Development and validation of NMDA receptor ligand-gated ion channel assays using the Qube 384 automated electrophysiology platform

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1 ABSTRACT

Ligand-gated ion channels are of particular interest to the pharmaceutical industry for the treatment of diseases from a variety of therapeutic areas including CNS disorders, respiratory disease and chronic pain. Ligand-gated ion channels have historically been investigated using fluorescence-based and low throughput patch-clamp techniques. However the development of the Qube 384 automated patch-clamp system has allowed rapid exchange of liquid and direct measurement of ion channel currents on a millisecond timescale, making it possible to run HTS campaigns and support SAR with a functional readout.

Here, we have used the Qube platform to develop an assay against the NR1/NR2A receptor, which is part of the N-methyl-D-aspartate (NMDA) glutamate receptor family. For this assay we utilized stacked liquid addition which enabled us to assess the open state kinetics of the channel and to investigate the effects of antagonists with multiple modes of actions.

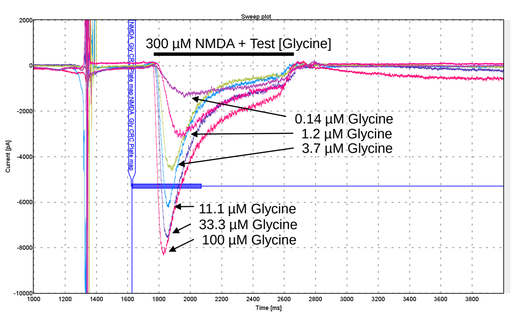
2 MATERIALS AND METHODS

Cell Culture: HEK-NMDA NR1/N2A receptor cells were produced at Charles River Laboratories and are commercially available. All cells were grown according to their respective SOPs as developed by Charles River, except for the use of D-(-)-AP-5 as antagonist during induction. Cells were kept in a serum-free medium in the cell hotel on the Qube instrument for up to 4 hours during experiment.
Induction: Cells were induced 24 h prior to use using 1 µg/mL tetracycline and 100 µM D-(-)-AP-5 in neurobasal medium + 10% dialysed FBS.
Solutions: The following extracellular saline solution was used (mM): 145 NaCl. 4 KCl, 10 HEPES, 10 Glucose, 2 $CaCl_2$, pH7.4. Intracellular solution (mM): 70 KCl, 70 KF, 10 HEPES, 1 EGTA, pH7.2.
Qube experiments: All experiments were carried out using the Qube platform which performs 384 parallel and independent patch-clamp recordings on a disposable multi-hole QChip. The holding potential used for this assay was -70mV.
Analysis: Data analysis was performed using Qube Analyzer software and GraphPad Prism (7.0).

3 RESULTS

3.1 High success rates were achieved in the NMDA NR1/NR2A receptor assay

(A)



(B)

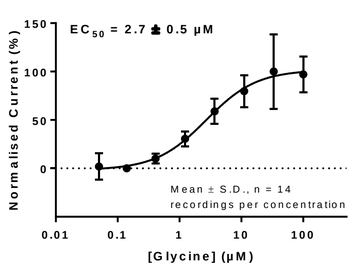
	NMDA NR1/NR2A
Mean Seal Resistance (MΩ ± SD)	81 ± 25
Mean Current Amplitude (nA ± SD)*	4.7 ± 1.8
No. wells > 2 nA, >20 MΩ (Success rate)	374 (97%)

* Response to EC_{80} NMDA + EC_{80} Glycine

Figure 1. NMDA receptor assay example traces and success rates. (A) NMDA receptor current response to increasing concentrations of glycine in the presence of 300 µM NMDA. (B) Mean seal resistance, mean current amplitude and success rates for the assay using minimum current and seal resistance criteria of >2 nA and >20 MΩ, respectively.

3.2 Dual agonist concentration-response curves

(A)



(B)

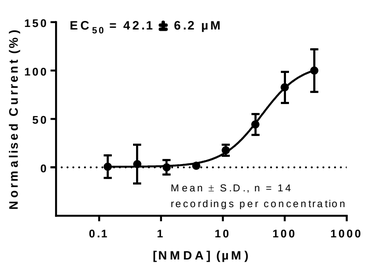


Figure 2. Agonist concentration response curves. (A) Glycine concentration response curve in the presence of 300 µM NMDA. (B) NMDA concentration response curve in the presence of 100 µM glycine.

3.3 The NMDA receptor assay was stable for over 40 min (18 liquid additions)

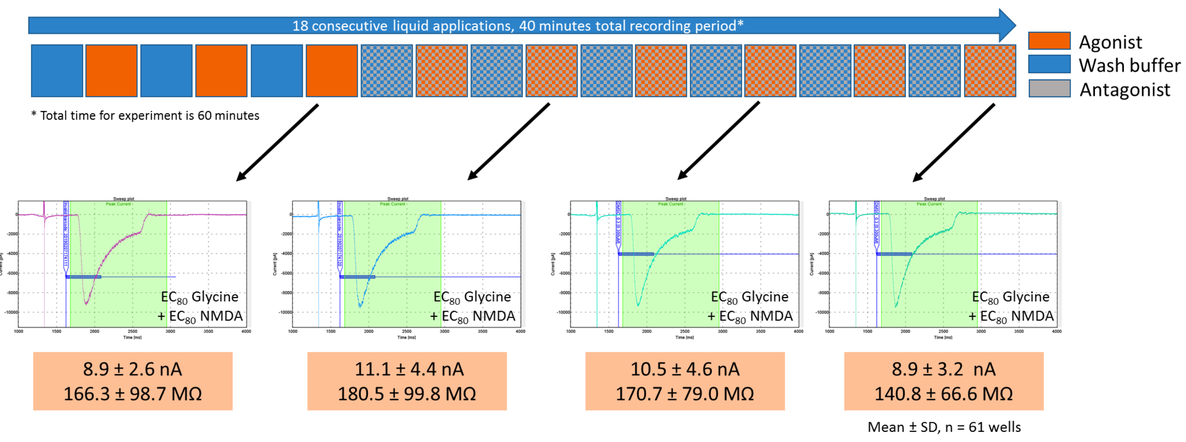
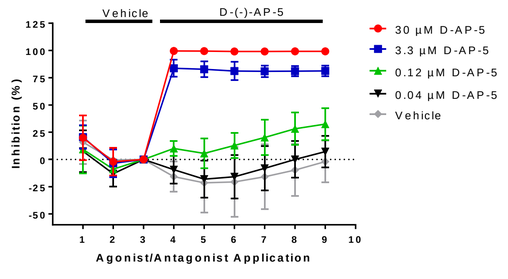


Figure 3. Final assay format for assessing use-dependent compounds. Schematic diagram demonstrating the consecutive addition of 3 agonist periods, followed by 6 agonist ±antagonist additions, with alternating wash ± antagonist periods . Example traces from selected agonist periods and associated mean current amplitude and mean seal resistance values.

3.4 NMDA receptor currents were inhibited by competitive antagonist D-(-)-AP-5

(A)



(B)

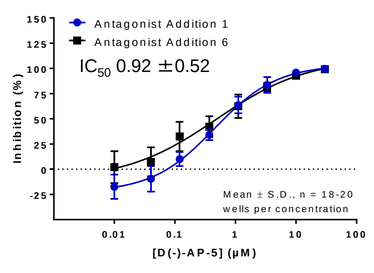
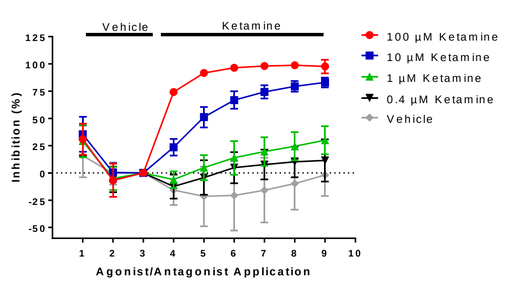


Figure 4. D-(-)-AP-5 concentration response curve and inhibition response over consecutive antagonist applications. (A) Percent inhibition by increasing concentrations of D-(-)-AP-5 over consecutive agonist ± antagonist applications. (B) D-(-)-AP-5 concentration response curve after 1 versus 6 agonist + antagonist applications.

3.5 NMDA receptor currents were inhibited by the use-dependent pore blocker ketamine

(A)



(B)

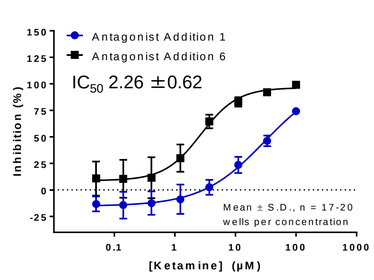
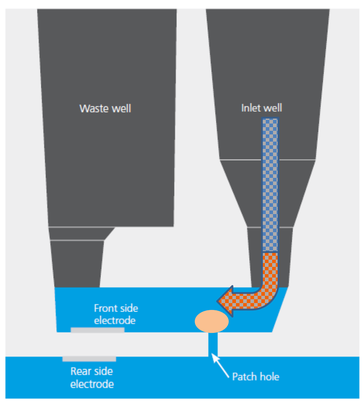


Figure 5. Ketamine concentration response curve and inhibition response over consecutive antagonist applications. (A) Percent inhibition by increasing concentrations of ketamine over consecutive agonist ±antagonist applications. (B) Ketamine concentration response curve after 1 versus 6 agonist + antagonist applications.

3.6 Compound inclusion in stacked addition washout improved antagonist potency

(A)



(B)

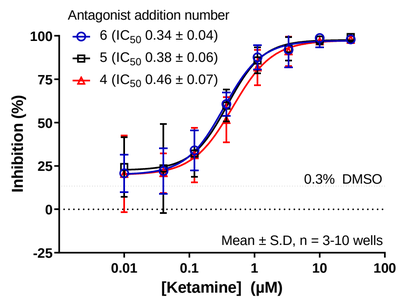


Figure 6. Optimizing the stacked liquid addition protocol for assessing use-dependent compounds. (A) Schematic diagram demonstrating the inclusion of antagonist compound in the agonist washout step during the stacked liquid addition protocol (B) Concentration response curve of ketamine with ketamine included in the agonist washout step during the stacked liquid addition. Dotted line

4 SUMMARY

Here we present a novel NMDA NR1/NR2A assay for the assessment of use-dependent compounds on the Sophion Qube 384 automated electrophysiology platform.

Initial recordings of the NMDA NR1/NR2A receptor using a multi-hole QChip demonstrated an average of 4.7 nA of current with a mean seal resistance of 81 ± 25 MΩ, and success rate of 97%. Agonist concentration response curves were produced for both glycine and NMDA at top concentrations of their respective co-agonist, EC_{50} values for both agonists reflect that of literature values [1,2].

The NMDA NR1/NR2A receptor assay was stable up to 18 consecutive liquid additions, with minimal variation in current amplitude and seal resistance from the 6th liquid addition to the 18th.

Inhibition of the NMDA NR1/NR2A receptor by D-(-)-AP-5 inhibition was stable over multiple consecutive antagonist applications. D-(-)-AP-5 IC_{50} values reflected literature values [3].

The percentage inhibition by ketamine increased over consecutive antagonist applications and the concentration response curve after 6 antagonist applications was left-shifted compared to only 1 antagonist application. The inclusion of ketamine in the agonist washout during the stacked liquid addition caused a 7-fold increase in the potency of ketamine against the NMDA NR1/NR2A receptor.

In conclusion, we have produced a novel, highly robust assay with high success rates for testing use-dependent compounds against the NMDA NR1/NR2A receptor.

Thank you to the cell culture team at Charles River for their time and expertise.

(1) Cummings, K.A. & Popescu G.K. (2015) *J. Gen. Physiol* (2) Fedorov, N. *et. al.(2015) Biophys. J. *(IonWorks Barracuda assay)*

(3) Feng *et. al.* (2005) *Neuropharmacology*