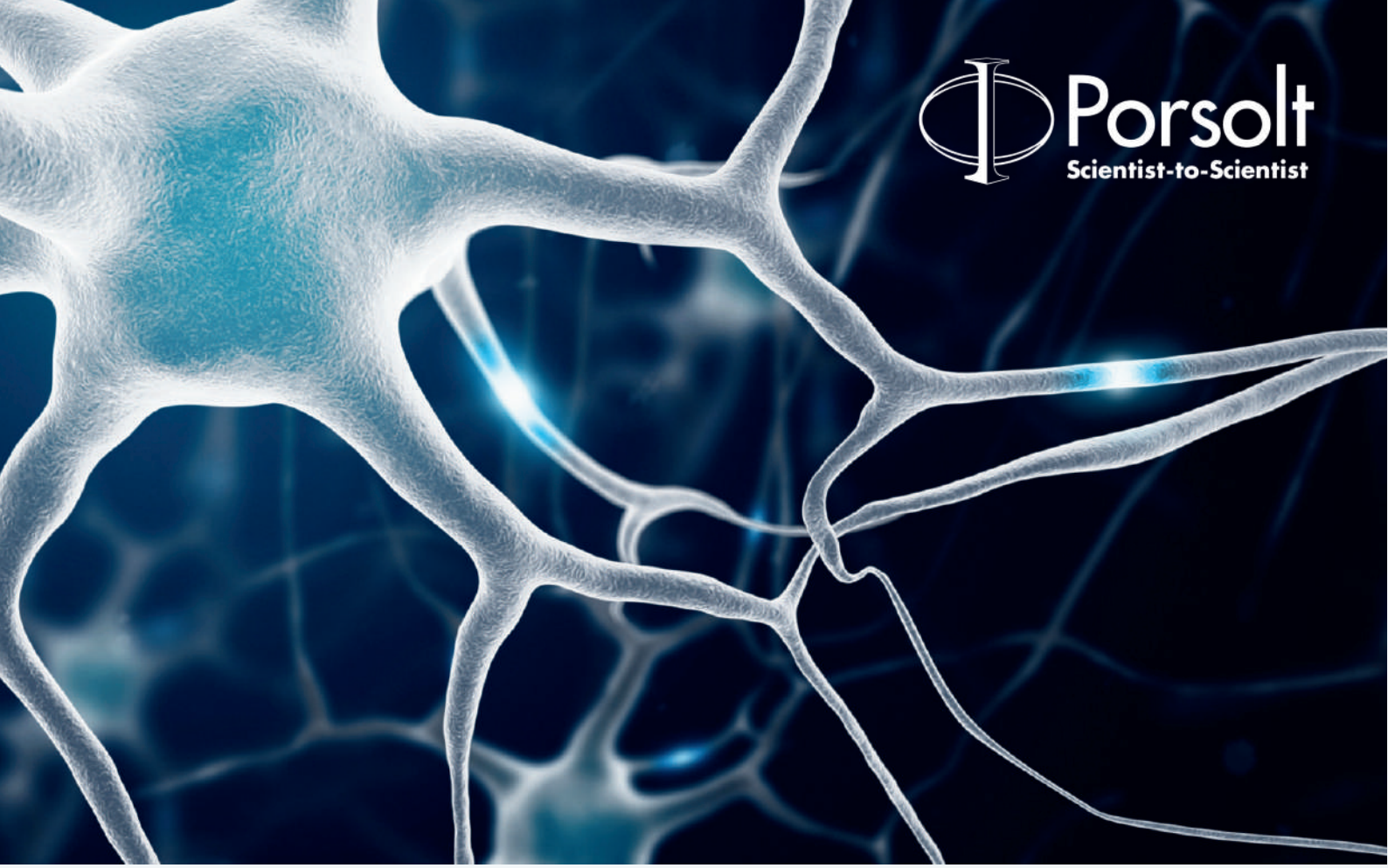


Evaluating Future **Antiepileptic Drugs**

Screening Models and Mechanisms

WHITEpaper

Porsolt

WHITEpaper

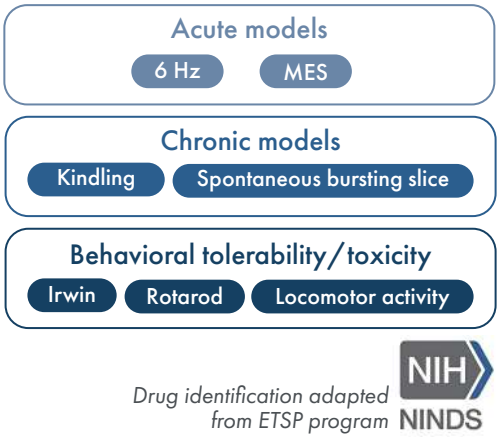
Abstract

This white paper provides an overview of screening methodologies for new antiepileptic drugs (AEDs), detailing the process of using various *in vitro*, *in vivo*, and *ex vivo* models.

This paper focuses on the critical role these models play in identifying promising drug candidates and examines their translational value in assessing efficacy against pharmacoresistant epilepsy. This paper draws on research studies that evaluate Carbamazepine (sodium-channel blocker), Levetiracetam (SV2A modulator) and Retigabine (Kv7 potassium channel opener), and demonstrates the importance of using a combination of assays.

Introduction

Epilepsy is a prevalent and debilitating neurological condition affecting millions worldwide. Despite advancements in treatment, approximately 30–40% of patients continue to experience drug-resistant seizures, underscoring the need for more effective antiepileptic drugs (AEDs). Historically, AED development has relied on simple seizure models, but the inclusion of more complex pharmacoresistant models is now preferred for their greater predictive accuracy in drug discovery.



Drug identification adapted from ETSP program NIH NINDS

Background

The National Institutes of Health (NIH) and the National Institute of Neurological Disorders and Stroke (NINDS) have implemented the Epilepsy Therapy Screening Program (ETSP) to encourage the development of new treatments, particularly for pharmacoresistant epilepsy *(Kehne et al., 2017 and Wilcox et al., 2020)*.

This white paper outlines key methodologies currently used to evaluate potential AEDs and explains how Porsolt's expertise in applying these models can assist with the discovery of new therapies and accelerate drug development.

1 Methodologies for Screening AEDs

In vitro assays

In vitro assays provide a valuable first line high throughput screening tool for new drug candidates.
Cortical or hippocampal neurons are isolated and seeded in 384-well plates, allowing for the evaluation of up to 30 experimental conditions per plate (8 wells per condition). After 10 days of culture, calcium flux indicative of neuronal activity is evaluated using a calcium probe on the cells for 1 hour at 37°C. During incubation, the calcium probe passes through the cell membrane and binds to Ca2+ ions. Variations in intracellular fluorescence are recorded using a FlipR platform prior to and after the addition of 4-aminopyridine (4-AP), a non-selective blocker of potassium channels, inducing epileptiform activity.

Internal studies recorded basal spontaneous calcium oscillations on cortical or hippocampal neurons over a 12-minute period **(Figures 1a and 2a)**. 4-AP addition increased calcium oscillation frequency while Retigabine, a Kv7 potassium channel opener, decreased 4-AP-induced calcium oscillations **(Figures 1b and 2b)**.
The inhibitory effects of Retigabine were of higher amplitude on cortical neurons compared to hippocampal neurons when the same doses were tested (10 and 30 µM) **(Figures 1c and 2c)**. Carbamazepine also dose-dependently decreased 4-AP-induced calcium oscillations on hippocampal neurons (not tested on cortical neurons) while Levetiracetam at 100 and 300 µM was devoid of activity in this assay.

These results confirm the value of in vitro models for high-throughput screening of compounds. However, acute *in vivo* models, such as the 6 Hz test, have more relevance for compounds with a different mechanism of action, such as Levetiracetam.

Primary Cortical Neurons

Figure 1a: Control conditions

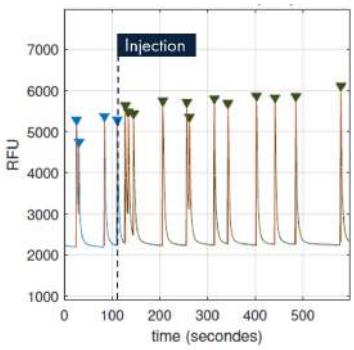


Figure 1b: 4-AP (100 µM)

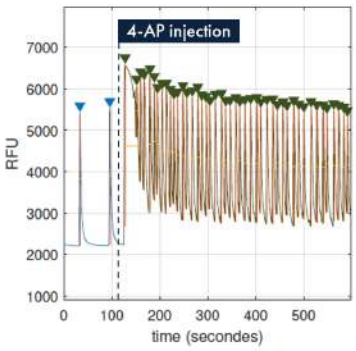
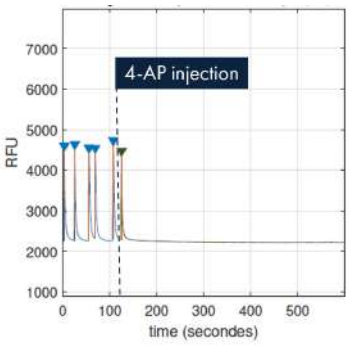


Figure 1c: 4-AP/Retigabine (30 µM)



Primary Hippocampal Neurons

Figure 2a: Control conditions

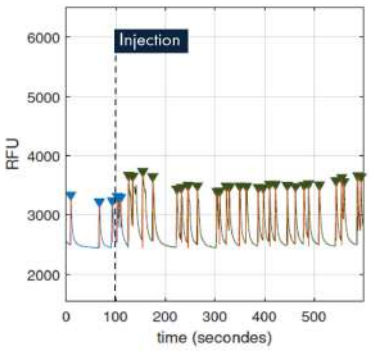


Figure 2b: 4-AP

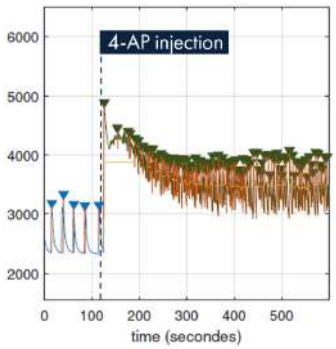
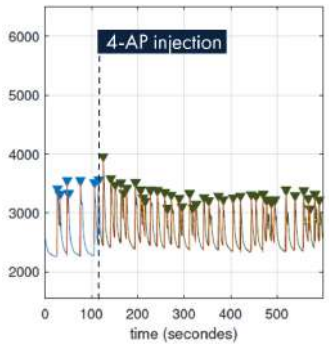
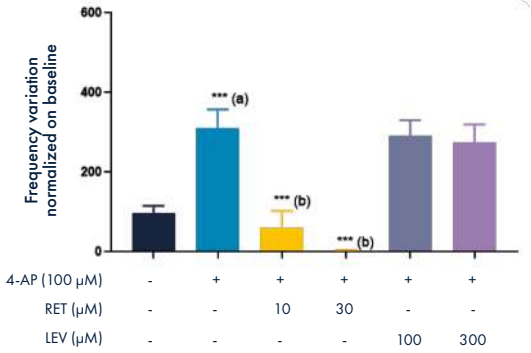


Figure 2c: 4-AP/Retigabine (30 µM)



Primary Cortical Neurons

Figure 1d: Evaluation of Retigabine and Levetiracetam on 4-AP induced calcium oscillation frequency on rat cortical neurons



Primary Hippocampal Neurons

Figure 2d: Evaluation of Carbamazepine, Retigabine and Levetiracetam on 4-AP induced calcium oscillation frequency on rat hippocampal neurons

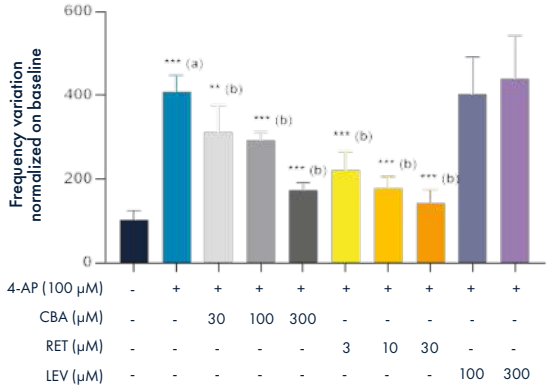


Figure 1: Calcium oscillations recorded on primary cortical neurons in baseline conditions (shown by blue arrows) and after injection of 0.1% sterile water (a), 4-AP at 100 µM (b) and Retigabine at 30 µM in presence of 4-AP at 100 µM (c) (shown by green arrows).
Effects of Retigabine at 10 and 30 µM and Levetiracetam at 100 and 300 µM on frequency variation on primary rat cortical neurons (normalized on baseline conditions, 100%) (d).

Figure 2: Calcium oscillations recorded on primary hippocampal neurons in baseline conditions (shown by blue arrows) and after injection of 0.1% sterile water (a), 4-AP at 100 µM (b) and Retigabine at 30 µM in presence of 4-AP at 100 µM (c) (shown by green arrows).
Effects of Carbamazepine at 30, 100 and 300 µM, Retigabine at 3, 10 and 30 µM and Levetiracetam at 100 and 300 µM on frequency variation on primary rat hippocampal neurons (normalized on baseline conditions, 100%) (d).

One-way ANOVA followed by Dunnett's test, ** = $p < 0.01$ and *** = $p < 0.001$, N= 8/condition for cortical neurons and N=4 per condition for hippocampal neurons. (a): compared with non-treated conditions and (b): compared with 4-AP conditions.

In vivo acute models

The Maximal Electroshock (MES) test remains the most used *in vivo* test for screening new drug candidates. Most AED's that are clinically efficacious for treating generalized tonic-clonic seizures, are observed to be active in MES. This test is based on an electrical corneal stimulation (50 mA) delivered at high frequency (50 Hz in the mouse) for a short duration *(Swinyard, 1972)*.

Porsolt's data demonstrate that **Carbamazepine** dose-dependently decreases the number of mice exhibiting tonic convulsions and reaches a significant effect from 25 mg/kg **(Figure 3a)**.
Retigabine is also protective against tonic convulsions from 10 mg/kg **(Figure 3b)**.
Levetiracetam, while tested at high dose (250 mg/kg) in the mouse does not protect against tonic convulsions **(Figure 3c)**.
This compound is described as not active as the effective dose in the MES test is similar to TD50 in the mouse *(dose higher than 500 mg/kg, Barton et al., 2001)*.

Figure 3a: CBA (mg/kg)

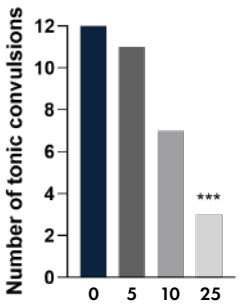


Figure 3b: RET (mg/kg)

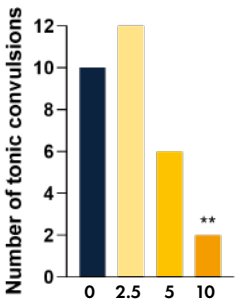


Figure 3c: LEV (mg/kg)

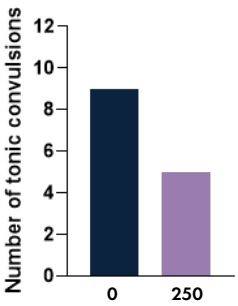


Figure 3: Effects of Carbamazepine at 5, 10 and 25 mg/kg, administered p.o. 60 minutes before MES in the mouse (a). Effects of Retigabine at 2.5, 5 and 10 mg/kg, administered i.p. 15 minutes before MES in the mouse (b). Effects of Levetiracetam at 250 mg/kg, administered i.p. 60 minutes before MES in the mouse.
Fisher's Exact Test, ** = $p < 0.01$ and *** = $p < 0.001$, N=12/group.

The 6 Hz test was initially set up to mimic partial seizure *(Brown et al;, 1953)*. Its lack of phenytoin sensitivity initially led to the suggestion that this test was not predictive. It was later reintroduced and proposed as a useful model for evaluating new AEDs against drug-resistant therapy *(Barton et al., 2001)*. The test can be conducted at different intensities (24, 32 or 44 mA) but most AEDs lose their protective efficacy or show decreased efficacy with increasing intensities.

At 44 mA, **Carbamazepine** decreases the forelimb seizure score from 25 mg/kg **(Figure 4a)**. Anticonvulsant activity is also observed with **Retigabine** at 15 and 20 mg/kg and with **Levetiracetam** from 200 mg/kg **(Figures 4b and 4c)**.

Figure 4a: CBA (mg/kg)

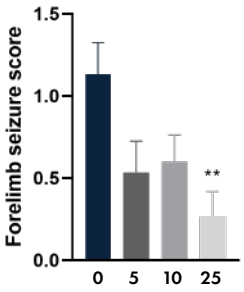


Figure 4b: RET (mg/kg)

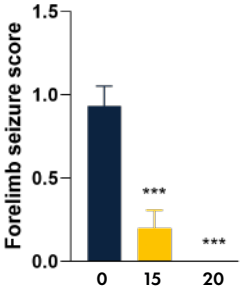


Figure 4c: LEV (mg/kg)

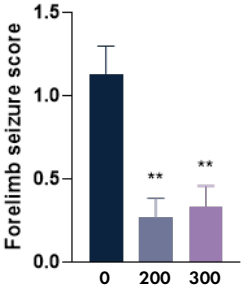


Figure 4: Effects of Carbamazepine at 5, 10 and 25 mg/kg, administered p.o. 60 minutes before 6 Hz stimulation in the mouse (a). Effects of Retigabine at 15 and 20 mg/kg, i.p. 15 minutes before 6 Hz stimulation in the mouse (b). Effects of Levetiracetam at 200 and 300 mg/kg, administered i.p. 60 minutes before 6 Hz stimulation in the mouse (b). Kruskall-Wallis test followed by Dunn's test, ** = $p < 0.01$ and *** = $p < 0.001$, N=15/group.

In vivo chronic models

Chronic models, such as the electrical amygdala kindling model, offer a more complex and clinically relevant assessment of AEDs. The electrical kindling model is based on focal and repeated electrical stimulations that progressively lead to seizure activity after several days of stimulation *(Goddard et al., 1969)*.

In addition to seizure occurrence, the kindling model recapitulates many aspects of human epilepsy, including neuroinflammation, cognitive and behavioral disturbances *(Barker-Haliski and White, 2020)*. Several models have been developed in the rat, including implantation of a depth electrode into the amygdala or hippocampus, along with constant or threshold current protocols for sensitizing the animals. The frequency of stimulations can also be varied during the sensitization phase. The model has since been adapted to the mouse using corneal electrical stimulations, which reduces the quantity of compound needed.

Porsolt's scientists have developed expertise in implementing these complex protocols, allowing for the accurate evaluation of AED efficacy over extended periods :

Electrode Implantation

- **Amygdala:** stimulation + EEG recording
- **Cortex:** EEG recording

Kindling

- Sensitized to a constant current (300µA): *Twice daily for two weeks*
- Seizure threshold intensity determination: *Once daily for two weeks*

ADT (After Discharge Threshold) stimulation produces spikes with at least double the pre-stimulus EEG amplitude for 3 seconds or more

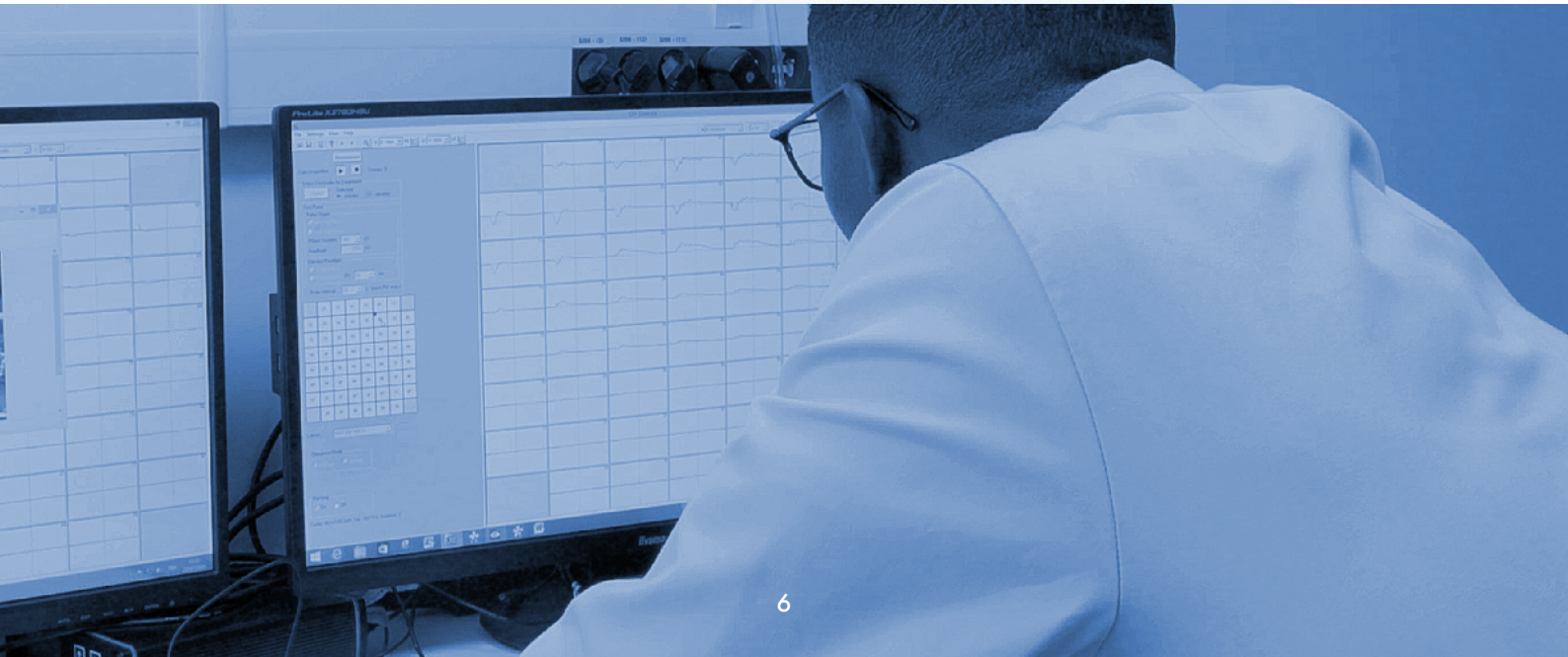
GST (Generalized Seizure Threshold) stimulation corresponds to motor seizure.

AED Testing *(over several weeks)*

- Baseline session
- Test session

Endpoints

- Seizure score based on Racine's scale
- **ADD (After Discharge Duration) of the amygdala and the cortex**



Porsolt's data demonstrate that :

- **Carbamazepine at 500 mg/kg** slightly decreases the After Discharge Threshold **(ADT)** seizure score **(Figure 5c)** compared to baseline without modifying ADT intensity **(Figure 5a)** and the ADT duration in the amygdala **(Figure 5e)**.

- **Retigabine at 10 mg/kg** decreases both the ADT seizure score **(Figure 5c)** and ADT duration **(Figure 5e)** while increasing ADT stimulation **(Figure 5a)**. ADT duration was also decreased with Retigabine at 5 mg/kg compared with baseline **(Figure 5e)**.

- **Levetiracetam at 500 and 1000 mg/kg** seems the most efficacious compound in this model with a dose-dependent effect on the seizure score and ADT duration **(Figures 5c and 5e)**.

In addition, the 3 AEDs increase Generalized Seizure Threshold **(GST)** at the highest dose accompanied with a decrease of the GST seizure score and GST duration in the amygdala **(Figures 5d and 5f)**.

Figure 5a

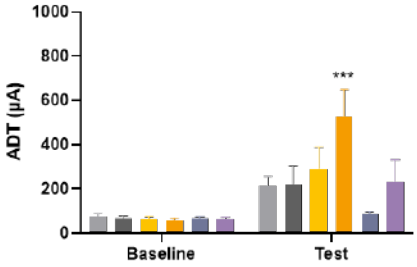


Figure 5c

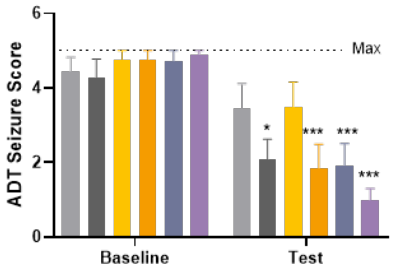


Figure 5e

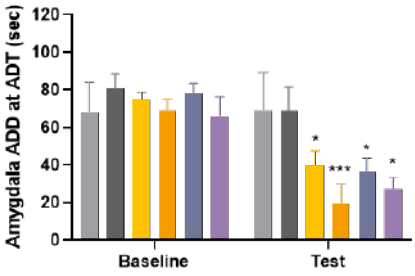


Figure 5b

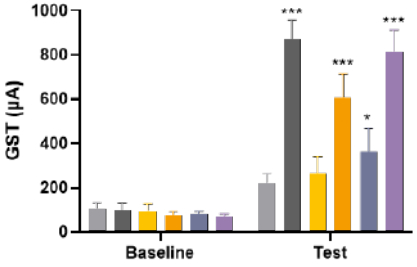


Figure 5d

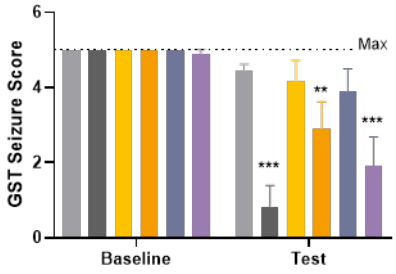
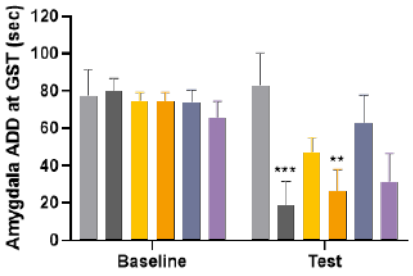


Figure 5f



CBA (250 mg/kg)
CBA (500 mg/kg)
RET (5 mg/kg)
RET (10 mg/kg)
LEV (500 mg/kg)
LEV (1000 mg/kg)

Figure 5: Effects of Carbamazepine at 250 and 500mg/kg (p.o. 60 minutes before the test), Retigabine at 5 and 10 mg/kg (i.p. 30 minutes before the test) and Levetiracetam at 500 and 1000 mg/kg (i.p., 60 minutes before the test) in the electrical amygdala kindling test in the rat. Evaluation of afterdischarge threshold (ADT) (a), generalized seizure threshold (GST) (b), seizure score at ADT (c) and GST (d), afterdischarge duration at ADT (e) and GST (f). Two-way ANOVA followed by Bonferroni's test, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$, N=12 rats/group.

Ex vivo assays

Ex vivo assays, such as the hippocampal slice model, provide complementary data and additional mechanistic insights into the efficacy of drug candidates. These are also valuable tools for detecting antiseizure effects of treatments such as gene therapy, which often targets a specific brain region (such as the hippocampus).

Epileptiform activity may be induced in the presence of Mg^{2+}-deprived artificial cerebrospinal fluid, 4-AP or bicuculline addition and is based on recording of electrical activity with **MicroElectrode Arrays (MEA)**. This test may also be classified as a chronic model when hippocampal slices are used from animals showing spontaneous seizures.

Porsolt's *ex vivo* models include hippocampal slices (400 µm) obtained from male C57BL/6 mice. After 1 hour of recovery, slices are placed in an MEA well with 64 electrodes. Electrical activity is monitored in the CA1 region of the hippocampus prior to and during 4-AP induction in the presence/absence of AEDs and the firing rate is calculated.

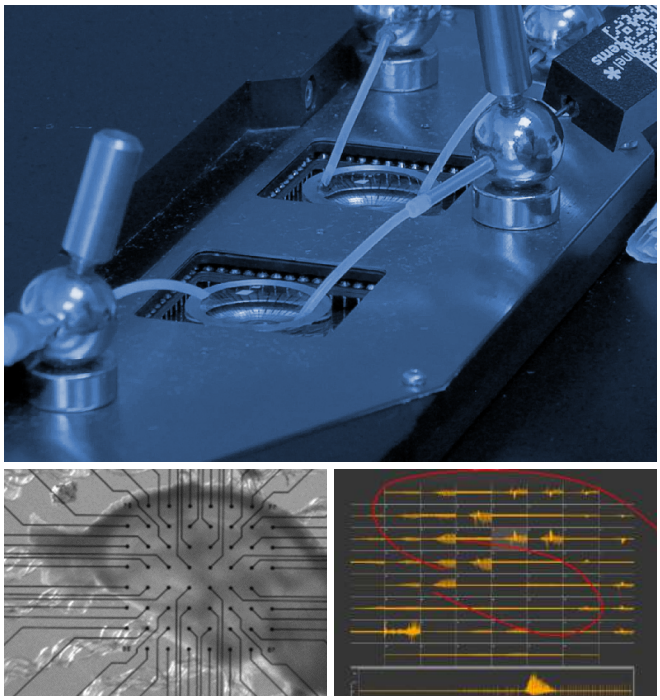


Figure 6

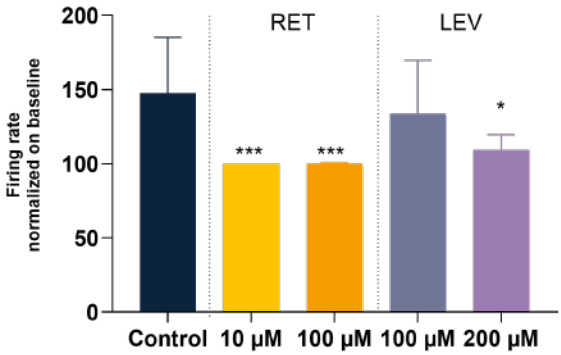


Figure 6: Effects of Retigabine at 10 and 100 µM and Levetiracetam at 100 and 200 µM on 4-AP induced firing rate on mouse hippocampal slice. Kruskal-Wallis test followed by Dunn's test, * = $p < 0.05$ and *** = $p < 0.001$, N=6-10 slices/condition.

As expected, Porsolt's data show that **Retigabine** (10-100 µM) fully antagonizes 4-AP-induced epileptiform activity while the effects with **Levetiracetam** were more moderate **(Figure 6)**.

However, a significant decrease in firing rate is observed with Levetiracetam at high concentration (200 µM).

Porsolt's ability to generate high-quality data from this model helps clients better understand the drug's mode of action, as demonstrated by the efficacy of Retigabine and Levetiracetam in antagonizing 4-AP-induced epileptiform activity.

2 Porsolt's Expertise in Epilepsy Models

Discussion

The use of reliable and translational epilepsy models needs to be considered when screening and evaluating drugs that are active against refractory epilepsy. *In vitro* models such as 4-AP-induced calcium oscillations on primary neurons are useful for high throughput screening of compounds and are good precursors to acute *in vivo* models such as the MES and the 6 Hz tests which are recommended by the Epilepsy Therapy Screening Program.

Porsolt's data with Levetiracetam confirm the importance of using a pharmacoresistance model such as the 6 Hz test at an early stage of drug development followed by a chronic model such as the electrical amygdala kindling in the rat.
Ex vivo models, including hippocampal slices also demonstrate a good level of reliability even when normal animals (non-SRS) are evaluated, as can be seen by the efficacy of both Retigabine and Levetiracetam in antagonizing 4-AP-induced epileptiform activity.

Further evaluation in additional chronic models is recommended by the ETSP once promising drug candidates have been identified in the above assays and included in a "differentiation phase" *(Kehne et al., 2017 and Wilcox et al., 2020)*. Models of spontaneous recurrent seizures following status epilepticus induced by systemic or intra-hippocampal injection of a chemical substance (Pilocarpine or Kainate) or lamotrigine-resistant amygdala kindling are considered as the most relevant models for differentiation.

These additional chronic models require an investment of time and effort to perform, however they replicate physical features of human temporal lobe epilepsy, including spontaneous recurrent seizures following status epilepticus or the development of focal seizure that become secondarily generalized. The use of drug-resistant models also allow for the evaluation of new compounds in situations where traditional AEDs fail.

Conclusion

The development of AEDs requires a careful combination of *in vitro, in vivo,* and *ex vivo* models to ensure thorough evaluation of drug candidates. Porsolt's history of validating and in implementing these models, along with a deep understanding of epilepsy, provides a reliable foundation and partner for identifying and advancing new AEDs.

With a deep understanding of epilepsy's complexity, Porsolt has developed unique expertise in appreciating the complexities of epilepsy, and offers a wide array of validated and innovative *in vitro, in vivo,* and *ex vivo* models to help its clients identify promising drug candidates for pharmacoresistant epilepsy.

Porsolt capabilities include:

***In vitro* Screening Platforms**

That allow for high-throughput evaluation of AED candidates using neuronal cultures.

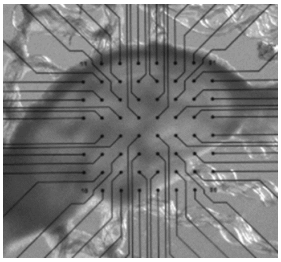
***In vivo* Acute Seizure Models**

Such as MES and 6Hz which provide early-stage efficacy data.

***In vivo* Chronic Epilepsy Models**

Like the amygdala kindling model, which enable the study of AED effects in drug-resistant epilepsy.

***Ex vivo* Assays**



Which provide mechanistic insights using hippocampal slices and MicroElectrode Arrays (MEA) to analyze epileptiform activity.

Porsolt's team of scientists delivers high-quality, reproducible results through rigorous experimental design, helping clients make informed decisions during the drug discovery process, with expertise that spans the entire drug development process, from early screening to advanced preclinical studies, ensuring that potential AEDs are evaluated comprehensively.

This unique multifaceted approach assists researchers with developing more effective treatments for epilepsy, particularly for patients with pharmacoresistant forms.

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Scientist-to-Scientist

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