Development of an Ultra High-Throughput Screen to Identify Antagonists for GluK1

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Overview

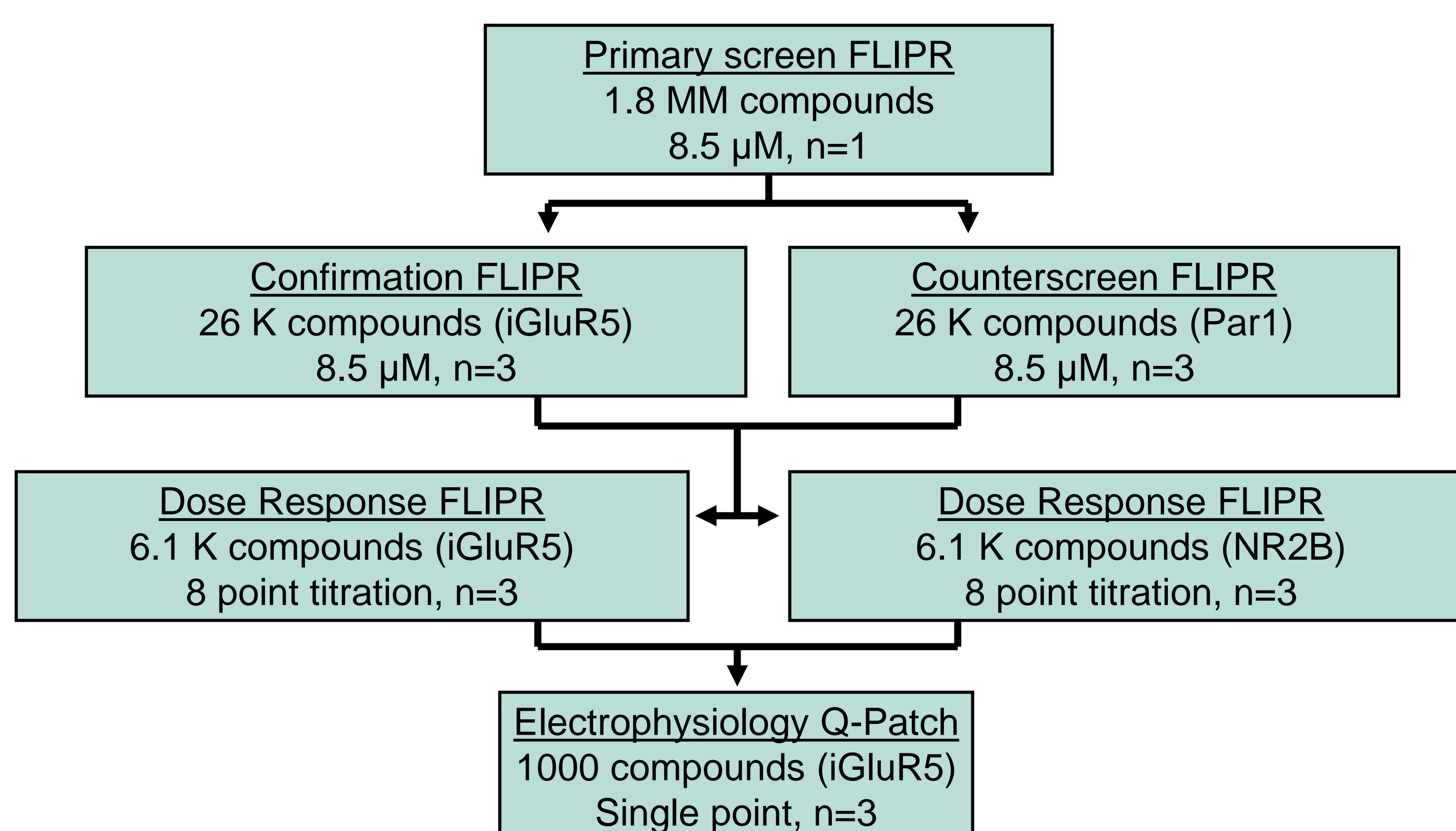
A FLIPR-based ultra high-throughput assay, using a calcium-permeable form of the GluK1 receptor and the calcium-indicator dye Fluo-4, was developed to screen for antagonists for GluK1. The assay was used to screen a multi-million member small molecule library in 1536-well plate format. Following confirmation of primary actives and counterscreens to eliminate off target inhibitors of GluN2B, a selection of confirmed GluK1 selective antagonists was evaluated in an automated electrophysiology assay. Approximately 20% of the selected 1000 compounds confirmed activity on the Q-Patch-HT. Additionally, various mechanisms of inhibition were identified though the electrophysiology assay, helping to further characterize the compounds.

Introduction

The ionotropic glutamate receptor GluK1 (previously known as GluR5) is a kainate subtype of the glutamate receptor family and is comprised of a ligand binding domain and an associated ion channel. The receptor is expressed throughout the central nervous system with particularly strong expression in the dorsal root ganglion and trigeminal ganglion; antagonists for the receptor could be treatments for pain disorders, as well as acute migraine.

Methods

FLIPR: Due to rapid desensitization of the receptor, it was not possible to observe a response by addition of agonist alone. The assay required the addition of concanavalin A (ConA), a lectin known to decrease GluK1 desensitization. A frozen cell FLIPR protocol was optimized to include ConA, and kainate as an agonist. The assay ran a primary screen of a small molecule compound library in 1536-well plate format. Briefly, HEK cells transfected with the GluK1 channel were thawed from cryovials and plated the day before an assay. On the day of the assay the cells were washed and loaded with fluo-4, a calcium indicator dye. After dye loading, the cells were washed and incubated with ConA and test compounds. During a kinetic FLIPR read, the cells were stimulated with the agonist kainate at an EC_{80} concentration, and the resulting fluorescence was measured. A decrease in fluorescence signal indicated a block of the channel. Q-PATCH: The voltage protocol maintained the cells in voltage-clamp at -80mV throughout the compound testing period. For each cell, three current sweeps of 16 sec duration sampled at 10 kHz were recorded: 1) extracellular (EC) solution wash; 2) 500 μM Kainate (KA) (in EC+ 0.3 mg/mL ConA) followed by 3 washes with EC, reference for total amplitude; 3) a preapplication of the test compound (in EC+ConA) followed by a second KA application (in EC+ConA) in the presence of the same compound.



Results

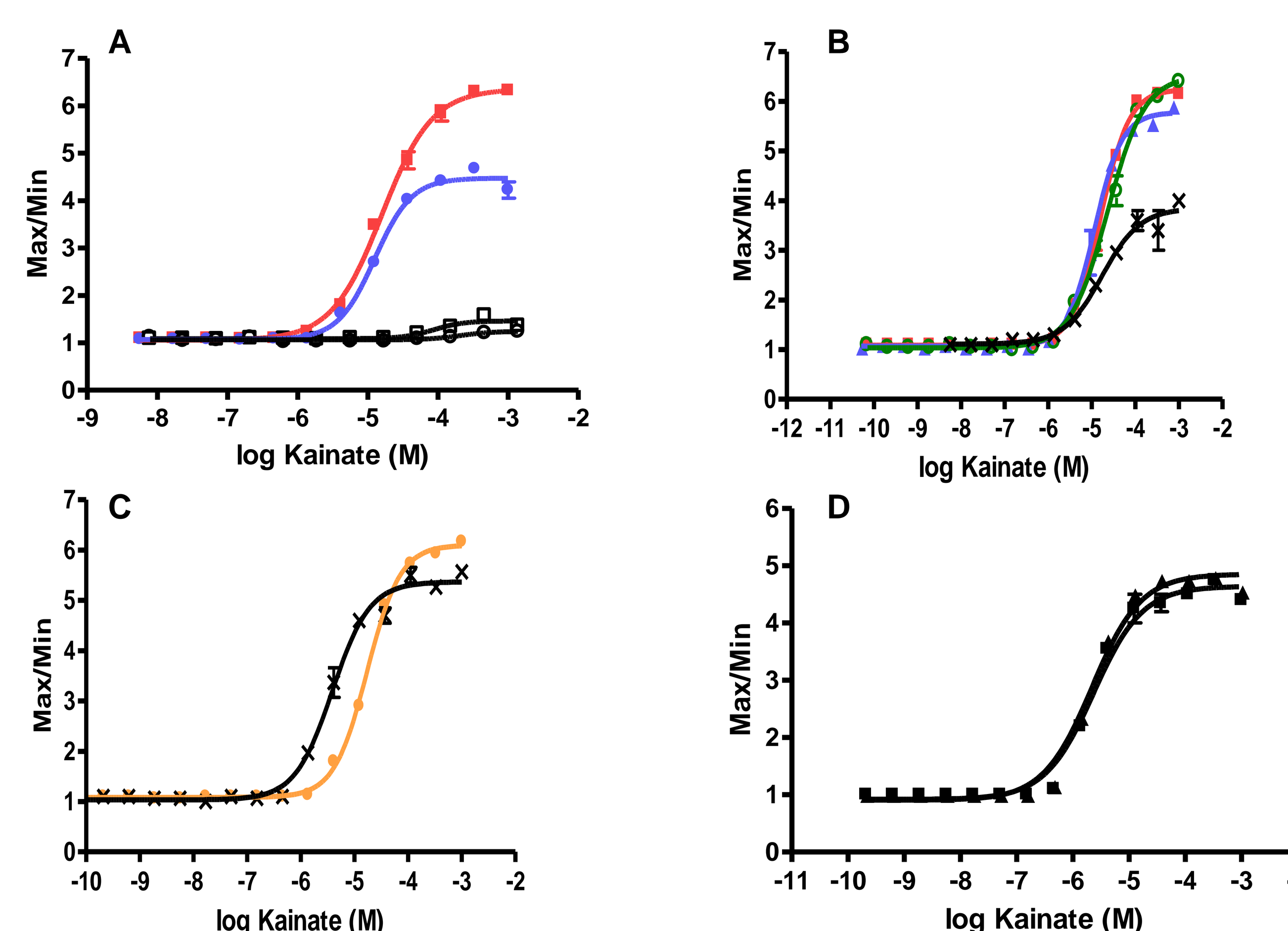
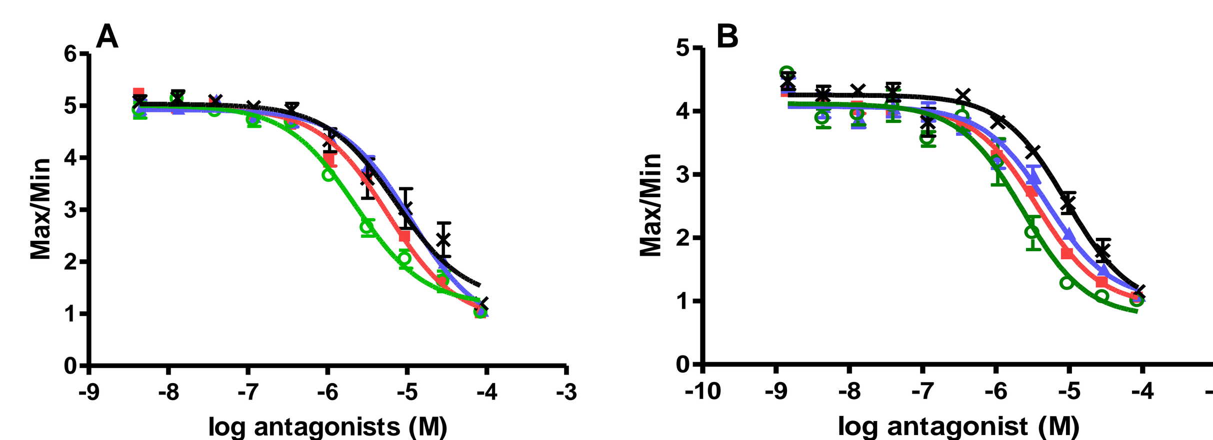


Figure 1: Assay Development: A. Kainate titration in FLIPR assay. Results in the presence (■) and absence (□) of concanavalin A with a media and dye washout, and in the presence (●) and absence (○) of concanavalin A with a dye washout only. **B. Effect of calcium chloride**. Varying the final concentration of calcium chloride in the dye washout buffer. 10 mM $CaCl_2$ final (■), 8 mM (▲), 5 mM (○), 1 mM (X). **C. Effect of probenecid.** Absence (X) or presence (●) of 2.5 mM probenecid in the wash buffers and fluo-4 dye buffer. **D. Fresh vs frozen cells**. Freshly prepared cells from a triple flask (■), and cells previously frozen in cryovials and thawed on the day of plating (▲).



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	IC50 value (μM)			
	DNQX	CNQX	UPB302	NBQX
Fresh cells	5.7	11.0	2.2	7.2
Frozen cells	3.5	4.9	2.3	9.1
Literature	na	8.0	0.4	9.1, 25

Figure 2: Pharmacology: A. Fresh cells tested in the presence of increasing concentrations of inhibitors added via pintool. An EC_{80} concentration of kainate, 20 μM, was used to stimulate calcium flux in the FLIPR. **B. Frozen cells**. DNQX (■), CNQX (▲), UBP302 (○), NBQX (X). Error bars (SD) are indicated and represent n=4. **C. Potency values.**

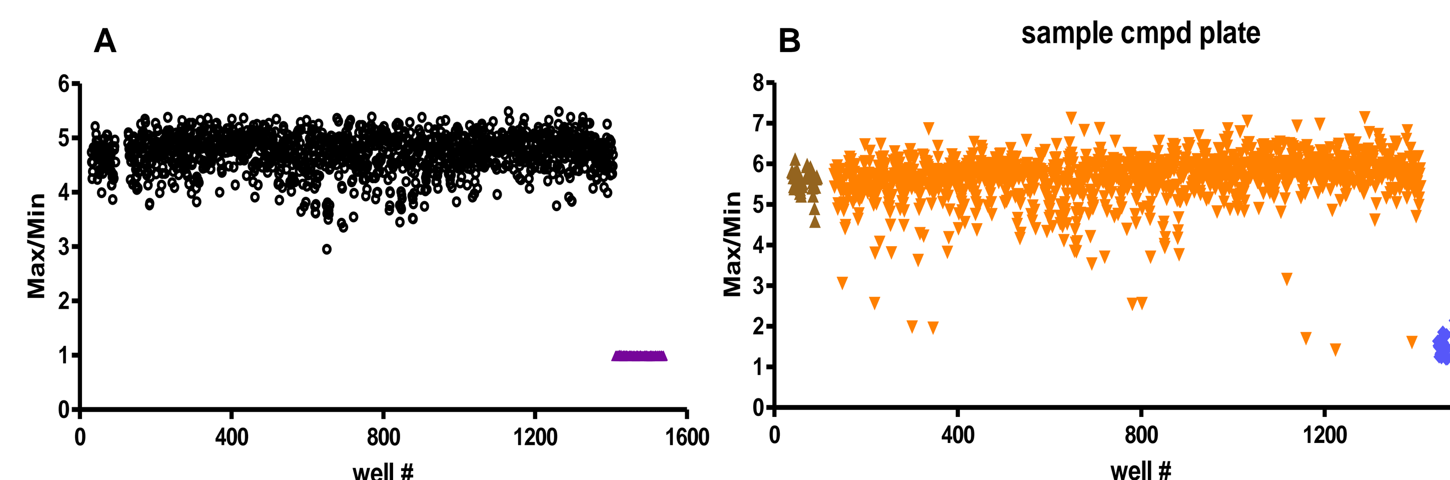
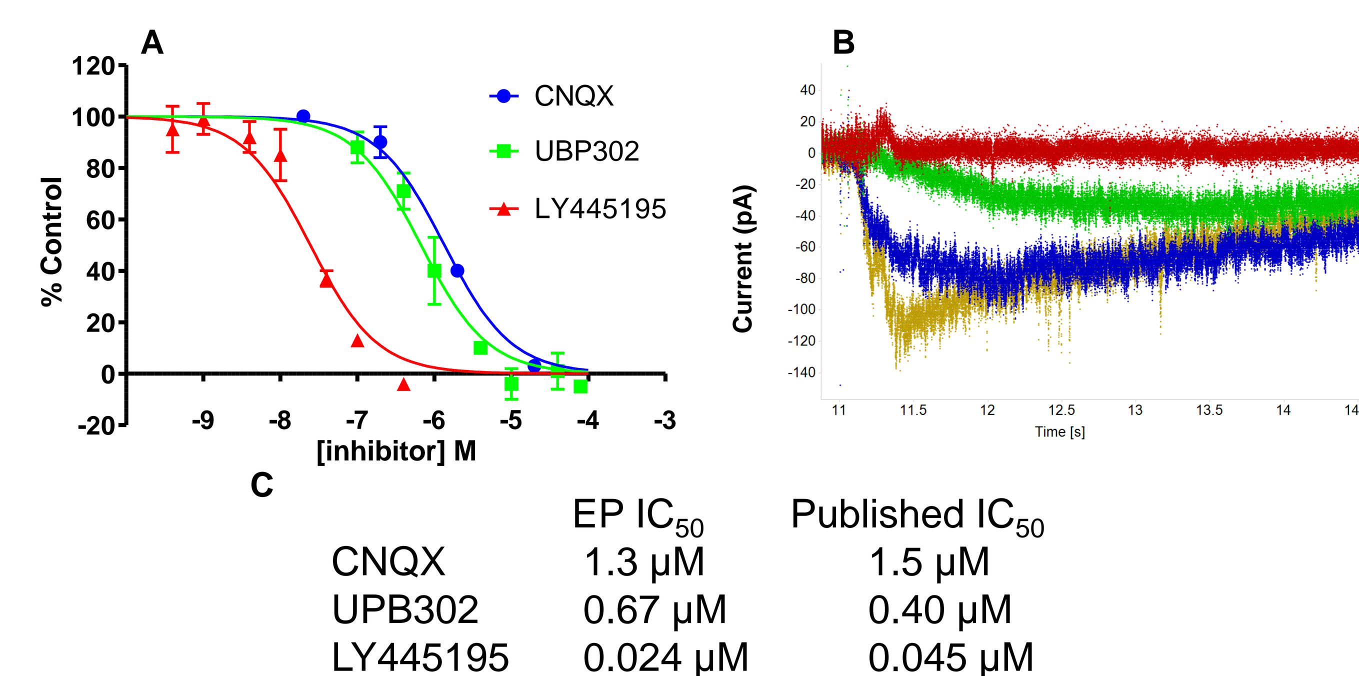


Figure 3: 1536-Well Plate Assay: A. DMSO plate with 30 nL DMSO added via pintool. EC_{80} kainate was added to promote calcium flux (O) or a buffer control (▲). The plate has a 4.7-fold S:B, 7.4% CV, and a 0.75 Zprime. **B. Sample compound plate.** 30 nL of either DMSO (▲), sample library compounds (▼), or 50 μM CNQX (♦) was added to the cell plate. All of these wells also contained an EC_{80} concentration kainate, (●) represents buffer addition.



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	EP IC50	Published IC50
CNQX	1.3 μM	1.5 μM
UPB302	0.67 μM	0.40 μM
LY445195	0.024 μM	0.045 μM

Figure 4: Electrophysiology Assay Validation: A. Titrations of standard GluK1 inhibitors on Q-Patch. B. UBP302 overlay current trace from Q-Patch. Gold (DMSO); Blue (100 nM); Green (1000 nM); Red (10000 nM). **C. IC_{50} values** of standard compounds from Q-Patch electrophysiology versus manual patch clamp published values.

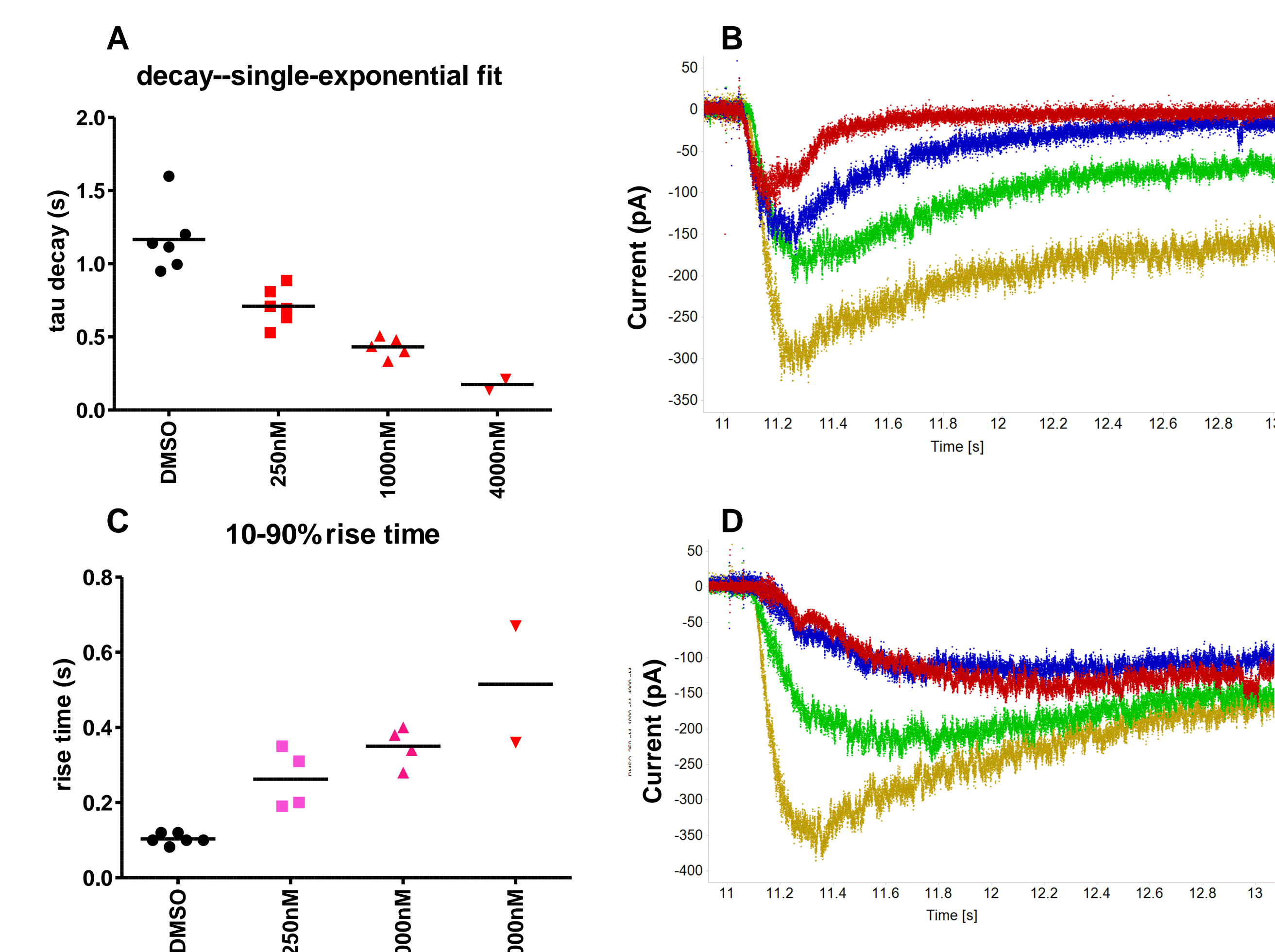

Figure 5: Compounds That Alter Kinetic Properties: **A and B.** Representative current traces and tau decay data for Compound A. Compound shows faster decay rates compared to standard GluK1 inhibitors. **C and D**. Representative current traces and rise time data for Compound B. Compound shows slower time to peak (delay in rise time) compared to standard GluK1 inhibitors. Legend for trace graphs in B and D: Gold (DMSO); Green (250 nM); Blue (1000 nM); Red (4000 nM).

Conclusions

In conclusion, we have developed an ultra high-throughput, robust assay to screen for small molecule inhibitors of GluK1. A screening campaign evaluated a library of 1.8 million compounds and confirmed activity in over 6000 compounds. An electrophysiology assay on the Q-Patch was developed to screen 1000 compounds for functional GluK1 activity. Activity was confirmed in 20% of the compounds; in addition, the functional assay was able to identify compounds that altered the kinetic properties of the current trace. This information could be useful when characterizing development candidates for potential mechanism of action. This screening approach highlights the importance of introducing high-quality whole-cell electrophysiology assays at early steps in HTS triage for ion channel screening programs.

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