF_63),[58](#_ENREF_58),[36](#_ENREF_36),[37](#_ENREF_37). The study design for Aim1A and 1B is shown in Figure 8.

*Target Investigation* willinclude established immunohistochemistries or Western blots (WB) to or qRT-PCR for mRNA expression in the ipsilateral and contralateral hippocampus, perilesional cerebral cortex, and

thalamus as in **Table 1**[17](#_ENREF_17),[18](#_ENREF_18),[32](#_ENREF_32),[48](#_ENREF_48),[93](#_ENREF_93). We will use 5 rats/group/timepoint and drug, several targets per brain.

*Drug Treatments***:**Drugs will be delivered through a bolus injection 30min after LFPI, to initiate fast increase in drug levels, and implantation of SC osmotic minipumps immediately after, which will deliver a constant infusion of the drug till end of week 1. We will use the starting doses in **Table 2**.

**Table 1. Targets studied in Aim 1A and 1B.**

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| **Drug** | **Targets (antibody, source)** |
| *Sodium selenate* | Total tau (Tau-5, Millipore), p-Ser198 tau, p-Ser202-tau (AT8, ThermoFisher Scientific), p-Thr231-tau (AT180, Pierce), and p-Ser262-tau (ab64193, Abcam), PR55 (539521, Millipore)[49](#_ENREF_49), [93](#_ENREF_93). |
| *Deferiprone* | H&E stain[114](#_ENREF_114), Modified Perls stain, total iron assay[10](#_ENREF_10), ceruloplasmin (ABN39, SIGMA) [10](#_ENREF_10). |
| *Kineret (rrIL-1ra) / VX-765* | IL-1ra (PA5-21776, ThermoFisher Scientific), IL-1NBP1-42767, NFkB (8242S, Cell Signaling Technology), glial fibrillary astrocytic protein (GFAP) (MS1376P1, ThermoFisher Scientific), MBP (SMI-94, Covance Research products), Iba1 (016-20001, Wako Chemicals) [40](#_ENREF_40), [75](#_ENREF_75), [87](#_ENREF_87), [95](#_ENREF_95). |
| *Z944* | qRT-PCR protocols mRNAs for Cav3.1, Cav3.2, Cav3.3 (as in [17](#_ENREF_17)). |
| *Levetiracetam* | anti-SV2 antibody (Developmental Studies Hybridoma Bank) [67](#_ENREF_67). |

**Table 2. Drug treatments in Aim 1B.**

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| **Drug** | **Doses** |
| *Sodium selenate* | Bolus: 1mg/kg i.p.; Minipump delivery rate: 0.3 - 1mg/kg/day (see Figure 4). |
| *Deferiprone* | Bolus: 200 mg/kg i.p.; Minipump delivery rate: 70 vs 200 mg/kg/day [106](#_ENREF_106) |
| *Kineret / VX-765* | Kineret Bolus: 100 mg/kg i.p. followed by Minipump VX-765 delivery rate: 70vs 200mg/kg/day [68](#_ENREF_68), [71](#_ENREF_71) |
| *Z944* | Bolus: 30mg/kg i.p.; Minipump delivery rate: 10 vs 30mg/kg/day [17](#_ENREF_17). |
| *Levetiracetam* | Bolus: 200mg/kg i.p. Minipump delivery rate 70 vs 200mg/kg/day [39](#_ENREF_39) |

*Drug levels during treatment (RX ON) and following drug washout (RX OFF):* In Aim 1B, blood will be collected for drug levels at baseline, Rx ON period (30min post i.p. bolus, 24hr, 72hr, 7d, post-minipump placement) and Rx OFF (at 30min, 2hr, 4hr after minipump removal) and will be sent to the Minnesota site for Pharmacokinetics (PKs). Based on the PKs, we will adapt the doses to generate steady levels during the Rx ON period. Confirmation of low-to-undetectable levels at Rx OFF will allow the evaluation of persistent effect beyond exposure to treatment. If Rx OFF levels are detectable, additional later endpoints will be added to determine the washout period and interpret the effects of the drug in Aims 1C-D and 2.

Aims 1A-1B: Data analysis and expected results**:** We will utilize the harmonized methodology of data collection, evaluation, scoring measures and outcomes, and reporting across the collaborating sites, as above. Aim 1A will determine whether the expression of the targets of the selected drugs is altered in the LFPI and will define the optimal treatment window. We will not screen sodium selenate, which has already been characterized by the O’Brien group (section C1) but will only define its PKs. We expect to find hemorrhages by H&E stain ipsilateral to the lesion and increased iron deposits post-LFPI (by iron stains) by 2d, based on[114](#_ENREF_114) and preliminary data (see *Project 1*), suggesting that treatment needs to start early after LFP. Iron and ceruloplasmin-ir in the brain (Aim 1B) will be correlated with the time course of plasma ceruloplasmin (Aim 1D) to test if plasma ceruloplasmin reflects iron/ceruloplasmin changes in the brain. We expect an early (within 2d post-TBI) increase in pro-inflammatory markers (IL-1, Iba1, GFAP, NFkB) and reduction of MBP (~14d), consistent with the literature[74](#_ENREF_74),[75](#_ENREF_75). Cav3.1-3 channel expression will determine if their dysregulation (possibly increase in expression) may be an early dysfunction that might suggest a benefit from Z944. We will also study SV2A expression to determine if LFPI modifies the target of levetiracetam. The pathways that are disrupted are candidates for early intervention. Therefore, treatment will be started early (here at 30min post- LFPI). The treatment window will determine the duration of treatment in Aim2. In Aim 1B, we will determine the PKs of the tested drug and the levels that modify their targets; PK-PD modeling will therefore be used to generate steady levels. The targets that relate to the drugs’ mechanisms, are listed in Table 1. We will select then two doses (**Table 2**) adapted to reproduce the active levels that modify the targets. Brain and plasma PKs will be done. Persistence of the drug effect during the Rx OFF period (when PKs confirm no drug levels) may suggest enduring modifying effect.

Aims 1A-1B: Caveats and alternative approaches:If the preselected timepoints (Aim 1A) do not demonstrate target relevance of a drug that is expected to alter the expression of the studied targets, it will be

a **NO GO** decision point for the drug. Z944 also functionally inhibits T channels and therefore its effect may not be necessarily dependent upon the change in T channel expression reported in Figure 6. If the selected doses of drugs in Aim 1B do not normalize the target, we will test a higher dose that is well tolerated. Blood levels of the drugs at the time of minipump removal will exclude the possibility of faulty delivery. It is possible that a drug may not show evidence of target engagement during the period of observation because it may require longer than 1week to act. Then, we may prolong the duration of treatment. Levetiracetam will be tested in Aim 1C-D, regardless of SV2A expression, because it is commonly used in TBI patients during the acute 7d post-TBI period. If drug levels are not undetectable by 14d, we will prolong the washout period till the drug is cleared.

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|  |
| **Figure 9.**  EEG electrode placement (**A, B**) and study design for Aim 1C-1D (**C**). In panel **A**, the blue electrodes are epidural screw electrodes and the pink are microelectrodes targeting the cerebral cortex (layers 2/3 or layers 5/6) or dentate gyrus of the hippocampus. **B.** We will use the full montage in Aim1C and Aim 2 at 7months and minimal montage in Aim 2 at 12months post-TBI. |

## Aim 1C – 1D: Experimental design and specific methods:

*Study Design* (Figure 9*):* Einstein, Melbourne, and UCLA sites will be involved. A blinded, randomized, vehicle controlled study design will be done[63](#_ENREF_63),[58](#_ENREF_58),[36](#_ENREF_36),[37](#_ENREF_37). EEG electrodes will be placed after the LFPI, and wide band video-EEG recordings (Full montage) will be done for 2 weeks. An osmotic minipump (vehicle vs drug dose with best effects on targets in Aim 1B) will be placed after EEG implantation and will be removed at 7d. Blood will be collected at 2d, 7d, and 14d for plasma targets and drug levels (7d only, sent to Minnesota site). Rats will be sacrificed at 14d (half of the brains will be perfused, half will be fast frozen) and stored in the *BioBank* (see Aim 3). *Exclusion criteria:* (1) >15% weight loss during first post-TBI weeks, (2) loss of electrode headset more than once, (3) poor well-being assessed using standard Animal Center protocol, (4) undetectable drug levels in 1 blood draw in rats assigned to drug-treatment. Methods are as in section 3.3. EEG review will be blinded to group by two readers per site and unbiased automated analysis of EEG will be done by the *Informatics and Analytics Core (IAC)* EEG Core. We will use 57 rats/group to reach 90% power to detect a decrease in pHFO incidence from 58% to 29% of rats. The outcome measures and endpoints are in **Table 3**.

**Table 3. OUTCOME MEASURES AND ENDPOINTS IN EPIBIOS4RX – PROJECT 2**

|  |  |  |
| --- | --- | --- |
| **Aim** | **OUTCOME MEASURES** | **ENDPOINTS** |
|  | **A. PRIMARY EFFICACY MEASURES** | **A. PRIMARY EFFICACY ENDPOINTS** |
| 2 | Presence of PTE at the 7th and 12th month post-TBI | ***RR50%- (Seizure-freedom)*** at 7mo or 12mo post TBI A |
| 2 | Duration of seizures monitored at the 7th and 12th month post-TBI | ***RR50%- (Seizure duration)*** at 7mo or 12mo post TBI B |
| 2 | Frequency of seizures, monitored at the 7th and 12th month post-TBI | ***RR50%-(Seizure frequency***) at 7mo or 12mo post-TBI C |
| 2 | Type of seizures, Racine scale monitored at the 7th and 12th month post-TBI | ***Maximal Racine scale*** at 7mo or 12mo post-TBI  ***Average Racine scale*** at 7mo and 12mo post-TBI |
|  | **B. SECONDARY EFFICACY MEASURES** | **B. SECONDARY EFFICACY ENDPOINTS** |
| 1B | Presence of early seizures | ***RR50%- (Early Seizure-freedom)*** |
| 1B | Time to 1st early seizure | Time to 1st early seizure |
| 1B | Frequency of early seizures | ***RR50%-(Early Seizure frequency)*** |
| 1B | Duration of early seizures | ***RR50%- (Early Seizure duration)*** |
|  | **C. OTHER MEASURES (for biomarkers / targets)** | **B. OTHER ENDPOINTS (for biomarkers)** |
|  | ***Other EEG measures*** | ***Other EEG endpoints*** |
| 1B | Presence of pHFOs | ***RR50%-(pHFOs-freedom)*** at 1week, 7mo, 12mo post-TBI |
| 1B | Frequency of pHFOs (pHFOs /hr) | ***RR50%-(pHFOs frequency)*** at 1week, 7mo, 12mo post-TBI |
| 1B | Presence of rHFOSs | ***RR50% (rHFOSs-freedom)*** at 1week, 7mo, 12mo post-TBI |
| 1B | Frequency of rHFOSs (rHFOSs /hr) | ***RR50%-(rHFOSs frequency)*** at 1week, 7mo, 12mo post-TBI |
| 1B, 2 | Presence of epileptiform discharges (EDs) (EDs /hr) | ***RR50%-(EDs-freedom)*** at 1st week, 7mo, 12mo post-TBI |
| 1B, 2 | Frequency of EDs | ***RR50%-(EDs-frequency)*** at 1st week, 7mo, 12mo post-TBI |
|  | ***Imaging measures*** | ***Imaging endpoints*** |
| 2 | Volumes and shape statistics of the:  hippocampus, thalamus, remaining ipsilateral cortex, and ventricles (ipsilateral, contralateral) | 20% change in ipsilateral region of interest (ROI) volume from vehicle-treated rats, time-adjusted.  Contralateral ROI volumes will be reported. |
| 2 | For white matter changes: quantitative MTR values, phase, diffusion anisotropy measures: fractional, linear, planar, and spherical, diffusivities: axial, radial, and mean in selected atlas-aligned ROIs. | 20% change in WM values (MTR, phase, diffusion anisotropy) from vehicle-treated rats, time-adjusted |
| 2 | Number and extent of hemorrhages as detected in gradient echo and phase images, quantitative magnetization transfer ratio, scalar DTI parameter, and phase maps | Significant reduction from vehicle-treated LFPI rats |
| 2 | Fiber-orientation distribution tractography and high angular resolution diffusion imaging (HARDI) using high-order spherical harmonics | Significant change from vehicle-treated LFPI rats |
|  | ***Plasma target measures*** | ***Plasma target endpoints*** |
| 1D, 2 | Relative Intensity of target (-fold change vs control) D | Change in relative intensity vs control |
|  | ***Tissue target measures*** | ***Tissue target measures*** |
| 1A | Relative Intensity of target (-fold change vs control) E | Change in relative intensity vs control |
|  | **D. TOLERABILITY MEASURES** | **C. TOLERABILITY ENDPOINTS** |
| 1B, 2 | Survival, time course | ***Survival rate*** at end of 1st week, 7mo, 12mo post-TBI.  Reduction in survival rate vs vehicle: **NO GO** |
| 2 | Neuroscore, time course (baseline, 2d, 7d, 15d) | Change in Neuroscore, time-adjusted.  Reduction in time-adjusted Neuroscore vs vehicle: **NO GO** |
| 1B, 2 | Body Conditioning Score (BCS) | % of Rats with BCS ≤2 , time adjusted  ***BCS of 2 is a criterium for euthanasia***  Increase in % of rats with BCS ≤2 vs vehicle: **NO GO** |
| ***From Aim 1 to Aim 2 (with input by DSMB, Public Engagement Core, Consultants):*** | | |
| ***GO CRITERIA****: (A) best performance on endpoints of EEG / plasma biomarkers validated by* ***Projects 1 and 2****, (B) best tolerability, (C) evidence of target relevance / engagement of known target.*  ***NO GO CRITERIA:*** *(A) fails 1 tolerability endpoint, (B) lack of target relevance/engagement of known target (Aim 1A-B).* | | |
| ***From Aim 2 to clinical trial (with input by DSMB, Public Engagement Core, Consultants):*** | | |
| ***GO CRITERIA****: (A) best performance on primary efficacy endpoints,* ***AND*** *(B) best performance on endpoints on EEG / plasma biomarkers validated by* ***Projects 1 and 2*** *(Year 3), (C) best tolerability, (D) evidence of target relevance / engagement of known target.*  ***NO GO CRITERIA:*** *(A) failure to meet 1 tolerability endpoint,* | | |
| **EXPLANATIONS OF ENDPOINTS:**  A: *RR50%- (Seizure-freedom):* Reduction in proportion of rats with epilepsy by ≥50% vs vehicle-treated of same timepoint  B: *RR50%- (Seizure duration):* Reduction in seizure duration by ≥50% vs vehicle-treated of same timepoint  C: *RR50%-(Seizure frequency*): Reduction in seizure frequency by ≥50% vs vehicle-treated of same timepoint  D: ***Plasma targets*** include: (1) Protein targets for: *Neuronal and glial cell death* (NSE, neuron specific enolase; CK-BB, creatine kinase BB; S100B, S100 calcium binding protein B; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein); A*xonal Injury* (Tau; P-tau; NF-H, neurofilament-H; SNTF, calpain-derived -II spectrin N -terminal fragment); *Inflammation* (IL-1 IL-6, interleukin-6; IL-8, interleukin-8; TNF-, tumor necrosis factor alpha; IFN-, interferon gamma); *Metabolic Changes* (Ceruloplasmin; HIF-1a, hypoxia-inducible factor 1 alpha). (2) miRNAs: selection of miRNAs and power analysis will be done based on results of *Project 1*.  E: ***Tissue targets*** will be as in **Table 1**. | | |
| **DEFINITIONS:** Early seizures: Seizures within the first 7 days post-TBI. Late seizures: Seizures after the 7th post-TBI day.PTE: At least 1 late seizure after TBI.Epileptiform Discharge (ED): isolated spikes/ polyspikes/ spike wave discharges (SWDs) with no clinical correlate. Electrographic seizure 1: rhythmic spikes above baseline activity that rapidly progresses into high amplitude rhythmic spikes, spike-wave or polyspike-wave complexes, and eventually regresses into arrhythmic spike-waves with decreasing amplitude lasting ≥10 s [55](#_ENREF_55). Electrographic seizure 2: high-amplitude rhythmic discharges that represent a new pattern of activity (repetitive spikes, spike-and-wave discharges, slow waves) lasting ≥10 s [55](#_ENREF_55). Motor (Convulsive) seizure: Racine scale score ≥3 [84](#_ENREF_84). | | |
| **CLASSIFIERS: Seizures will be described as: electrographic vs electroclinical; convulsive vs nonconvulsive; focal vs generalized; If focal: onset at X region.** | | |

Aim 1C – 1D: Data analysis and expected results: Confirmation of the validity of these EEG/plasma candidate biomarkers will be at the end of Year 3, when *Project 1* and *Project 2*-Aim1 will be completed. We expect that ~58% vehicle-treated LFP rats will demonstrate pHFOs and rHFOSs, as described in[15](#_ENREF_15" \o "Bragin, 2016 #31458) and ~40% of rats will have early seizures, during the 1st post-TBI week. If *Project 1* validates the findings of Bragin et al.[15](#_ENREF_15) that pHFOs and rHFOSs predict epileptogenesis, we will select the drugs that suppress these EEG biomarkers in Aim 1C for possible advancement to Aim 2. Drugs that show persisting suppression of pHFOs / rHFOSs beyond the treatment exposure window will be preferred. Drugs that prevent early seizures will be investigated as possible antiseizure drugs, regardless of the effect on pHFOs / rHFOSs. If *Project 1* indicates certain plasma biomarkers as biomarkers of PTEgenesis, we will select drugs that normalize these plasma biomarkers for Aim 2, giving priority to those with persisting effects (during both Rx ON and Rx OFF period). In this Aim, we will screen all 16 plasma biomarkers against the tested drugs to determine if they are target specific or more general biomarkers of treatment response. The latter would be more promising as potential biomarker predicting AEG effect. We will determine whether the levels of plasma biomarkers and the frequency or presence of pHFOs / rHFOSs depend upon recent seizure activity. Drugs that modify plasma biomarkers of PTEgenesis independent of acute antiseizure effects will be preferred for Aim 2. If a drug has antiseizure effect, we will compare its effects against levetiracetam. We will use the *GO / NO GO* criteria in **Table 3** to select the lead compound(s) and biomarkers to advance to Aim 2 testing will be done in consultation with the *DSMB* experts, our expert consultants, and the *Public Engagement Core*, which will provide the consumer’s perspective on efficacy and tolerability issues, appropriateness of a biomarker for use in the clinical practice.

Aim 1C – 1D: Caveats and alternative approaches:It is possible that the frequency of pHFOs and rHFOSs may vary significantly, rendering the associations with recent seizures challenging. Automated analysis by the *IAC* of large samples of EEGs may solve this issue. We will measure drug levels at the end of week 1 to confirm that drug delivery was reliable and to compare the drug levels across rats. Rats assigned to the treatment arm displaying undetectable levels at 7d will be excluded. We anticipate 30% mortality acutely, which has been addressed by increasing the number of planned LFPI surgeries. If unexplained mortality is observed, the trial for the drug will be stopped and unmasked to determine if this may be due to drug toxicity. If none of the candidate biomarkers is validated for PTEgenesis by *Project 1* at the end of Year 3, we will select for Aim 2 testing a drug with proof of target relevance and engagement to advance to Aim 2. Sodium selenate may be a candidate based on the preliminary data for an AEG effect (section C1) that we will attempt to validate. The inclusion of three centers in the Aim 1C-D study (Einstein, Melbourne, UCLA) will require procedures for harmonization of methodologies, data collection and reporting and data analyses to minimize the variability in results. We will create CDEs, standardized protocols, and across sites visits to minimize these issues. TBI inherently demonstrates significant variability in pathology[113](#_ENREF_113) and therefore, if successful, the results of a multicenter trial will be more likely to be robust and translatable.

3.2.2. Specific Aim 2:To determine whether an optimized targeted treatment selected by the early stage post-TBI multi-modality screening process can:

**Specific Aim 2A:** have AEG effects in adult rats with LFPI when given during defined therapeutic windows.

**Specific Aim 2B***:*modify the MRI/ EEG/plasma biomarkers of PTEgenesis identified in *Project 1*, in a manner that can predict its AEG effect.

**Hypothesis 2:** Targeted early stage treatments that have lasting modifying effects on relevant targets and MRI/EEG/plasma TBI biomarkers also have lasting AEG mitigating effects against PTEgenesis.

**Deliverable: (1)** Identify at least 1 AEG treatment in the LFPI rat model of PTE that is for suitable for clinical trials. (2) Validate at least 1 early stage biomarker of PTE epileptogenesis predicting AEG treatment response.

**Rationale:** Identification of AEG treatments is a priority in epilepsy research [30](#_ENREF_30), [37](#_ENREF_37), [81](#_ENREF_81). The costs and time

required for clinical AEG trials is considerable and can be further multiplied when failures to replicate preclinical findings are considered[28](#_ENREF_28),[35](#_ENREF_35),[72](#_ENREF_72),[94](#_ENREF_94). The adoption of rigorous and unbiased standards of practice and the creation of multicenter preclinical phase II trials, as done in clinical studies, may also reduce the risk of advancing treatments that are likely to fail[72](#_ENREF_72),[94](#_ENREF_94),[107](#_ENREF_107). In this Aim, we therefore plan to create a multicenter preclinical study to test the AEG potential of the lead treatment from Aim 1 in the LFP model of PTE, incorporating and possibly guided by the biomarkers identified by *Project 1* and Aim 1 of *Project 2.*

**Aim 2: Experimental design and specific methods:** (please see General Methods, section 3.3).

*Study design*(**Figure 10**): (Einstein, Melbourne, UCLA, UEF) A rigorous, blinded, randomized, vehicle controlled study design will be done[63](#_ENREF_63),[58](#_ENREF_58),[36](#_ENREF_36),[37](#_ENREF_37). Rats will receive LFPI and Neuroscores will be recorded. A bolus i.p. injection of drug or vehicle will be given 30min after LFPI to raise levels acutely followed by minipump implantation. Minipumps will be removed at 6wk and replaced with a second pump till the 12th week, as follows: (vehicle x 6wk) + (vehicle x 6wk); (drug x 6wk) + (vehicle x 6wk); (drug x 6wk) + (drug x 6wk). Structural MRI (T2 weighted, DWI, DTI, SWI) will be done at the best predictive early timepoint (2d or 9d or 1month) (as per *Project 1*) and at 5 months. Blood for plasma biomarkers (protein, miRNA) will be collected at the best predictive early timepoint (2d or or 7d or 9d or 1month) (as per *Project 1* and *Project 2-Aim 1*) and at end of the 7th month video-EEG. Blood collection for drug levels will be done after each minipump is removed to confirm delivery of drug. EEG electrodes will be implanted at the 6th month and video-EEG monitoring will be done in the 7th (full EEG montage, Figure 9A) and 12th months (Minimal seizure montage, Figure 9A) using wide band video-EEG recordings. Rats will be perfused at end of 12th month for ex vivo MRI to assess injury and will be stored at the *BioBank* (Aim 3). *Exclusion criteria:* as in Aim 1C-1D. We will use 121 rats/group (Power Analysis, section 3.3). The outcome measures and endpoints are in **Table 3**.

Aim 2: Data analysis and expected results**:** We have powered the study to identify a drug that reduces the risk for PTE by 50% (from 30% to 15% at 7months), based on prior studies[55](#_ENREF_55). Please note that we define PTE rates based on the electrographic seizure type 1 definition (**Table 3**), because such seizures have not been seen in sham controls at this age, according to[55](#_ENREF_55) but also according to data from the Einstein, Melbourne, and UCLA groups. These seizures are first detected in the ipsilateral ventral hippocampus and can be associated with a Racine score 3.3±1.4 (range 1-5, median 4)[55](#_ENREF_55). The definition of electrographic seizures type 2 (**Table 3**, from[55](#_ENREF_55)) may also include SWD bursts, which have been reported in certain rat strains in sham control animals. We include them to differentiate the effects of drugs in SWD bursts vs other seizures. Future studies may determine whether drug effects are different in SWD bursts seen in PTE vs sham controls. We will record the type of seizures (i.e., nonconvulsive vs convulsive, see **Table 3**) using the Racine scale[84](#_ENREF_84). Our primary outcomes will consider epilepsy in general (all types included) but we will also study the trends for convulsive vs nonconvulsive seizures, which will be useful for future studies but also the clinical trial design. The presence of convulsive seizures in the standardized LFPI model presents an advantage, given that motor seizures in patients may be more impactful than nonconvulsive seizures. We will include the plasma biomarkers that predict treatment response relevant to the tested drug (i.e., they are either drug-specific or general biomarkers of treatment response). Blood collection will be done at the end of each video-EEG session (1st week, 7months) to account for prior seizures. Results will be analyzed as a function of recent seizures to observe trends that will inform future studies. We will also select the timepoints and MRI sequences that provide information on extent of injury and/or serve as biomarkers of PTEgenesis (from *Project 1*). We expect to identify at least 1 AEG drug, based on preliminary data and rationale (section C1). It is unlikely that levetiracetam will be the lead drug since it has not shown such an effect in clinical practice[61](#_ENREF_61). Project 2 will also validate the biomarkers with high sensitivity/specificity identified in *Project 1* in an independent animal cohort. If highly predictive biomarkers of PTEgenesis are identified in *Project 1 (*MRI, plasma), we will use them to select out the LFPI rats at risk for PTE (i.e., biomarker-positive) to minimize the sample size needed to observe the AEG effect. If a plasma (protein and/or miRNA) biomarker has shown high predictive value for both PTEgenesis and treatment response, we will test it for its ability to predict AEG effect. If confirmed, it may shorten the duration of future AEG studies in the model and accelerate the screening. With the *DSMB,*

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|  | consultants, and *Public Engagement Core,* the investigators of *Project 3,* and the GO/NO GO pre-set criteria (**Table 3**), we will determine the best drug and study design to prepare a future clinical trial on the lead compound. |

Aim 2: Caveats and alternative approaches: The LFPI model shows 30% acute mortality and 25% follow up mortality or headmount loss; we have adapted the numbers of rats accordingly. Treatments that show antiseizure effects will be compared with levetiracetam. We are testing tolerability using a minimal battery of assessments and did not include behavioral tests that can inform on *comorbidity* modification, because of cost and logistic issues. We do plan however to apply for future research funding to support such studies and determine the disease-modifying effect of these compounds. *We also recognize the importance of testing both sexes. However, because of the high sample size demands to meet power criteria, we chose to exclude females and plan for future grants to support such important studies. Alternatively, if the predictive value of the biomarkers is high, we may be able to reduce samples sizes and incorporate some of these studies in this project*. We have not included video-EEG monitoring in the 1st week, because of concerns about headmount losses till the 7th month that could significantly limit our ability to confirm an AEG effect. If the EEG biomarkers are highly predictive of PTE and or treatment effect, we will incorporate early (1st week) video-EEG monitoring, to determine if the treatment effects on early EEG biomarkers are predictive of AEG effect. We also do not test early vs late initiation of treatment, due to sample size issues, but future studies will address this issue. The inclusion of 4 centers in the Aim 2 study requires procedures for harmonization of methodologies, data collection and reporting and data analyses to minimize the variability in results. We intend to create CDEs, standardized protocols, and across sites visits for training. However, robust results of the multicenter trial will more likely be translatable to the clinic. This approach has already been implemented in stroke research through collaborative multicenter preclinical trials, e.g. MultiPART[26](#_ENREF_26), [107](#_ENREF_107). We will analyze the results as a function of injury (Neuroscore, MRI) to determine if these affect the AEG effect. Here, we focus on early onset treatments. Different therapeutic windows (i.e. early vs late) may be addressed in the future.

## 3.3.3. Specific Aim 3: Create a Rodent Biospecimen Repository (BioBank)

**Hypothesis 3:** A Rodent Biospecimen Repository will serve as a resource for future research to identify

targets and biomarkers that will support future AEG trials.

Deliverable:A Rodent Biospecimen Repository (BioBank) for future AEG target/biomarker discovery in PTE

Rationale**:** The availability of biospecimens from rodents with specific phenotypes (with or without epilepsy,

responders vs nonresponders to treatment) is invaluable and requires long observation periods and high cost and efforts to produce. However, the creation of a BioBank that provides such well characterized specimens to investigators with advanced technologies or novel testable hypotheses would be unique and would significantly

advance research progress and collaborations beyond the group of centers involved in this consortium.

Aim 3: Experimental design and specific methods:No further experiments will be done. Blood and brains

(perfused or frozen) from Aims 1 and 2 will be stored at each site, catalogued and linked to the data of the rat and uploaded to a central database at the *IAC***.** Biospecimens will be stored at the site of collection (if brains) or analysis (if plasma). The PIs of the relevant sites will be the contact persons to communicate with interested investigators who seek research collaborations. The data sharing resource policies and publication policies of this grant application will apply to these biospecimens and users.

Aim 3: Data analysis and expected results: No data are planned in this Aim. The creation of the BioBank will however allow for new research to stem out of the stored biospecimens. Interested investigators will submit proposals to collaborate and utilize these specimens. Such proposals will be evaluated by the PIs of our grant for their promise and value and will be made available to the interested parties.

Aim 3: Caveats and alternative approaches**:** None foreseen.

# 3.3. general METHODS

*Animals*: Adult male 12-week old Spraque-Dawley rats (300-350 g) will be purchased from local vendors. 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. Only male rats will be used in all experiments of this project to minimize sample size and reduce the variability[6](#_ENREF_6),[33](#_ENREF_33),[34](#_ENREF_34),[38](#_ENREF_38),[76](#_ENREF_76),[78](#_ENREF_78). *Recognizing the importance of investigating sex as a variable*[*19*](#_ENREF_19)*,*[*33*](#_ENREF_33)*, we will plan to seek funding for similar studies in adult female rats****. Study design:*** A blinded, randomized, vehicle controlled study design will be followed, in accordance to the Landis et al[63](#_ENREF_63), ARRIVE[58](#_ENREF_58), and AES/ILAE Translational Task Force guidelines[36](#_ENREF_36), [37](#_ENREF_37). ***LFPI*** [49](#_ENREF_49" \o "Jones, 2012 #31606), [55](#_ENREF_55" \o "Kharatishvili, 2006 #31809), [93](#_ENREF_93" \o "Shultz, 2015 #2411), ***subcutaneous (SC) osmotic minipump placement***[***49***](#_ENREF_49)***,*** [***93***](#_ENREF_93)***:*** Details are in Vertebrate Animal section.Rats are anaesthetized with isoflurane. 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. 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 (≈30% mortality). SHAM animals receive anesthesia and all surgical procedures without LFPI. Minipumps (ALZET 2004 or 2006) will be placed SC immediately after LFPI.  ***Neuroscore:*** Neuroscore will be done 1d prior, at 2d, 7d, 14d post-LFPI[79](#_ENREF_79)**. *Blood collection*** will be doneunder isoflurane anesthesia through tail vein.*Short-term PK studies (Aim 1B):* Drugs will be administered via subcutaneous minipumps. For each drug, 2 doses will be tested, 3 samples/drug/timepoint/dose. Blood samples will be collected at 7 time points: 30min post bolus drug i.p., 24hr, 72hr, 7d, and at 30min, 2hr, 4hr after minipump removal. PK analysis of plasma and brain samples will be done at the Minnesota site, according to standard procedures for drug levels[64](#_ENREF_64),[65](#_ENREF_65), using a high performance liquid chromatography (HPLC) system coupled to a triple quadrupole mass spectrometer (HPLC-MS/MS). PK modeling will use nonlinear mixed-effects model, a one-compartment PK model to model the data and two- and three-compartment models will be evaluated as needed. Goodness of fit criteria will be applied. The PK models described above will be used to help explain results of the short-term studies and to simulate dosing regimens for the longer-term mini-pump studies completed during Years 4-5. *Plasma biomarkers:* Plasma for proteinmicroarray assay will be shipped to Dr. Agoston for analysis of the 16 protein markers (See **Table 3**) as in reference[5](#_ENREF_5) (see also *IAC*). Differences from vehicle-treated LFP rats will be analyzed with independent t-tests. A Bonferroni adjusted significance level of 0.0125 will be used. Relative intensity (y-cept) will be used. Plasma for miRNA analysis will be sent to UEF and shipped to Exiqon (Denmark) for further data analyses (see methods and analyses in *Project 1*). ***MRI (in vivo, ex vivo):***In vivo MRI will be done under isoflurane anesthesia and monitoring vitals at an early (most predictive of 2d, 9d, 1mo timepoint, per *Project 1*) and late timepoint (5months) and ex vivo MRI will be done at end of the 12th month to assess lesion. The following sequences will be done: anatomical, MT and SWI/phase imaging,Diffusion tensor MRIdata for microstructural characterization and fiber tracking, Diffusion MRI tractographyfor structural connectivity between regions of interest (ROIs), andex vivo MRI and histology**.** Outcome measures are in **Table 3**. Anatomical high-resolution data collected with FISP will be transferred to the *IAC*for group level morphometric analysis that will be aligned with the methodology in *Project 3.* Other data analysis will be done at each site using identical analysis tools and procedures. The data will be compared between drug and vehicle treated LFP rats, with or without epilepsy. To determine the biomarker value for diagnosing epileptogenesis correctly, area under curve (AUC), sensitivity, and specificity will be assessed using ROC analysis for each parameter alone and in combination with other measures. *EEG electrode / microelectrode placement and long-term video-EEG monitoring*(See Vertebrate Animal section for methods): Placement of electrodes will be as in Figure 9. EEG review for seizures will be done blinded to group, at the 4 sites, by experienced readers. Automated analysis and pHFOs / rHFOSs and unsupervised EEG analysis will be done through the *IAC*-EEG Core and as in[15](#_ENREF_15). *Electrographic seizure* definitions are in **Table 3***.* Epileptic events occurring with an interval < 5 s without return to baseline are defined as one seizure. Harmonization of methods and criteria for seizures will be done in Year 1, months 1-3 across centers to optimize the inter-rater variability for seizure detection and pHFOs / rHFOSs studies[15](#_ENREF_15). *Histology, Western blots, qRT-PCR:*These will be done by established procedures as in[17](#_ENREF_17), [18](#_ENREF_18), [32](#_ENREF_32), [48](#_ENREF_48), [93](#_ENREF_93).

***Statistics:*** Generalized linear mixed models accounting for repeated observations from each animal will be used to determine the effects of treatment on % of rats with specific responses (seizure-freedom, seizure frequency, seizure duration, Racine scale score, spike cessation) across the timepoints of assessment. Effects of treatment on survival will be analyzed with log-rank tests and Cox proportional hazard models to estimate the hazard ratio for the treatment groups compared to the vehicle. Linear mixed models will be used to compare rates of weight gain across groups. Neuroscores will be analyzed by the Kruskal-Wallis test. To determine the effect of duration of treatment (6 vs 12 weeks) on % of seizure freedom, a Mantel-Haenszel test will be performed stratified by dose. Histology measures (% area and number of positive cells) will be compared with generalized linear mixed models accounting for repeat measures observations from each rat.

*Power Analyses:* For the AEG trial, with 121 rats/group, we have 80% power to detect a 30% vs 15% difference in epilepsy rate at 7months between vehicle group and the treatment group at (=0.05, 2-tail). Epilepsy rate at 12months is 50% and therefore, we will have a 90% power to detect a change from 50% to 25% risk for epilepsy at 12 months, with a sample size of 76 rats / group (=0.05, 2-tail). Considering a 25% attrition (mortality, headmount dislocation) we expect to have sufficient sample size (~90 rats/group) to show this effect at 12 months. In the MRI studies, 121 rats / group will give us 93% power to detect a change from 60% to 80%, using nonlinear calculations; 80% power to detect a change from 60% to 76.5%; and an 89% power to detect a change from 50% to 70%. For pHFOs incidence, with 57rats / group, we are 90% powered to detect a reduction in pHFOs incidence at 1 week from 58% (as in[15](#_ENREF_15)) to 29% (=0.05, 2 tail). With an estimated rHFOSs frequency of 3.03 ± 0.94 rHFOSs/min (Staba, preliminary data) we will need ~9 rats / group to detect a 50% reduction in frequency with a power of 90%, assuming rHFOSs are detected in ~58% of rats during the 1st week. To observe an effect of treatment on the early seizure incidence, we are weakly powered for 50% rate reduction, but reach 90% power to detect early seizure rate reduction from 42% to 11% (i.e. 75% reduction) with 38 rats/group (a=0.05, 2-tail). Sample sizes for plasma target measures are based on the following:  (1) sample sizes that have yielded statistically significant findings from previous experiments conducted in our laboratory and in the literature; and (2) power analyses of the dependent measures based on data from ongoing experiments as well as data reported in related literature following the procedures in references[52](#_ENREF_52), [53](#_ENREF_53) . For proteomics outcomes, n = 8 / group will have 80% power to detect differences of 1.5 SDs. These calculations are based on t tests for independent samples with a 5%, 2-sided significance level and effect sizes similar to those observed in previous studies. For histology we will need ≥ 6 rats/group to detect a change from 100 to 80 densitometric units with an SD of 12 per region of interest, (power of 80%, =0.05).

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| **ABBREVIATIONS:** AEG: antiepileptogenesis; AES: American Epilepsy Society; CDE: common data element; DSMB: Data and Safety Monitoring Board; FDA: Food and Drug Administration; h-tau: hyperphosphorylated tau; IAC: Informatics and Analytical Core; IL-1: interleukin-1; IL-1ra: IL-1 receptor antagonist; ILAE: International League Against Epilepsy; -IR: -immunoreactivity; LFPI: Lateral Fluid Percussion Injury; pHFOs: pathologic high frequency oscillations; PK: pharmacokinetics; PD: pharmacodynamics; p-tau: phosphorylated tau; PP2A: Protein phosphatase 2A ; PTE: posttraumatic epilepsy; PTEgenesis: PTE epileptogenesis; rHFOSs: repetitive HFOs and Spikes; TBI: traumatic brain injury. |