Developing a functional in vitro assay of neuronal hyperexcitability for anti-epileptic drug discovery

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2025

PSTR008.08

Abstract

Neuronal hyperexcitability is a hallmark of epilepsy and some neurodevelopmental disorders. This phenomenon arises from an imbalance between excitatory and inhibitory signaling in neural networks. Currently, therapeutic interventions, include antiepileptic drugs and neuromodulation approaches to restore excitatory-inhibitory balance. Understanding the mechanisms of E-I balance and the dysregulation in pathological states is critical for developing new therapies. Primary cultures comprised of glutamatergic and GABAergic neurons form functional excitatory and inhibitory connections. Electrophysiology assays and can be used to interrogate E-I balance in these cultures. Neuronal hyperexcitability *in vitro* can be provoked using experimental methods used to study epilepsy/seizure *in vivo* such as $GABA_A$ antagonists and kainic acid. Additional stimuli include KCl depolarization and the potassium channel blocker 4-aminopyridine. We will compare and contrast the network responses to these proconvulsant stimuli as well as the effects of known antiepileptic drugs in an effort to validate the *in vitro* model for assessing the efficacy of new therapeutic approaches for treating epilepsy and neurodevelopmental disorders.

Introduction

We sought to develop and validate an *in vitro* assay for assessing the efficacy and potency of anti-epileptic drugs (AEDs) on action potential firing in human iPSC-derived neurons and cortical rat neurons.

Approved AEDs for the treatment of epilepsy work by various mechanisms that mainly include the modulation of voltage-dependent ion channels, activation of GABA, or inhibition of glutamate receptors.

Valproic acid is a branched-chain carboxylic acid used to treat seizures, bipolar disorder, and migraine headaches. The medication's versatility stems from its multiple mechanisms of action, but its use is associated with serious risks, particularly liver damage and birth defects.

Carbamazepine is an anticonvulsant medication used to treat a variety of conditions, including epilepsy, nerve pain, and bipolar disorder. It works by calming overactive nerves in the body to control seizures and pain.

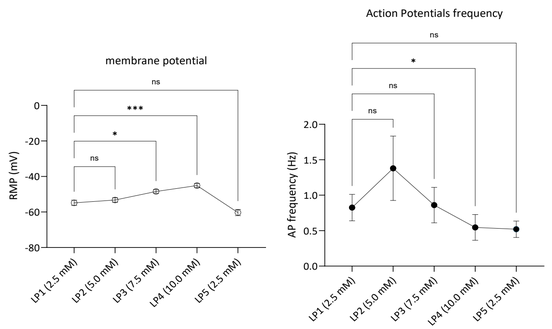
Phenytoin is an anti-epileptic drug (AED) used to prevent and control certain types of seizures. It works by calming overactive nerves in the brain. Its effect in blocking the fast inactivation state of voltage-dependent sodium channels in neuronal cell membranes represents its primary mode of action.

Neuroservices-Alliance uses electrophysiology techniques—including whole-cell patch-clamp recordings and multielectrode array (MEA) recordings from primary rat and human iPSC-derived neuron cultures to measure functional endpoints including resting membrane potential, action potential firing, and network excitability. These complementary systems allow us to compare pharmacological effects across species and developmental stages, providing insight into both preclinical and human-relevant mechanisms.

Methods

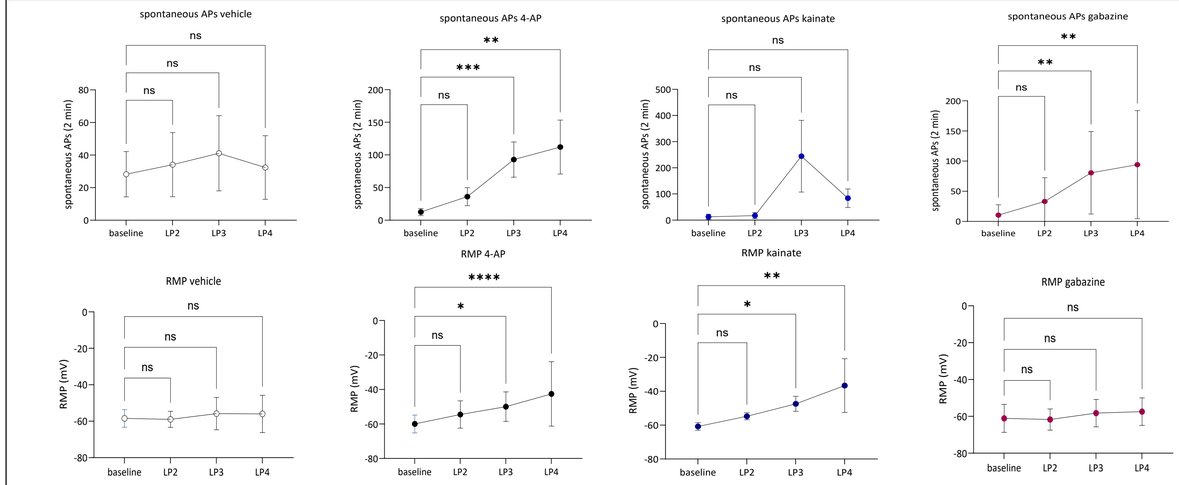
- Embryonic day 18 rat cortical neurons or human iCell Gluta neurons (Fujifilm) were plated at low density (50-100 cells/mm2) on monolayers of rat astrocytes.
- Whole cell patch clamp recordings were made using standard methods. The composition of external recording solution was: 140 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES pH 7.3. The composition of internal recording solution was: 120 mM K-gluconate, 20 mM KCl, 3 mM $MgCl_2$, 5 mM EGTA, 0.5 mM $CaCl_2$, 4 mM Na_2-ATP, 0.3 mM Li-GTP, 10 mM HEPES pH 7.3.
- Spontaneous action potentials were recorded in the current clamp configuration (10 kHz gap-free) at the "natural resting membrane potential" (RMP) of neurons. Neurons with RMP more depolarized than -50 mV were excluded.

Concentration-response of KCl on resting membrane potential and spontaneous APs in E18 rat cortical neuron cultures



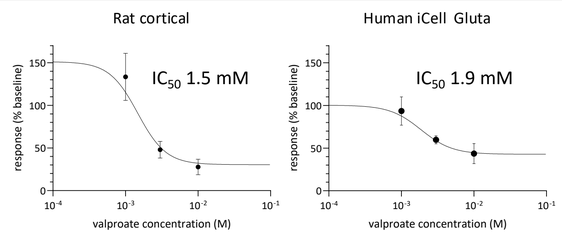
Extracellular KCl depolarized the resting membrane potential in a concentration-dependent fashion. The empirically measured resting membrane potentials closely matched the E_{rev} calculated from, the Goldman-Hodkin-Katz equation including K^+ and Na^+ with the relative resting permeability of Na^+ set at 1/10 of K^+. +2.5 mM KCl significantly increased the firing rate of spontaneous action potentials. Higher concentrations of KCl exhibited an "inverted-U" effect.

Proconvulsant effects of 4-aminopyridine (4-AP), kainic acid, and gabazine in E18 rat cortical neuron cultures



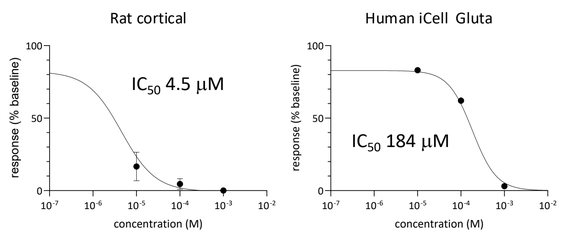
Increasing concentrations of test compounds were cumulatively applied over three 3-min liquid periods (LPs) following baseline: 4-AP (100, 300, 1000 uM), kainate (1, 3, 10 uM) and gabazine (1, 3, 10 uM). 4-AP, kainate, and gabazine increased the number of spontaneous action potentials in a concentration-dependent fashion. Resting membrane potential was depolarized by 4-AP and kainate but not by gabazine or vehicle.

Concentration-response of valproic acid (AED) on spontaneous action potentials in rat neurons and human iCell Gluta neurons



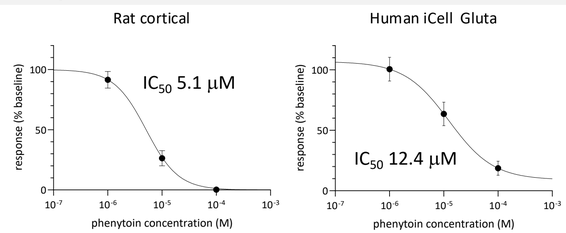
Valproic acid reduced spontaneous action potentials in rat cortical neurons with an Emax of 70% and an IC50 1.5 mM. Valproic acid reduced spontaneous APs in human iCell Gluta neurons with an Emax of 57% and an IC50 =1.9 mM.

Concentration-response of carbamazepine (AED) on spontaneous action potentials in rat neurons and human iCell Gluta neurons



Carbamazepine completely blocked spontaneous action potentials in a concentration-dependent fashion. Interestingly, carbamazepine was more potent in rat neurons (IC50 4.5 μM) than human neurons (IC50 184 μM). The published results for block of Na channels is 137 μM.

Concentration-response of phenytoin (AED) on spontaneous action potentials in rat neurons and human iCell Gluta neurons



Phenytoin completely blocked spontaneous action potentials in a concentration-dependent fashion. The IC50 was 5.1 μM in rat neurons and 12.4 μM in human neurons. This is consistent with the reported IC50 of 10-17 μM at Na channels.

Summary

- This study demonstrates the utility of our in vitro electrophysiology platform for evaluating neuronal excitability and drug responses in embryonic rat cortical neurons and human iPSC-derived glutamatergic neurons. We established a reproducible assay capable of assessing excitatory and inhibitory modulation relevant to epilepsy research.
- Increasing KCl concentrations depolarized the resting membrane. Moderate elevations in KCl enhanced spontaneous firing, whereas higher concentrations produced an inverted-U response, indicating depolarization block at excessive membrane depolarization.
- Exposure to known proconvulsant agents, 4-aminopyridine, kainate, and gabazine increased spontaneous action potentials in E18 rat cortical neuron cultures. Both 4-AP and kainate depolarized the resting membrane potential, while gabazine enhanced activity without altering membrane voltage, consistent with its disinhibitory action through $GABA_A$ receptor blockade.
- Classical sodium-channel–blocking antiepileptic drugs (valproic acid, carbamazepine and phenytoin) produced a concentration-dependent suppression of spontaneous action potential firing in both rat and human neurons. Their effects are consistent with stabilization of voltage-gated sodium channels in the inactivated state, reducing neuronal hyperexcitability. Carbamazepine and phenytoin were able to completely abolish action potential firing. In contrast, valproic acid did not completely block action potentials. Phenytoin and valproic acid exhibited similar potency in rat and human neurons. Carbamazepine was much more potent in rat neurons than human neurons.
- Collectively, these results demonstrate that the combined rat and human neuron platform faithfully reproduces key physiological and pharmacological signatures of excitability modulation. The concordance between empirical measurements and established biophysical predictions supports its reliability as a quantitative, mechanistically interpretable tool for screening and characterizing antiepileptic drugs. Moreover, the observed cell-type-specific responses to valproic acid highlight the importance of incorporating human iPSC-derived neurons into early-stage drug discovery. Overall, this work establishes a versatile and predictive in vitro system for advancing the development of therapies targeting neuronal hyperexcitability in epilepsy and related neurodevelopmental disorders.