

An Integrated Assay Platform for Mechanistic and Translational Profiling of K_v7.2/7.3 Activators

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Introduction

K_v7.2/7.3 (KCNQ2/3) potassium channels underlie the neuronal M-current, a key regulator of membrane excitability and action potential firing.¹ Pharmacological activation of these channels represents a validated strategy for dampening hyperexcitability in disorders such as epilepsy and neuropathic pain.² Activators of K_v7.2/7.3 exhibit differing mechanisms of action, including pore stabilisation and voltage sensor modulation dependent upon lipid cofactors such as phosphatidylinositol 4,5-bisphosphate (PIP₂).^{3,4}

We have developed an integrated assay platform combining high-throughput screening using a fluorescent thallium dye (FLIPR® Penta), automated voltage clamp (Qube 384) and current clamp (QPatch 48) electrophysiology, and manual patch clamp recordings from sensory neurons to characterise K_v7.2/7.3 activators.

Retigabine⁵, flupirtine⁶, ML213⁷, XEN1101⁸ and opakalim⁹ are gating modifiers, binding in the pore-forming domain (S5-S6). ICA 069673 binds within the voltage-sensing domain (S1-S4).¹⁰

Methods

Cell culture

A U2-OS cell line stably expressing concatemeric K_v7.2/7.3 channels was generated in-house. Cells were incubated at 30 °C for 48 h prior to experiments.

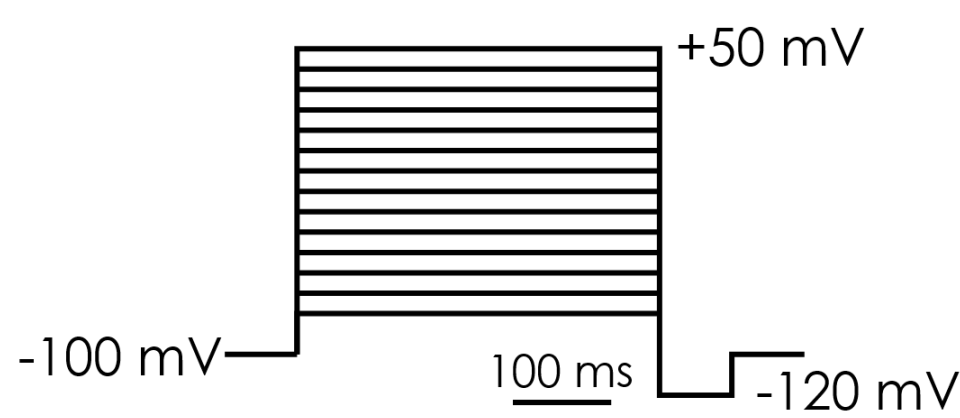
Fluorescent Imaging Plate Reader (FLIPR) Penta

10,000 cells per well were seeded into 384-well plates. Cells were dye-loaded with Potassium Assay Dye (Molecular Devices) prepared in a chloride-free assay buffer at 37 °C for 1 h. Compounds were diluted in assay buffer supplemented with 0.01 % v/v Pluronic F-127, 0.75 mM Ti₂SO₄ and 1.25 mM K₂SO₄. Following a 10-s baseline read, diluted compound was added to the dye-loaded cells on the FLIPR Penta (Molecular Devices) set to 37 °C. Final DMSO concentration was standardised to 0.5 % v/v for all conditions. Fluorescence change was monitored at 1-s intervals for 5 min using excitation 470-495 nm and emission 515-575 nm, and the data analysed using the Area Under Curve between reads 1 and 300. Data were then normalised to in-plate positive and negative controls consisting of 50 µM retigabine and 0.5 % DMSO, respectively.

Automated electrophysiology

Extracellular solution (in mM): NaCl (150), KCl (4), CaCl₂ (5), MgCl₂ (1), HEPES (10), glucose (10); pH 7.4 with NaOH. Intracellular solution (in mM): KF (120), KCl (20), EDTA (10), EGTA (10), HEPES (10), Na₂-ATP (5); pH 7.2 with KOH.

Qube 384 (Sophion Bioscience): voltage clamp



Protocol A – I-V voltage clamp protocol for Qube 384

U2-OS-K_v7.2/7.3 cells were stimulated using Protocol A. A current-voltage relationship (I-V) was recorded in 0.3 % DMSO followed by two increasing concentrations of compound per well. Recordings were conducted using multihole acquisition. These data were used to assess compound effects on channel V_{0.5} activation and conductance. Diluted compounds were supplemented with 0.01 % v/v Pluronic F-127.

QPatch 48 (Sophion Bioscience): current clamp



Protocol B – current clamp protocol for QPatch 48

Zero current was used to record the cell resting membrane potential (RMP) for 40 s per liquid period (Protocol B). Liquid addition occurred after 10 s recording and the effect on RMP was assessed 30 s thereafter. Vehicle (≤ 1 % DMSO) was applied, followed by four cumulative concentrations of compound. Recordings were conducted using single hole acquisition.

Manual patch clamp recordings from sensory neurons

Neonatal Wistar rat neurons (P4-P12) were isolated, dissociated and plated at appropriate densities onto poly-D-lysine- and laminin-coated glass coverslips. Manual patch clamp experiments were performed using a MultiClamp 700B amplifier (Molecular Devices) and an Axon Digidata 1400A digitiser (Molecular Devices).

Results

1. Potency assessment of K_v7.2/7.3 activators using a Fluorescent Imaging Plate Reader (FLIPR)

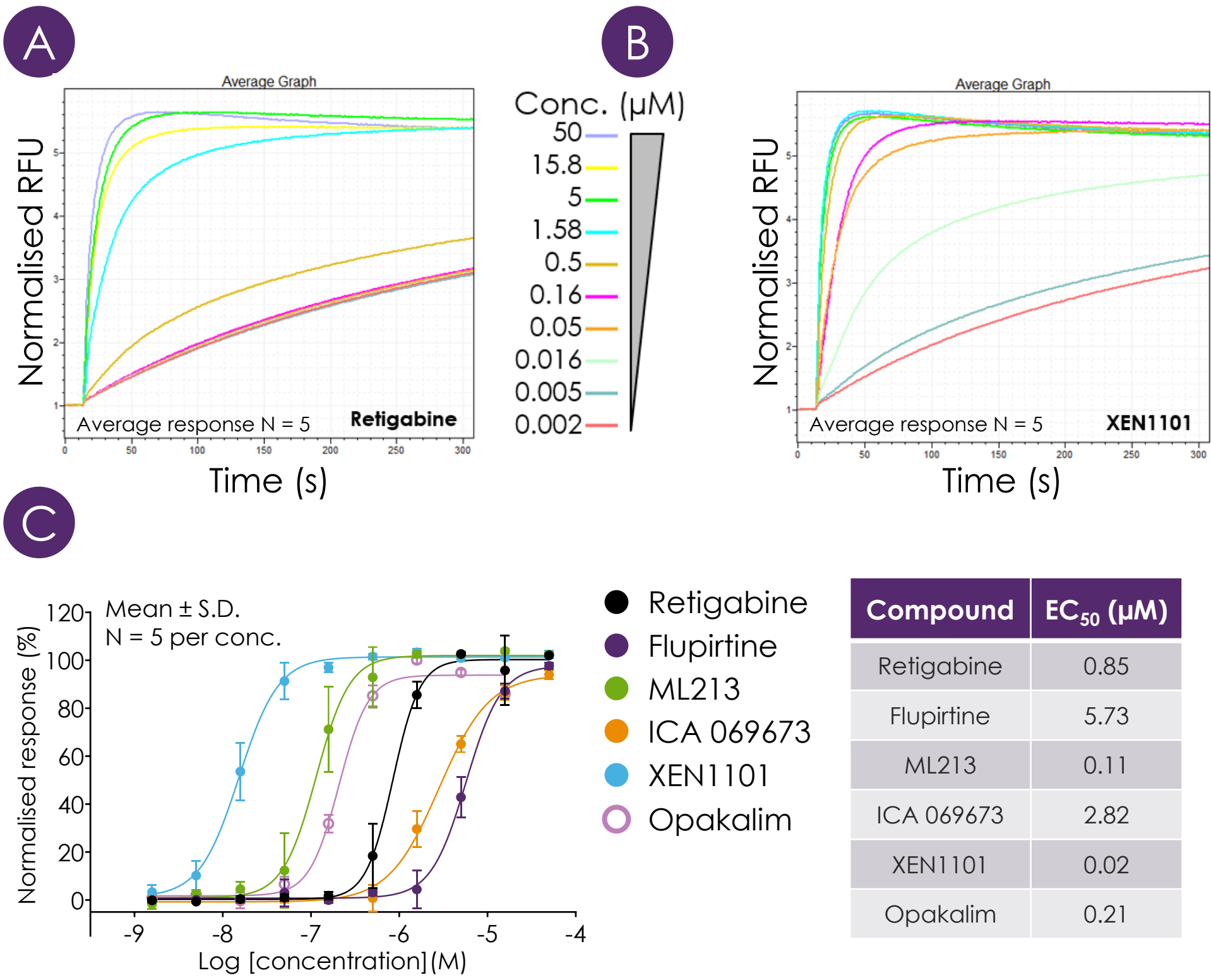


Figure 1 – Potency of K_v7.2/7.3 activators assessed using a FLIPR Penta thallium flux assay. Averaged (N = 5) raw data traces for retigabine (A) and XEN1101 (B). The legend for (B) is the same as for (A). RFU = relative fluorescence units. (C) EC₅₀ concentration-response curves normalised to 0.5 % DMSO and 50 µM retigabine.

2. Electrophysiological characterisation of K_v7.2/7.3 activator modalities using Qube 384

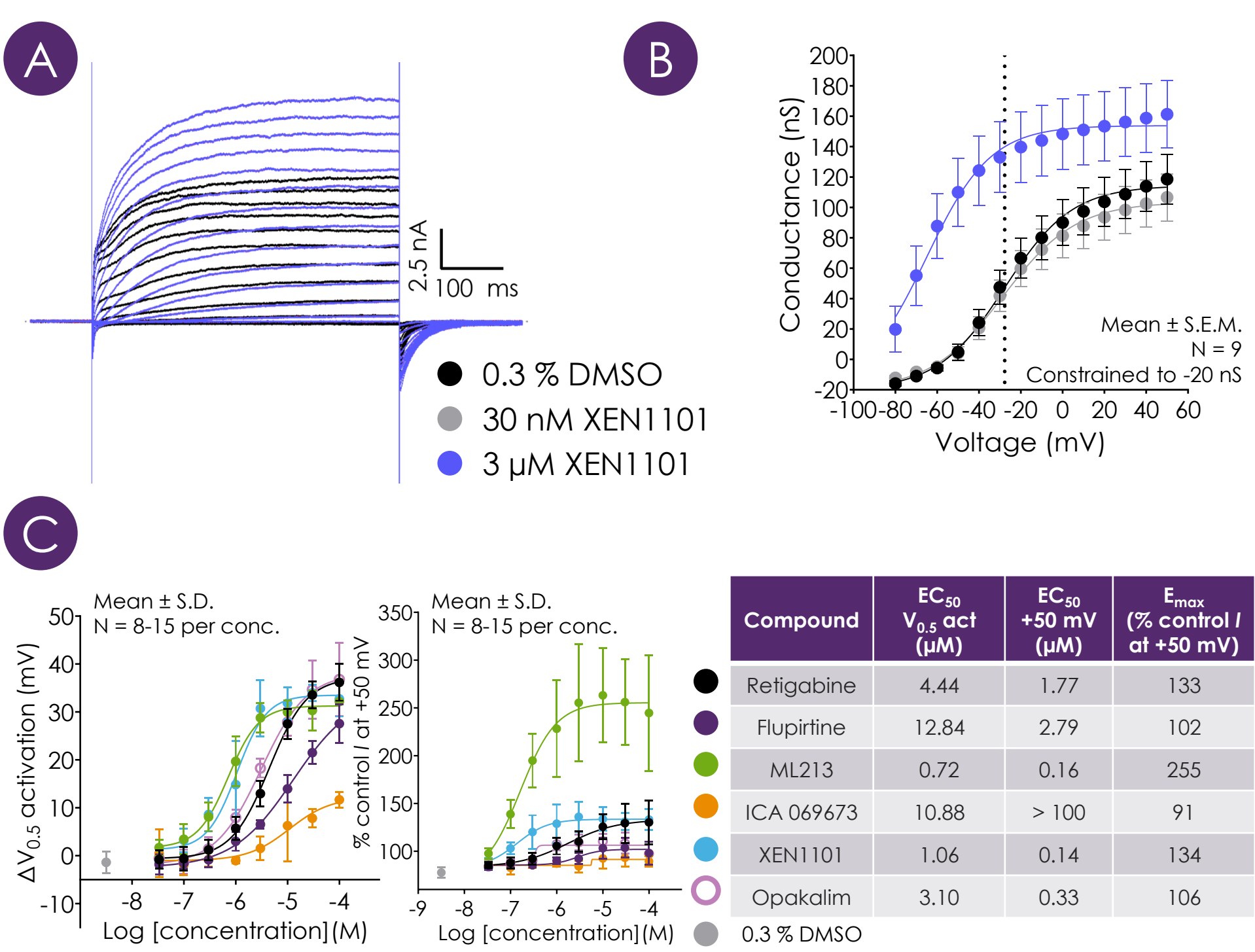


Figure 2 – Pharmacological effects of K_v7.2/7.3 activators characterised using automated voltage clamp electrophysiology. (A) Example I-V current traces of 0.3 % DMSO and 3 µM XEN1101. (B) Average conductance-voltage curves for 0.3 % DMSO (V_{0.5} = -27.70 mV – dotted line), 30 nM (V_{0.5} = -28.40 mV) and 3 µM (V_{0.5} = -65.15 mV) XEN1101. The legend for (B) is the same as for (A). (C) EC₅₀ potency assessment of compounds on K_v7.2/7.3 V_{0.5} activation and current (I) at +50 mV.

3. Physiological effect of K_v7.2/7.3 activators on cell resting membrane potential (RMP) using QPatch 48

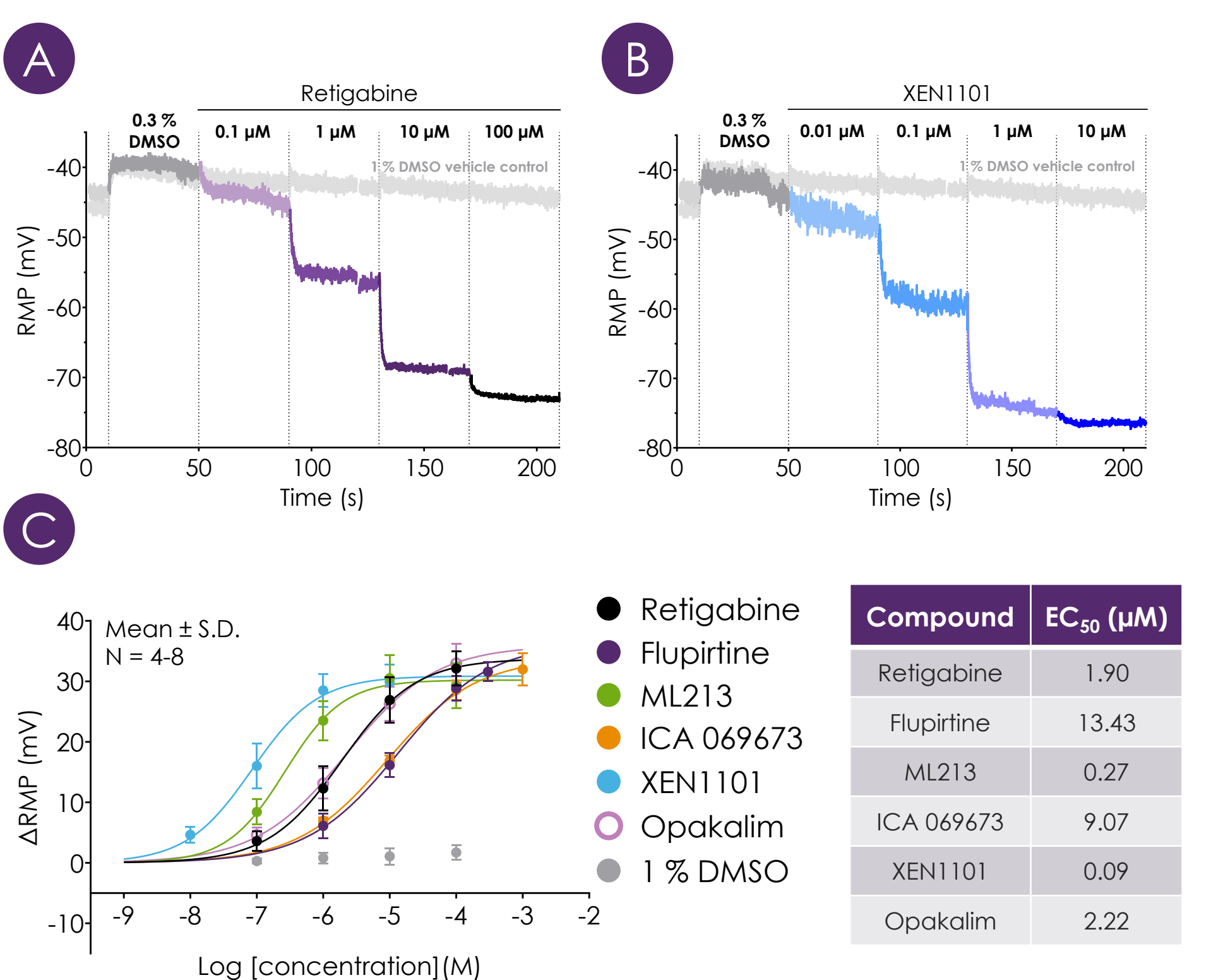


Figure 3 – K_v7.2/7.3 activators hyperpolarise the RMP of U2-OS-K_v7.2/7.3 cells. Raw current clamp traces from retigabine (A) and XEN1101 (B). Each panel also shows a time-matched vehicle control trace (1 % DMSO). (C) EC₅₀ potency assessment of K_v7.2/7.3 activators on RMP. Maximal hyperpolarisation approaches E_K.

4. Effects of K_v7.2/7.3 activators on sensory neuron resting membrane potential (RMP)

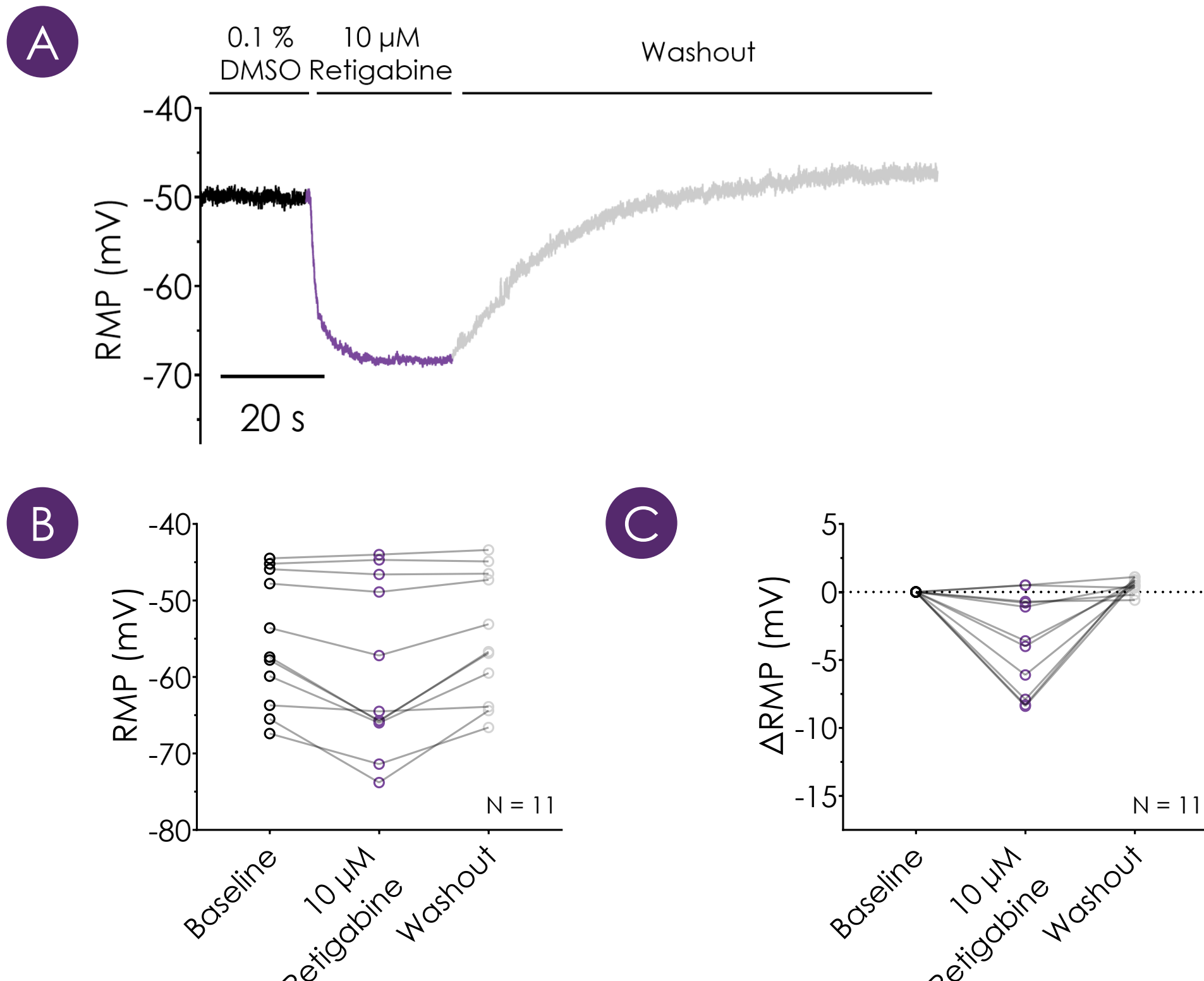


Figure 4 – Sensory neuron hyperpolarisation by retigabine. (A) Example trace showing sensory neuron RMP hyperpolarisation in response to 10 µM retigabine. Graphs illustrate raw RMP (B) and ΔRMP (C) in response to baseline (0.1 % DMSO), 10 µM retigabine and washout. Cell-to-cell variability in retigabine response was observed.

Conclusions

- The FLIPR Penta thallium flux assay provides a high-throughput method to determine potency and rank order of K_v7.2/7.3 activators
- FLIPR potencies robustly translate to automated voltage clamp electrophysiology (Qube 384), enabling more precise characterisation of compound mechanisms of action through shifts in V_{0.5} activation and changes in maximal conductance (G_{max})
- Automated current clamp assays on the QPatch 48 physiologically integrate V_{0.5} and G_{max} endpoints to assess compound effects on cell RMP. This can be used to triage key K_v7.2/7.3 activator compounds prior to testing in lower-throughput sensory neuron recordings
- Cultured sensory neurons provide a translational assay to assess the effects of K_v7.2/7.3 activators on RMP (targeting native K_v7 channels)
- Future work will investigate the properties of compounds with more distinct modalities and their effects on RMP

A comprehensive understanding of the pharmacology of K_v7.2/7.3 activators provides a route to developing more potent and efficacious K_v7.2/7.3-selective compounds for the treatment of pain and epilepsy.

We have developed an integrated workflow combining high-throughput screening using a FLIPR thallium dye assay, Qube 384 voltage clamp profiling to characterise biophysical effects of K_v7.2/7.3 activators, followed by QPatch 48 current clamp studies to assess physiological effects on resting membrane potential. Key compounds can then be triaged for validation in sensory neuron recordings, linking pharmacological mechanism to neuronal excitability.

References

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