Development and Validation of Automated Electrophysiology Assays for Voltage-gated Sodium Channels

L. Pym*, R. Helliwell*, J. Mann, A. Cook, H. Swain, C. Holdsworth, E. Maughfling, L. Webdale, A. Cooper, R. Reehal, U. Patel, D. Jarman, J. Clare

Millipore UK, 6-7 Technopark, Newmarket Road, Cambridge, UK; *Corresponding Authors: Luanda_Pym@millipore.com, Ray_Helliwell@millipore.com

Abstract

Voltage-gated sodium (Nav) channels are important targets for various indications including arrhythmia, pain, local anaesthesia, epilepsy and bipolar disorder. Despite their indispensable role in mediating action potentials in excitable tissues, drugs that inhibit these channels are remarkably well tolerated. This is thought to be due to their voltage- and use-dependent mechanism of action whereby the extent of block is greatly increased during periods of repetitive firing or sustained depolarisation as may occur, for example, during seizure activity or pain signalling.

Interest in NaV channels has been intensified by the discovery of human mutations in Nav1.7 that confer remarkable inability to sense pain in otherwise healthy individuals. Other subtypes have previously been implicated in pain signalling (e.g. Nav1.8 and 1.3) but the development of subtype selective inhibitors has proved extremely challenging. One reason for this is the limited nature of available high throughput screening assays (e.g. based on membrane potential dye / flux measurements) which resort to non-physiological manipulations to activate the channels, lack the necessary temporal resolution and are incapable of measuring the key properties of use- and voltage-dependence.

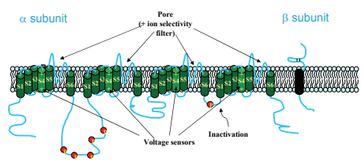
Millipore has developed a panel of robust electrophysiology-based assays for profiling compounds against each of the Nav subtypes, from 1.1 to 1.8, stably expressed in mammalian cell lines. These assays use two different automated platforms (IonWorks® and PatchXpress™ systems) which, like manual patch clamp, offer relatively accurate voltage clamp and the ability to directly record ionic current through the channel. Since the principles underpinning these technologies are so similar to manual patch clamp, the high false positive/negative rates typically associated with other screening methods are avoided and, crucially, they can be used to monitor use- and voltage-dependence.

Here we demonstrate how the improved robustness and reproducibility of population patch clamp (PPC) available with the IonWorks Quattro™ instrument can be exploited to reliably screen relatively large numbers of compounds against Nav channels.

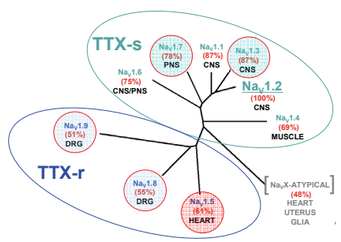
Introduction

Nav channels

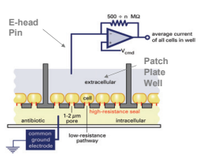
Nav channels are composed of a large pore-forming α subunit and one or more accessory β subunits. The α subunit contains 24 transmembrane spanning helices which are organised into four homologous domains. The α subunit alone is capable of forming a fully functional Na+ selective channel and various functional domains have been identified:



Ten different α subunit subtypes are known which share a high degree of sequence similarity but have distinctive tissue distribution. Historically these have been classified pharmacologically into those that are sensitive to tetrodotoxin (TTXs) and those that are resistant (TTXr):



Automated Patch Clamp Technology - IonWorks Quattro System



Schematic diagram of an E-head Pin in a Patch Plate Well

PRINCIPLE

The principal components of the instrument are the E (Electronics)-Head, F (Fluidics)-Head and patch plate. The patch plate contains 384 wells, each well having 1-2 µm diameter pores in the base. Cells, extracellular solution and drugs are added by the F-head.

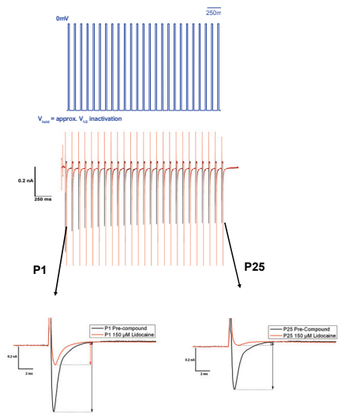
Cells seal over the holes at the bottom of the wells and electrical access is achieved by addition of the antibiotic, amphotericin, (which acts as a perforating agent) to the intracellular solution from the underside. This also enables dialysis of the cell interior with intracellular solution similar to manual perforated patch clamp. The IonWorks Quattro system uses patch plates that contain 64 holes per well, (c.f. single hole for Ionworks-HT) and currents recorded in each well are averaged from up to 64 cells. This population patch clamp (PPC) configuration provides significantly increased well-to-well reproducibility.

The voltage is controlled and currents recorded by a 48-channel E-head. Each channel has a separate Ag/AgCl2 pin connected to an amplifier which applies voltage across the cell membranes relative to a common ground electrode on the underside. With this arrangement currents from a maximum of 384 wells can be recorded using protocols lasting ~1 hr

Methods

Voltage Protocol and Data Analysis

A series of 25 depolarising pulses (20 ms at 10 Hz) to 0mV are applied from from a holding potential (V_{hold}) approximating to the $V_{1/2}$ of inactivation of the Nav channel under test, as shown in the blue upper trace. This voltage protocol is applied both prior to, and 300s after addition of, test compound evoking a series of inward Nav currents that fully inactivate during each pulse (pre-compound = black middle trace; post-compound = red middle trace). The example trace shows the effect of 150 µM lidocaine on Nav1.7 - also shown are traces for the 1st (P1) and last (P25) pulses on an expanded time-base (see bottom traces)



To assess compound effects the peak current amplitude after compound addition (red arrows, bottom trace) is divided by the peak current amplitude before compound addition (black arrows, bottom trace) for both P1 and P25.

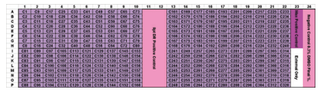
Comparing the relative extent of block for P1 and P25 provides a preliminary assessment of any accumulation of block (use-dependence) occurring during the train of pulses.

Selection of a V_{hold} that inactivates approximately half of the channels ensures that inactivated state blockers are readily detected (see below) and allowance is made for the different voltage dependencies of inactivation of each Nav subtype so that a similar number of inactivated channels are available for block.

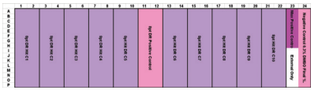
Controls and Compound Plate Layout

Compounds and controls are added to the assay plates from 384 well compound plates. These can be configured to test compounds at a single concentration in 'Screening Mode' (maximum of 320 compounds per run) or to establish dose-response relationships in 'DRC Mode' (e.g. 8 point curves for 10 compounds with a maximum number of 4 replicates per concentration). These plate arrangements are flexible though columns 11, 12 and 23, 24 are normally reserved for controls to monitor assay performance.

Screening Mode



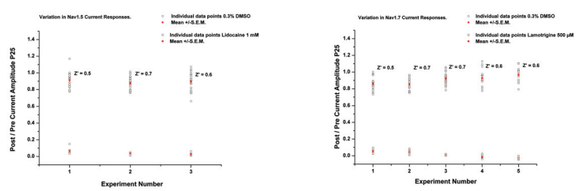
DRC Mode



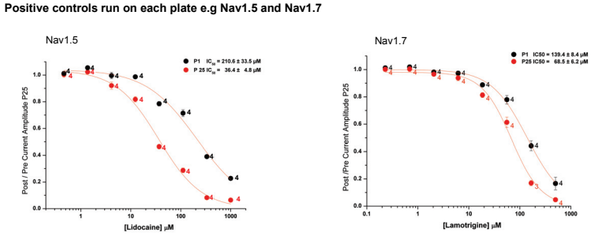
For each assay a positive control is tested, to obtain 8 point dose-response curve (columns 11 and 12) and also a Z' (column 23, A-H), calculated with 0.3% DMSO negative controls (column 24) – the highest concentration of vehicle used. In addition 8 wells are reserved for external saline only (column 23, I-P) to determine if the DMSO controls have any non-specific effects on current amplitude.

QC criteria: data from any given plate are accepted only if the following two basic criteria are met. 1) the IC_{50} value of the positive control must be within 3-fold of a mean value established during assay development (and validated against published values for 'inactivated state' block. 2) the Z' must be > 0.3.

Z' controls run on each plate e.g Nav1.5 and Nav1.7



Positive controls run on each plate e.g Nav1.5 and Nav1.7



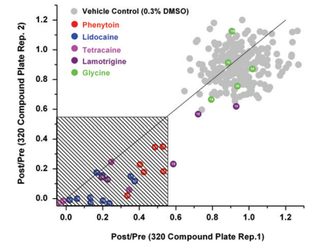
Results

Screening Mode: Nav1.7

320 wells of a 384-well plate containing vehicle (DMSO) were spiked with different concentrations of standard blockers or negative controls (glycine).

The assay was carried out twice and results compared between n1 and n2. Wells containing the standard blockers, were consistently identified (shaded area) whereas wells containing glycine gave responses that were similar to DMSO alone.

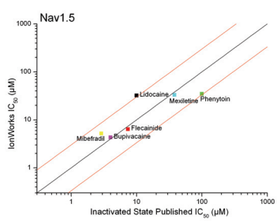
This assay design enables a potential 640 compounds to be screened / day at n=2.

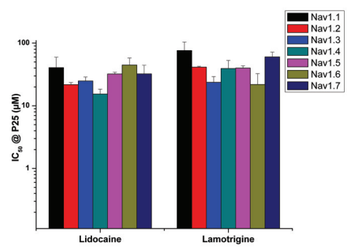


After the detection of Nav1.7 "hits", follow-up studies to screen these compounds against other Nav channels of interest e.g. Nav1.5, can be done to accurately determine IC_{50} values.

'DRC' Mode: Nav1.5

The IC_{50} estimations generated by the IonWorks system show a good correlation with published data (below. left). Estimated IC_{50} values have been determined for a range of sta
Representative data for lidocaine and lamotrigine are shown he





Summary / Conclusions

- **Automated electrophysiology-based assays for assessing the effects of compounds on Nav1.1 – 1.7 responses have been developed using Millipore's PrecisION® cell lines and IonWorks Quattro instrument.**
- **Using these assays, it is possible to estimate the efficacy and use dependence of antagonists against each of the Nav subtypes.**
- **These assays are suitable for screening test compounds at relatively high throughput with robust resolution and reproducibility.**

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