Targeting the Nav1.3 Sodium Channel in the Development of Novel Treatments for Neuropathic Pain

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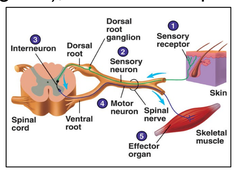
Introduction

Neuropathic pain occurs in a wide range of clinical patients, including those affected by diabetes, HIV, herpes, cancer and chemotherapy, as well as spinal cord injury. Neuropathic pain is characterized by chronic or spontaneous pain (hyperalgesia), as well as pain in response to normally innocuous stimuli (allodynia).



The increased excitability and ectopic action potential firing in damaged sensory neurons is thought to drive the development of neuropathic pain, and studies have shown that the expression of several Nav genes are altered in animal models of neuropathic pain. Whereas the expression of Nav1.7 and Nav1.8 decrease, the expression of Nav1.3 is up regulated in the DRG, spinal cord, and thalamus. Moreover, the TTX sensitivity and repriming kinetics of injury-induced Nav in the DRG match those of Nav1.3, suggesting that it underlies the increased firing in axotomised DRG neurons (Black et al., 1999). Significantly, antisense knockdown of Nav1.3 abrogates hyperexcitability and pain-related behaviours in rat models of neuropathic pain (Hains et al., 2003), whereas neuropathic pain develops normally in mice lacking both Nav1.7 and Nav1.8 (Nassar et al., 2005).

The off-label use of anticonvulsants and anti-epileptics known to affect sodium channels for pain management suggests that an Nav ion channel modulator could be an effective treatment. As existing compounds used for neuropathic pain are inadequate due to poor efficacy, tolerance, abuse liability and drug interactions, this major area of unmet clinical need could be well served by a drug selectively targeting the Nav1.3 channel.

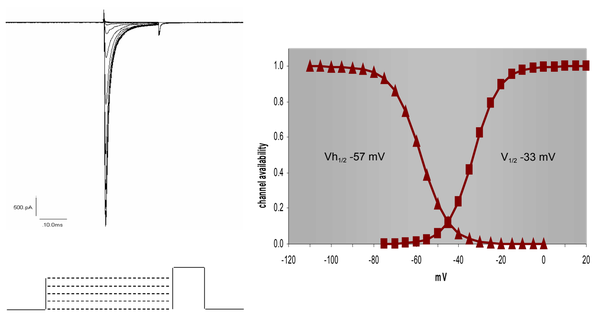
Methods

2. Tissue culture
   The full length human Nav1.3 gene (SCN3A) was stably expressed in a CHO K1 cell line. Antibiotic resistance was maintained using 400 μg/ml Geneticin, and stable sodium currents could be recorded up to passage # 50. Cells were grown in Iscoves modified Dulbecco medium containing L-glutamine and supplemented with 10% dialysed foetal calf serum, 2% HT, and 1% non-essential amino acids. Cells were grown in plastic T-75 flasks and routinely passaged after reaching 70% confluence, while cells for patch clamping were plated into 35 mm plastic petri dishes and used 2-4 days later.
3. HTS Flux assay
   Cells were grown in 96 well plates and used 12-36 hours later. Na^+-containing media was removed before pre-incubation in a Na^+-free solution in the presence or absence of test compounds. After 10 minutes stimulation buffer was added, containing tracer cation as well as a combination of channel activators and modulators to open the sodium channels and allow tracer flux into the cells. Flux was terminated after 30 minutes by removing the stimulation buffer and washing in Na^+-free solution, after which the cells were lysed in SDS and tracer cation accumulation measured.
4. Patch clamp electrophysiology
   Standard whole-cell patch clamp techniques were used to record Nav1.3 currents, using glass pipettes (2-4 MΩ) containing (in mM) 120 CsF, 15 NaCl, 10 HEPES and 10 Glucose; pH 7.2, 295 mOsm. Culture media was replaced with buffer containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES and 11 Glucose; pH 7.4, 315 mOsm. HEKA EPC amplifiers running Pulse and Autopatch software were used for data acquisition, and only cells exhibiting > 1 GΩ membrane resistance seals and stable series resistance (4-10 MΩ) were used for data collection. Series resistance was routinely compensated by 50-75%, and leak was subtracted using a P/4 protocol.

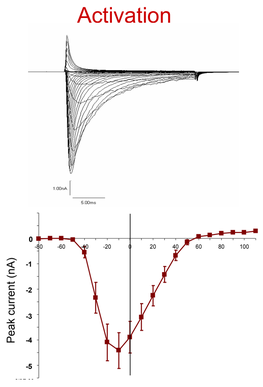
1. Patch Clamp Validation of Nav1.3 cell line

CHO cells stably expressing the human Nav1.3 channel were used throughout this study. Patch clamp recordings were made 2-5 days after plating, during which period >80% cells expressed peak inward current > -1 nA. Peak current and voltage-dependent properties "ran up" during whole cell perfusion, and steady state readings were made after 10 minutes. Nav1.3 currents exhibited typical voltage dependent values, and a small persistent component.

Voltage-dependent Inactivation



Activation

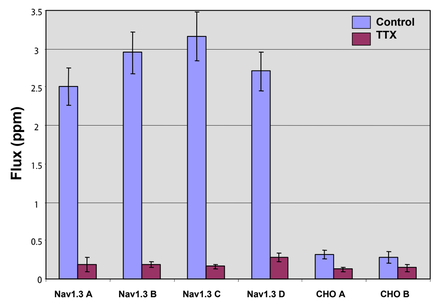


2. HTS Flux assay for Nav1.3 inhibitors

A non-radioactive HTS flux assay was developed to discover novel Nav1.3 inhibitors from our collection of compound libraries. A range of allosteric activators and inactivation modulators were utilized during the assay development phase, to create a 96 well format assay with large signal:background values and robust detection of known inhibitors (e.g. TTX).

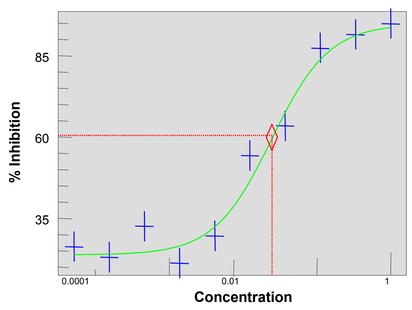
Testing Nav1.3 cell line clones

S:B > 10
z' factor > 0.6



TTX Dose-Response

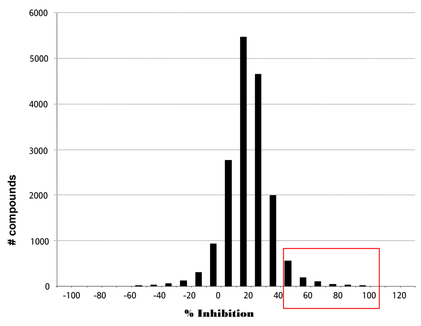
IC_{50} = 31 ± 3 nM



The optimized HTS flux assay was employed to screen a compendium of ion channel-focussed chemical libraries, yielding an average primary hit rate of 2.3%. Re-testing of compounds producing > 50% inhibition of flux in the primary screen produced a confirmed hit rate of 0.75%. All told, 160 chemical entities were identified based on their novel structure, amenable chemistry, and predicted ADME properties. This collection of primary hits represented 9 different lead series in addition to several singletons.

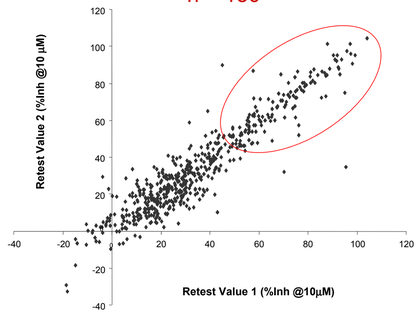
Distribution of Primary Hits

18.2 ± 15.6% inhibition



Confirmed Hits

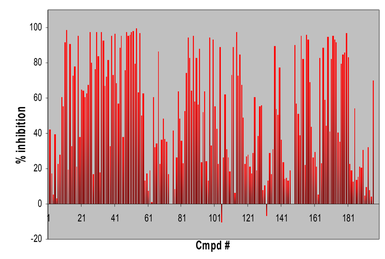
n = 130



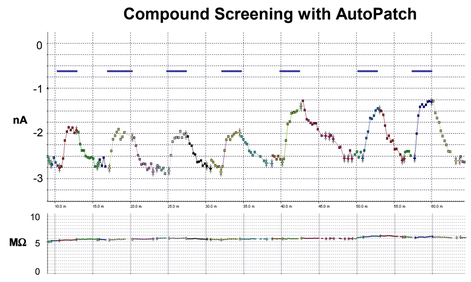
1. Electrophysiology Screening

As hits identified in the flux assay have an unknown mechanism of action, whole cell patch clamp was utilized to confirm their inhibitory effect on Nav1.3 ion channel currents. The slow throughput of manual patch clamp recording was increased through the use of our proprietary AutoPatch software suite, which co-ordinates data recording with compound delivery from an automated 96 well plate system. A typical cell produces 5-10 datapoints, either in the form of single concentration inhibition values (1 μM) or 3 point dose-response curves. This level of throughput is sufficient to confirm flux hits and support a medicinal chemistry-driven SAR programme.

PatchPilot confirmation of Flux hits



Compound Screening with AutoPatch



4. Determining the Mechanism of Action of Nav1.3 blockers

Inhibitors affect the Nav channel by several mechanisms, including phasic or open channel block (TTX), tonic or use-dependent block (Lidocaine), and stabilization of the inactivated state (Carbamazepine). We utilized voltage clamp protocols to assay these state-dependent processes, and categorise primary flux hits and subsequent SAR-derived compounds.

Phasic blocker

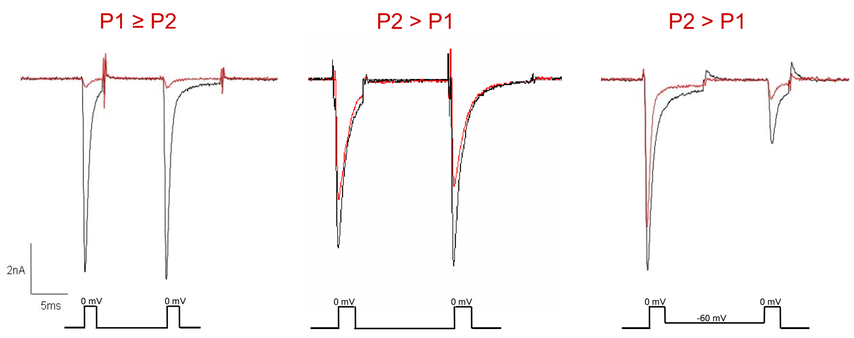
P1 ≥ P2

Tonic blocker

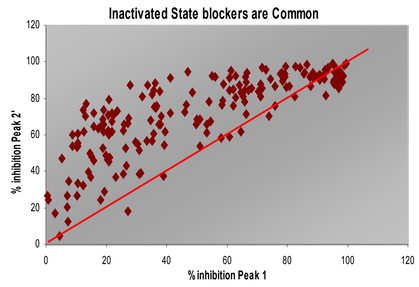
P2 > P1

Inactivated state

P2 > P1

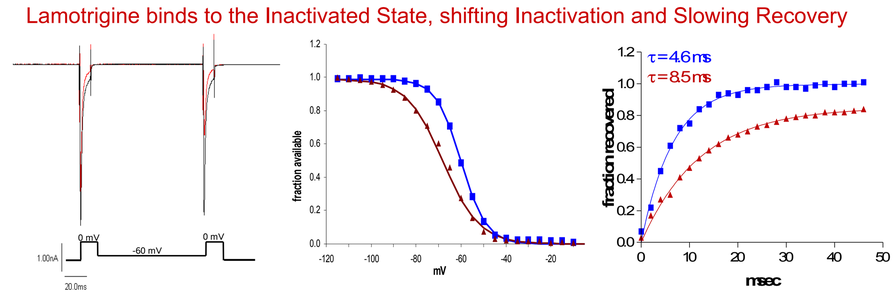


Inactivated State blockers are Common



Many clinically approved drugs currently used to treat neuropathic pain exhibit a preference for the inactivated state, such as Lamotrigine and Carbamazepine. We confirmed their mechanism of action against the Nav1.3 channel by measuring changes in the voltage dependence of inactivation, and slowing in the rate of recovery from inactivation, both indicative of stabilization of the inactivated state(s).

Lamotrigine binds to the Inactivated State, shifting Inactivation and Slowing Recovery



5. Medicinal Chemistry SAR

Below is an example of a lead series where substitutions were made to optimize binding to the inactivated state (P2/P1 ratio) while maintaining selectivity over the cardiac Nav1.5 channel. In some cases this was successful (bottom rows), while in others it was not (middle rows).

	% inhibition		ratio	Nav1.5 liability
XEN-ID	**P1**	**P2'**	**P2/P1**	**ratio 1.3:1.5 block**
Cmpd-1	37.1	68.0	1.83	1.0
Cmpd-3	34.0	58.7	1.73	1.0
	Increased P2 preference			**Increased Nav1.5 block**
Cmpd-5	17.5	39.6	2.26	1.0
Cmpd-2	15.0	54.0	3.60	-
Cmpd-8	10.6	38.3	3.60	- -
Cmpd-9	8.1	20.0	2.47	- - -
	P2 efficacy & preference			**Decreased Nav1.5 block**
Cmpd-4	29.3	49.8	1.70	+
Cmpd-6	22.4	42.3	1.89	+++
Cmpd-7	22.8	54.4	2.39	++

Conclusions

The high quality of data delivered by patch clamp electrophysiology is crucial for an ion channel drug discovery program. We utilize such data in every stage of developing an ion channel program, as illustrated here for the Nav1.3 channel:

1. Cell line validation

It is essential to determine if the ion channel of interest is expressed and functions correctly, prior to investment in HTS assays.

2. Medium throughput drug screening

We have developed a semi-automated suite of hardware and software which enables information-rich electrophysiological data to support our hit validation, lead expansion and lead optimisation phases. The use of precise voltage clamp protocols allows determination of a compounds' state-dependent binding properties, as well as overall blocking efficacy.

3. Mechanism of action

Tailored voltage clamp protocols enable us to measure drug binding to various conformational states of the Nav1.3 channel, and thus determine their mechanism of action. This approach has confirmed the efficacy of several lead series compounds that target the inactivated state of the Nav1.3 channel, thus holding the promise of delivering a treatment for neuropathic pain.

Black, JA *et al*. (1999) *J Neurophysiol* 82:2776-2785
Hains BC *et al*. (2003) *J Neurosci* 23:8881-8892
Nassar MA *et al*. (2005) *Molecular Pain* 1:24