



MILLIPORE

Targeting voltage-gated sodium channels for pain therapy

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***Ion Channel Retreat, Vancouver
June 2008***

Overview of presentation

- Introduction to Na_v channels as pain targets
- Development and validation of a panel of automated functional Na_v profiling assays
- Generation and characterisation of Na_v channels with mutations within the drug binding site

The New Millipore:

Two divisions serving Bioprocess & Bioscience Markets

Bioprocess



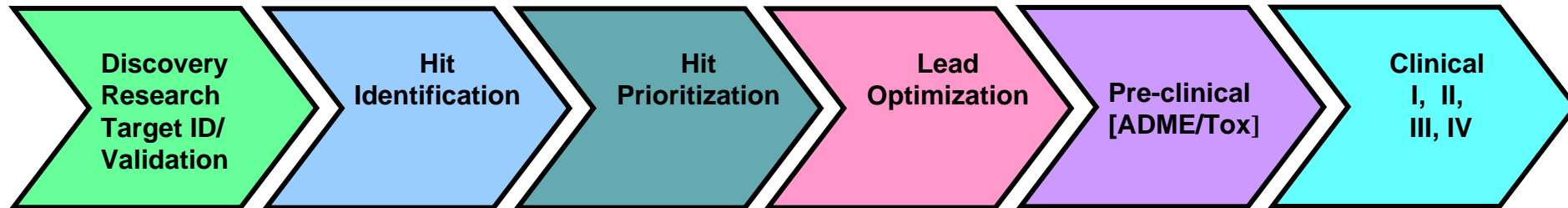
- Chromatography Columns & Media
- Microbiological Monitoring & Testing
- Ultrafiltration Cassettes
- Viral Clearing Devices
- EX-CYTE® growth enhancement media supplement
- Insulin, recombinant human
- Application specific grades of BSA
- Cell culture and diagnostic supplements

Bioscience



- Analytical sample prep devices
- Protein purification and detection
- Cell culture filtration
- Antibody based products
- Stem cell products
- Cell signaling products
- Respiratory/enterovirus detection products
- **Screening products and services**
- **Kinase/GPCR/Ion channel**
- Biomarker and protein detection services
- Lab Water Purification

Millipore and drug discovery



Profiling Reagents and Services:

GPCRs

Kinases

Ion Channels

Phosphatases

Biomarker
Immunoassay
Kits & Services

MultiScreen plates and ready-to-assay plates

Multiplexing
Kits & Services

Regulatory Compliant
Biomarker Assays

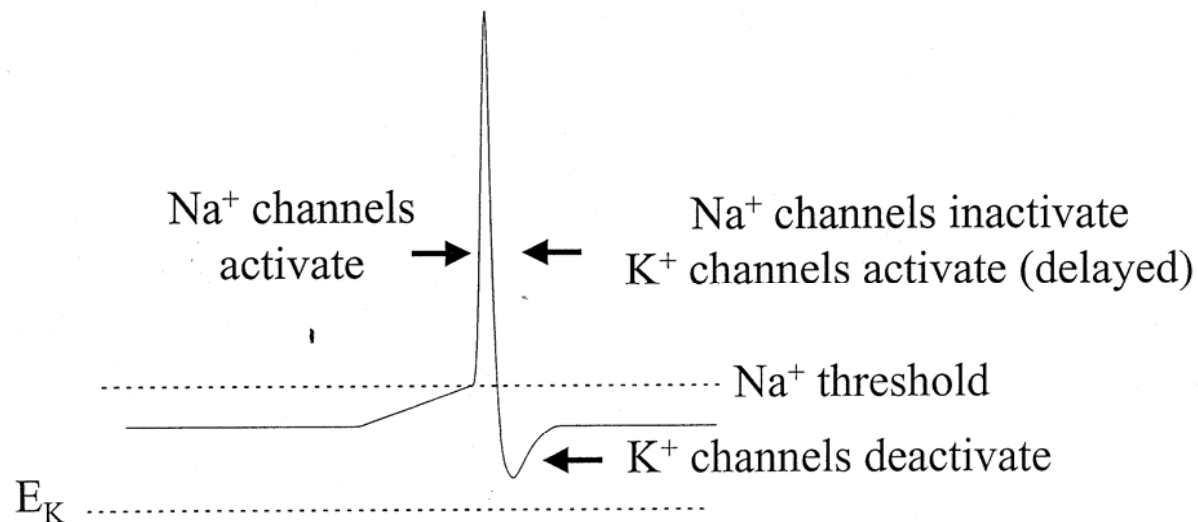
Antibody Reagents and Kits

HCS Reagents and kits

Predictive Tox assays

Voltage-gated sodium (Na_v) channels

- Present in most electrically excitable cells:
 - Widely expressed in neuronal, neuroendocrine, skeletal muscle and cardiac cells
- Activate in response to membrane depolarisation
- Responsible for rapid influx of Na^+ ions during rising phase of action potential:



Development of Na_v inhibitors – historical perspective

Disease indication	Drug	Discovered	Action at NaV identified
Local Anaesthesia	Procaine	1905	1959
Cardiac arrhythmia	Procaine	1936	1959
Epilepsy	Phenytoin	1937	1983
	Carbamazepine	1960s	1985
	Lamotrigine	1978	1986

NaV inhibitors subsequently shown to be clinically effective in *pain, bipolar disorder*

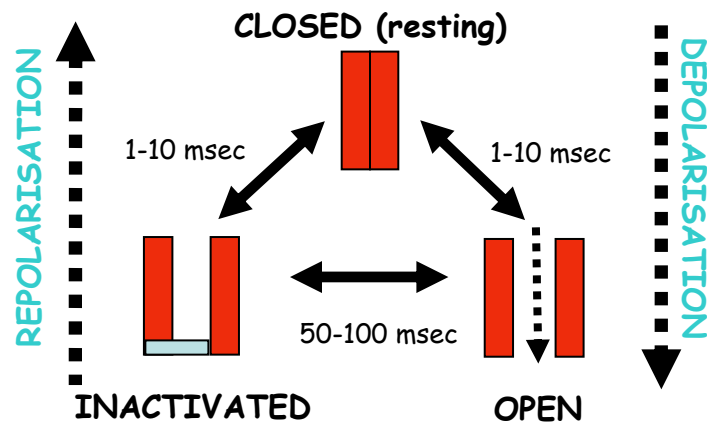
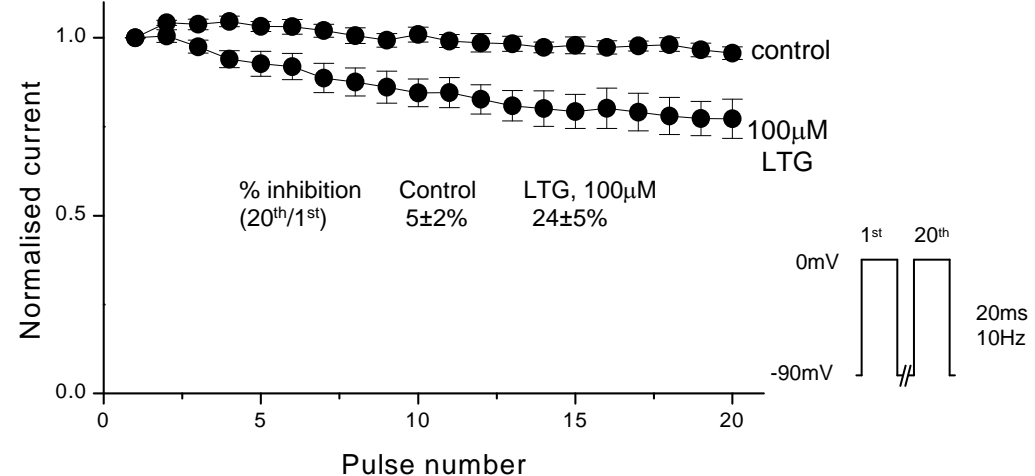
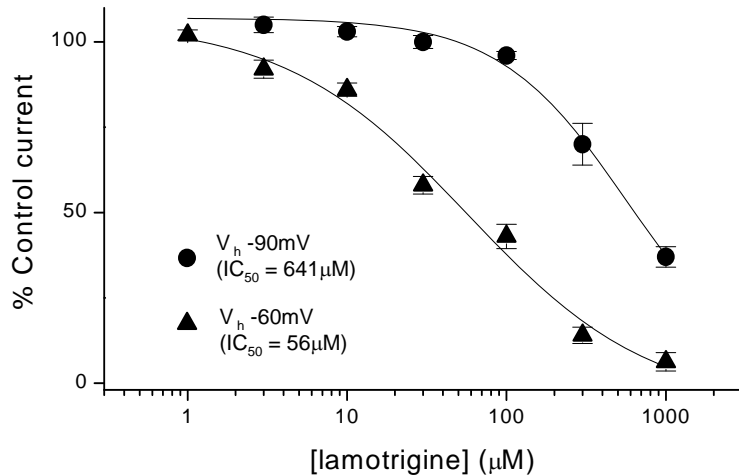
Other indications have also been implicated:

- *migraine, spinal cord injury, schizophrenia, cerebral ischemia, substance abuse, Parkinson's disease, prostate cancer, others....*

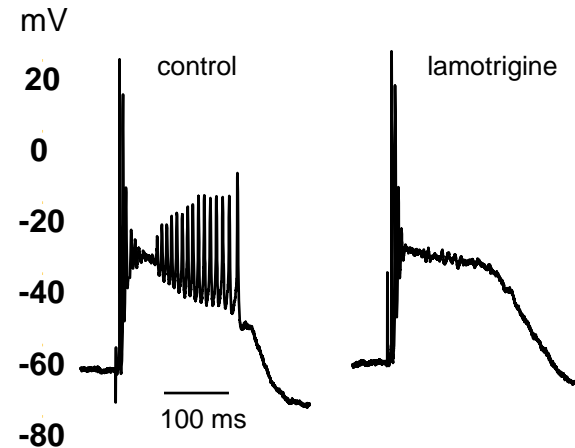
State-dependent block of Na_v channels

Voltage and use dependent action of lamotrigine:

Xie et al. (1995)
Pflugers Arch.
430:437-446



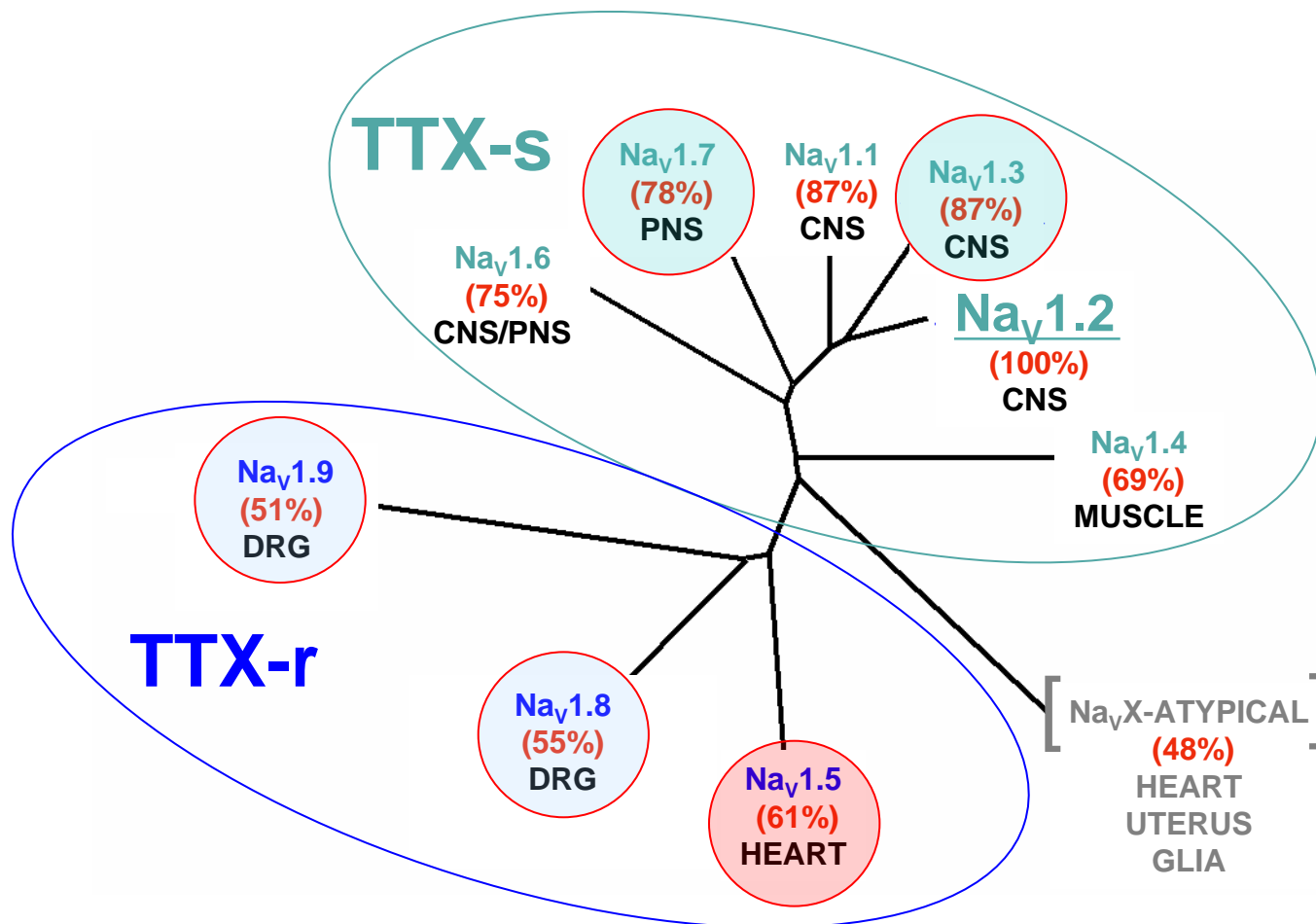
Stabilises an inactive state



Xie et al. (1995)
Pflugers Arch.
430:437-446

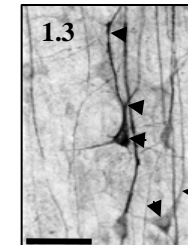
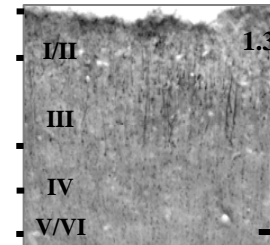
Selective blockade of repetitive firing

Multi-gene family of Na_v subtypes



Na_v1.3 as a target for pain therapy

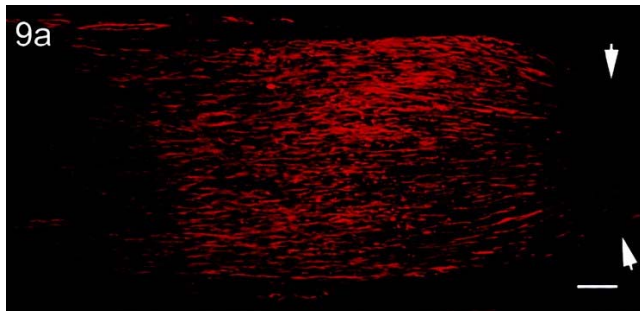
- Often referred to as an embryonic form (based on early rodent distribution studies)
- However, human studies showed it is expressed in normal adult brain, particularly cell bodies and dendrites (subsequently confirmed in rodents)



Somato-motor cortex

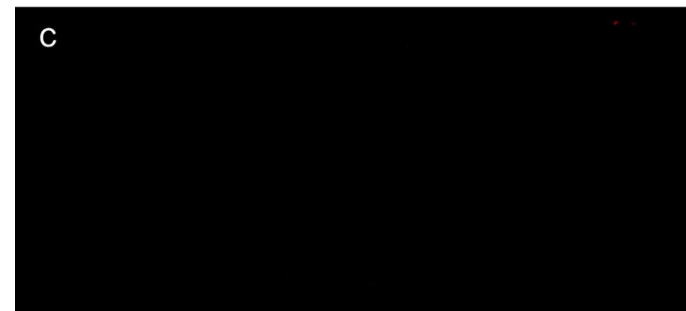
Whitaker et al. (2001)

- Normally not expressed in nociceptive neurons but upregulated after neuronal injury or inflammatory insult:-



Ligated sciatic nerve

Black et al. (2001)

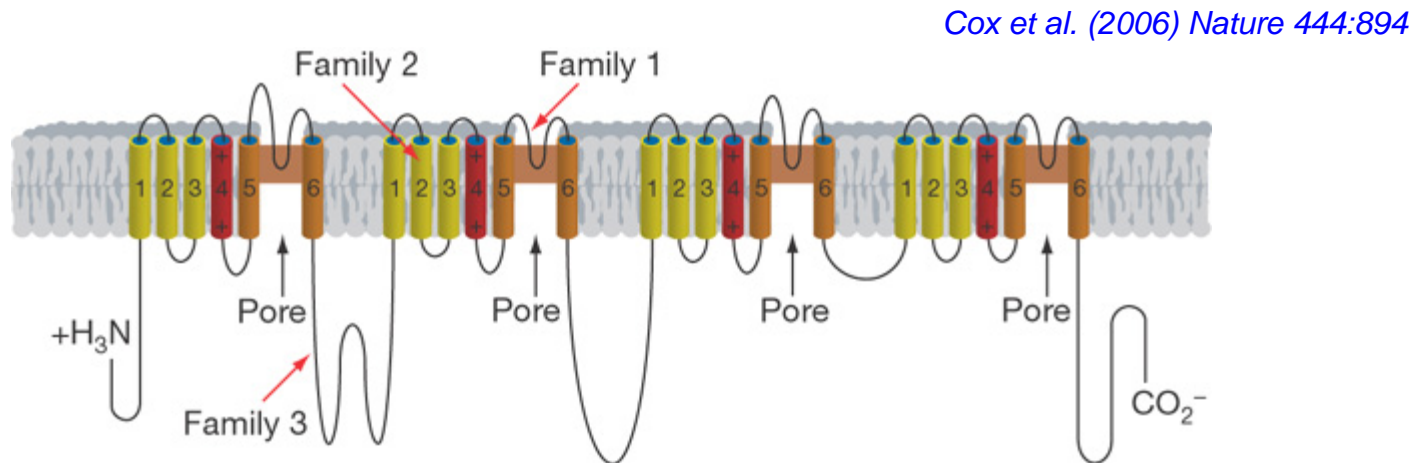


Control sciatic nerve

- Concomitant with emergence of rapidly re-priming TTXs sensitive current that correlates with hyperexcitability
- However, no effects on neuropathic pain in KO mice (Nassar *et al*, 2006) and conflicting data from anti-sense studies (Hains *et al*, , 2003, 2004)

Na_v1.7 as a target for pain therapy

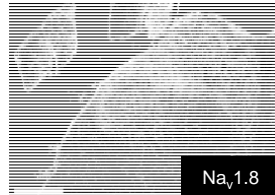
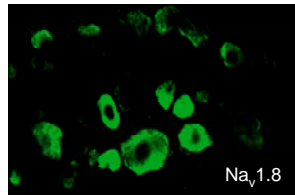
- Genetic validation from human mutations:
 - Gain of function, leading to Painful Erythermalgia or Paroxysmal Extreme Pain Disorder (Cummins et al., 2004; Fertleman et al., 2006)
 - Loss of function (homozygous null mutations), leading to Congenital Indifference to Pain (Cox et al., 2006; Goldberg et al., 2007; Ahmad et al., 2007)
 - Normal responses to touch, warm, cold



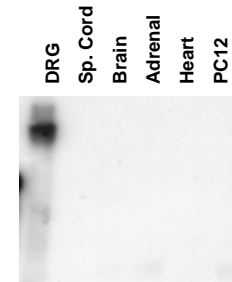
- Contrasts with KO mice - global null animals die after birth; nociceptor null animals show no effects in neuropathic pain (though some effects seen in inflammatory pain)

Na_v1.8 as a target for pain therapy

- Na_v1.8 is selectively localised to nociceptive sensory neurons:-

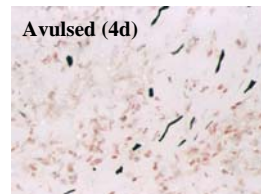
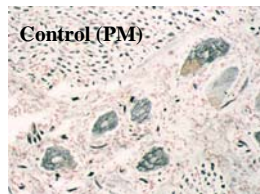


Amaya et al. (2000)

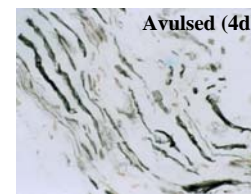


- Recombinant channel properties resemble predominant TTX-r currents in nociceptive DRG neurons (high activation threshold, slow kinetics, rapid recovery)
- Expression levels and properties altered in pain models and human pain states:-

DRG cell bodies:



Peripheral Nerves:



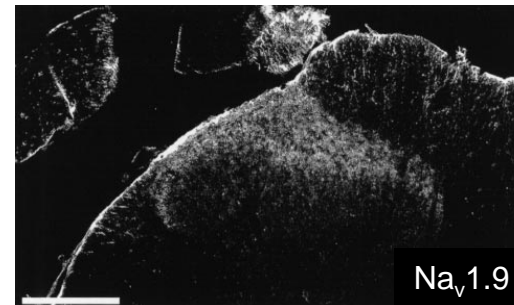
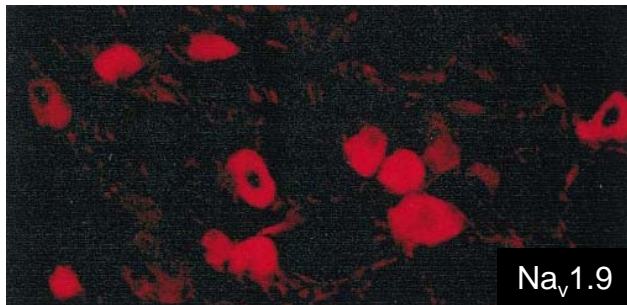
Coward et al, (2000)

*human brachial plexus
(spinal cord root avulsion) injury*

- Antisense oligonucleotides reverse mechanical allodynia and thermal hyperalgesia after peripheral inflammation or nerve injury
- KO mice show surprisingly limited pain phenotypes – decreased sensitivity to mechanical stimuli, delayed development of thermal hyperalgesia
 - Compensatory mechanisms?

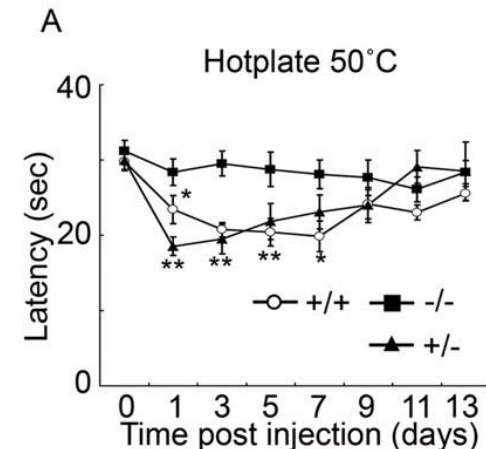
Na_v1.9 as a target for pain therapy

- Na_v1.9 is selectively localised to nociceptive sensory neurons:-



Amaya et al. (2000)

- Responsible for “persistent” TTX-r currents in nociceptive DRG neurons (voltage dependence of activation hyperpolarised compared to inactivation, large window current, very slow kinetics)
- Expression downregulated in injured neurons
- However KO mice have no neuropathic pain phenotype but do show reduced hypersensitivity to inflammatory stimuli



- N.B. Highly refractory to expression in recombinant systems*

Amaya et al. (2006)

Overview of presentation

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PrecisION™ human ion channel cell lines

Voltage-Gated:

Sodium

- Nav1.1
- Nav1.2
- Nav1.3
- Nav1.4
- Nav1.5
- Nav1.6
- Nav1.7
- Nav1.8 (rat)
- Nav1.8

Potassium

- | | | |
|---------|----------------|---------------|
| → Kv1.1 | → Kv2.1 | → Kv7.1/KCNE1 |
| → Kv1.2 | → Kv2.1/Kv9.2 | (KCNQ1/minK) |
| → Kv1.3 | | → Kv7.2/Kv7.3 |
| → Kv1.4 | → Kv3.1 | |
| → Kv1.5 | → Kv3.2 | → hERG-CHO |
| → Kv1.6 | → Kv3.3 | → hERG-HEK |
| → Kv1.7 | | |
| → Kv1.8 | → Kv4.2/KChip2 | → Kir2.1 |
| | → Kv4.3/KChIP1 | |
| | → Kv4.3/KChIP2 | |

Calcium

- Cav1.2/ β 2a/ α 2 δ 1
- Cav2.2/ β 3/ α 2 δ 1

Other

- HCN1
- HCN2
- HCN3
- HCN4

Ligand-Gated:

- | | | |
|---------|---|--|
| → TRPM8 | → GluR6 (GRIK2) | → nAChR α 1/ β 1/ δ / ϵ |
| → TRPV1 | | → nAChR α 3/ β 4 |
| → TRPV3 | → GABA _A α 1/ β 3/ γ 2 | |
| → TRPA1 | → GABA _A α 3/ β 3/ γ 2 | |

PrecisION™ human ion channel cell lines

Voltage-Gated:

Sodium

- Nav1.1
- Nav1.2
- Nav1.3
- Nav1.4
- Nav1.5
- Nav1.6
- Nav1.7
- Nav1.8 (rat)
- Nav1.8

Potassium

- | | | |
|---------|----------------|-------------------------------|
| → Kv1.1 | → Kv2.1 | → Kv7.1/KCNE1
(KCNQ1/minK) |
| → Kv1.2 | → Kv2.1/Kv9.2 | → Kv7.2/Kv7.3 |
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| → Kv1.5 | → Kv3.2 | → hERG-CHO |
| → Kv1.6 | → Kv3.3 | → hERG-HEK |
| → Kv1.7 | | |
| → Kv1.8 | → Kv4.2/KChip2 | → Kir2.1 |
| | → Kv4.3/KChIP1 | |
| | → Kv4.3/KChIP2 | |

Calcium

- Cav1.2/β2α/α2δ1
- Cav2.2/β3/α2δ1

Other

- HCN1
- HCN2
- HCN3
- HCN4

Ligand-Gated:

- | | | |
|---------|------------------------------|-------------------|
| → TRPM8 | → GluR6 (GRIK2) | → nAChR α1/β1/δ/ε |
| → TRPV1 | | → nAChR α3/β4 |
| → TRPV3 | → GABA _A α1/β3/γ2 | |
| → TRPA1 | → GABA _A α3/β3/γ2 | |

CardiacProfiler™ Panel

PrecisION™ human ion channel cell lines

Voltage-Gated:

Sodium

- Nav1.1
- Nav1.2
- Nav1.3
- Nav1.4
- Nav1.5
- **Nav1.6**
- Nav1.7
- Nav1.8 (rat)
- Nav1.8

Potassium

- | | | |
|---------|----------------|-------------------------------|
| → Kv1.1 | → Kv2.1 | → Kv7.1/KCNE1
(KCNQ1/minK) |
| → Kv1.2 | → Kv2.1/Kv9.2 | → Kv7.2/Kv7.3 |
| → Kv1.3 | | |
| → Kv1.4 | → Kv3.1 | |
| → Kv1.5 | → Kv3.2 | → hERG-CHO |
| → Kv1.6 | → Kv3.3 | → hERG-HEK |
| → Kv1.7 | | |
| → Kv1.8 | → Kv4.2/KChip2 | → Kir2.1 |
| | → Kv4.3/KChIP1 | |
| | → Kv4.3/KChIP2 | |

Calcium

- Cav1.2/ β 2a/ α 2 δ 1
- **Cav2.2/ β 3/ α 2 δ 1**

Other

- HCN1
- HCN2
- HCN3
- HCN4

Ligand-Gated:

- | | | |
|---------|---|---|
| → TRPM8 | → GluR6 (GRIK2) | → nAChR α1/β1/δ/ϵ |
| → TRPV1 | | → nAChR α3/β4 |
| → TRPV3 | → GABA_A α1/β3/γ2 | |
| → TRPA1 | → GABA_A α3/β3/γ2 | |

Neuronal and neuromuscular liability

PrecisION™ human ion channel cell lines

Voltage-Gated:

Sodium

- Nav1.1
- Nav1.2
- Nav1.3
- Nav1.4
- Nav1.5
- Nav1.6
- Nav1.7
- Nav1.8 (rat)
- Nav1.8

Potassium

- | | | |
|---------|----------------|---------------|
| → Kv1.1 | → Kv2.1 | → Kv7.1/KCNE1 |
| → Kv1.2 | → Kv2.1/Kv9.2 | (KCNQ1/minK) |
| → Kv1.3 | | → Kv7.2/Kv7.3 |
| → Kv1.4 | → Kv3.1 | |
| → Kv1.5 | → Kv3.2 | → hERG-CHO |
| → Kv1.6 | → Kv3.3 | → hERG-HEK |
| → Kv1.7 | | |
| → Kv1.8 | → Kv4.2/KChip2 | → Kir2.1 |
| | → Kv4.3/KChIP1 | |
| | → Kv4.3/KChIP2 | |

Calcium

- Cav1.2/ β 2a/ α 2 δ 1
- Cav2.2/ β 3/ α 2 δ 1

Other

- HCN1
- HCN2
- HCN3
- HCN4

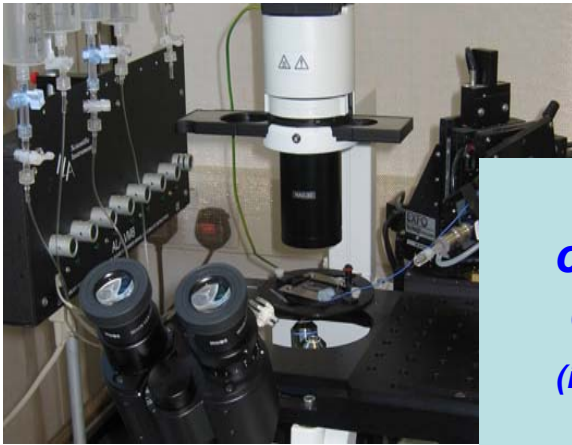
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| → TRPV3 | → GABA _A α 1/ β 3/ γ 2 | |
| → TRPA1 | → GABA _A α 3/ β 3/ γ 2 | |

Potential pain targets

Assay platforms available at Millipore

Manual Patch Clamp



- Very low throughput
- Versatile configurations
- “Gold standard”
- Detailed cell line characterization
- All types of channel
- Detailed assays and MOA studies

PatchXpress



N.B. Good success rate is critically dependent on high quality expression reagent (i.e. current amplitude, % cells expressing, high seal rate)

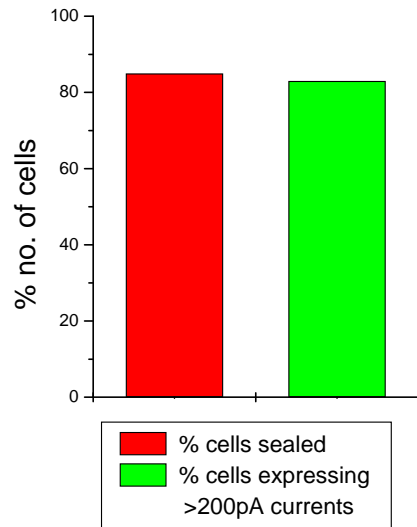
- Low/medium throughput
- Whole cell patch
- Continuous voltage clamp
- Series resistance compensation
- Fast compound addition and washout
- Some cell line screening
- Cell line characterisation
- Lower through-put assays
- Mechanism of action
- ‘Fast’ ligand-gated assays

IonWorksHT/Quattro (3 x)

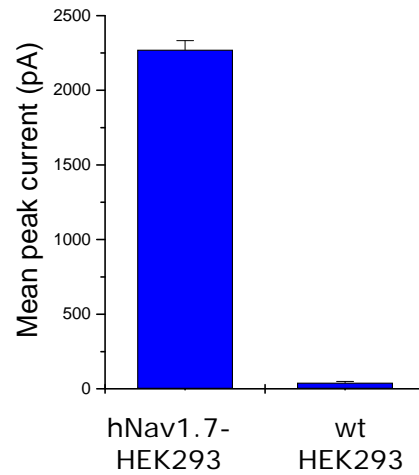


- High throughput
- Perforated patch
- Discontinuous voltage clamp
- No compound washout
- Low seal resistances
- Cell line screening and characterisation
- Higher through-put assays
- Some ligand-gated assays (‘slow’ kinetics)

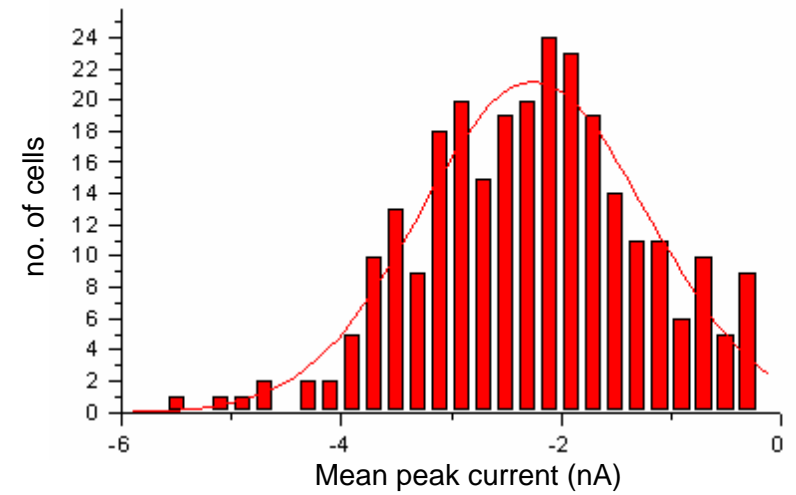
Na_v 1.7 cell line characterisation - Ionworks^{HT}



Seal and expression rate

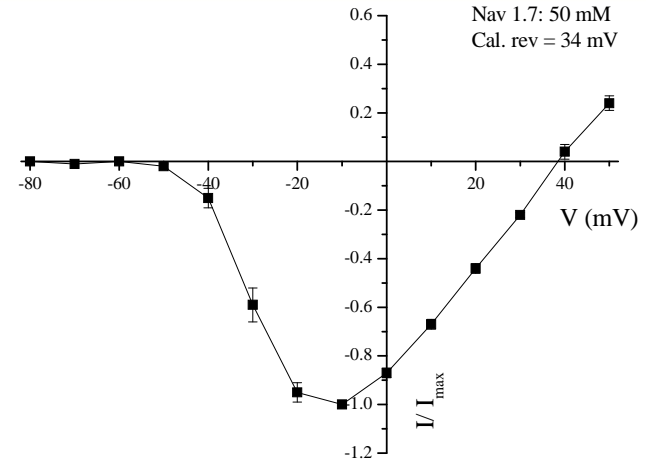
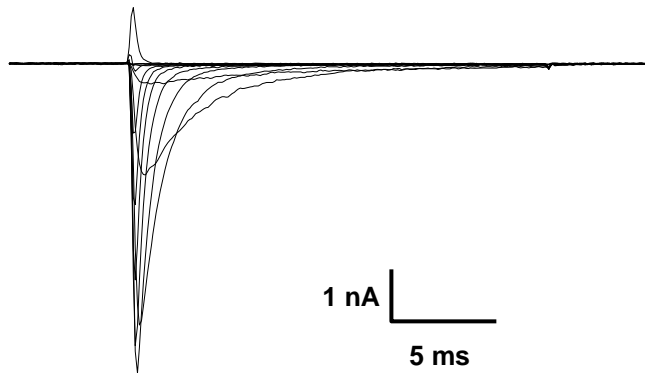


Current amplitude

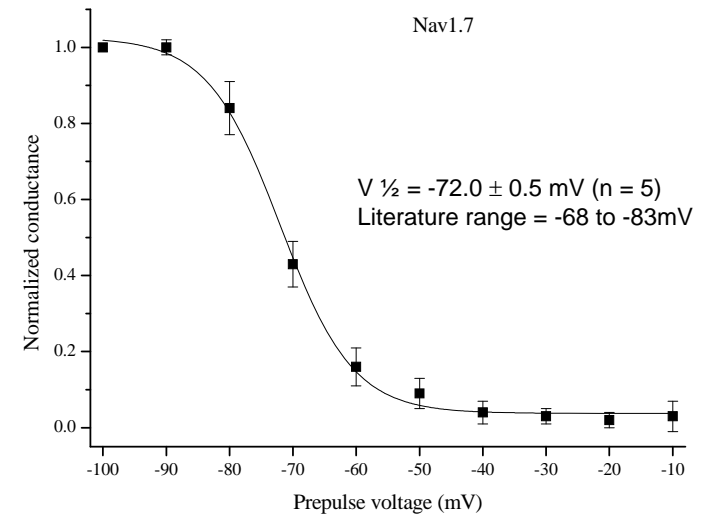
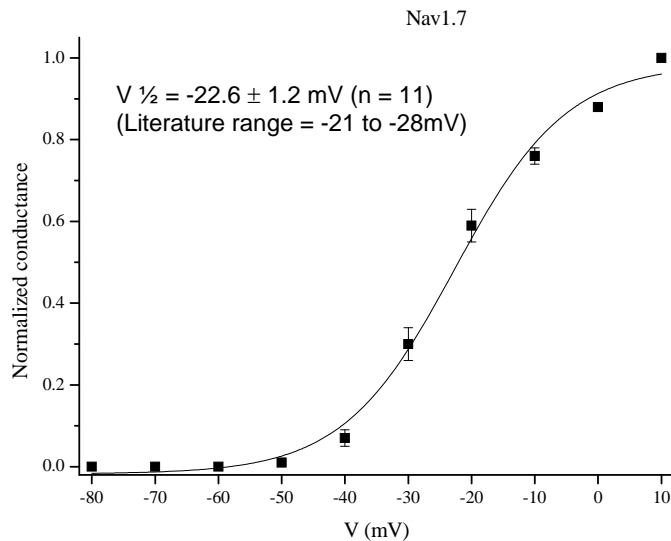


Expression profile within population

Nav_v 1.7 cell line characterisation - biophysics

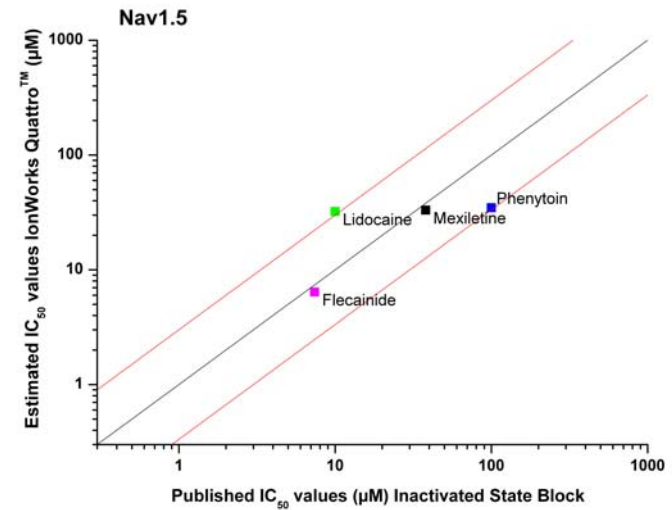
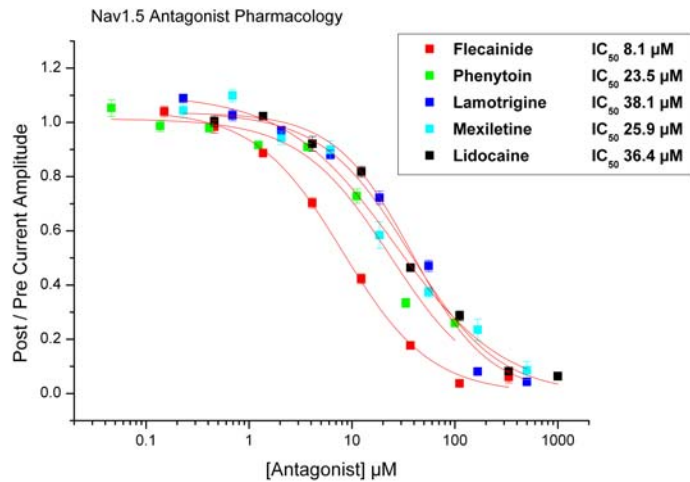


Mean current at -10 mV = 3.9 ± 0.8 nA

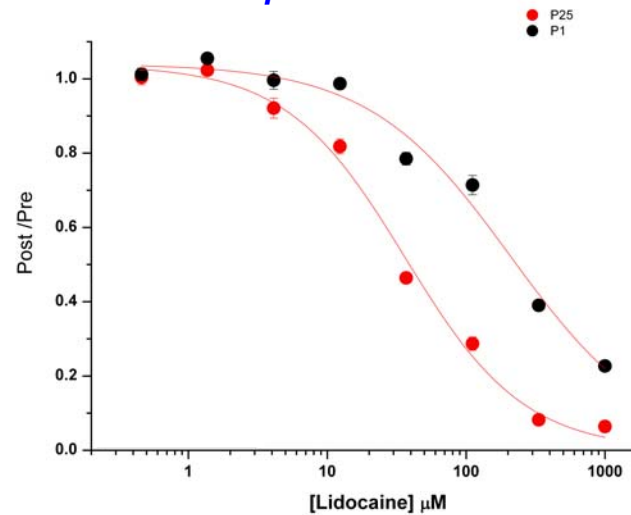


Na_v1.5 Ionworks assay (PPC)

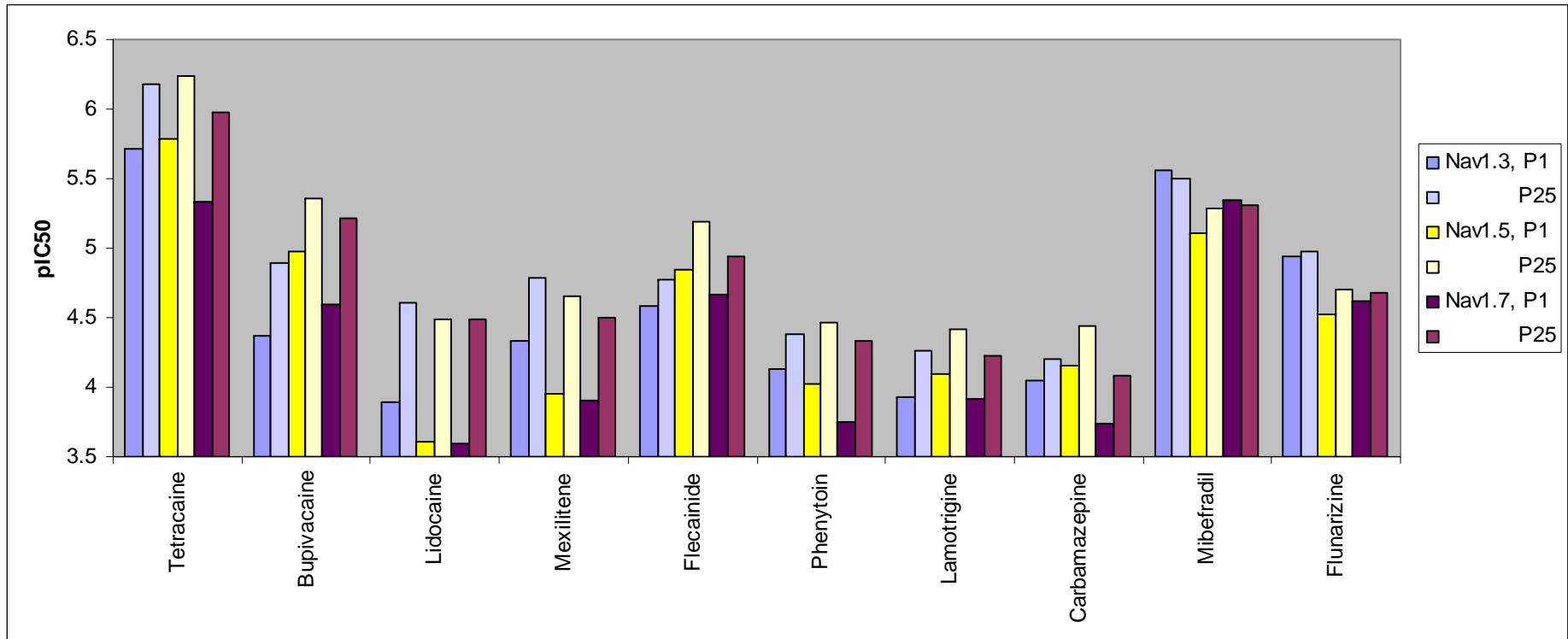
Inactivated state block



Use dependent block



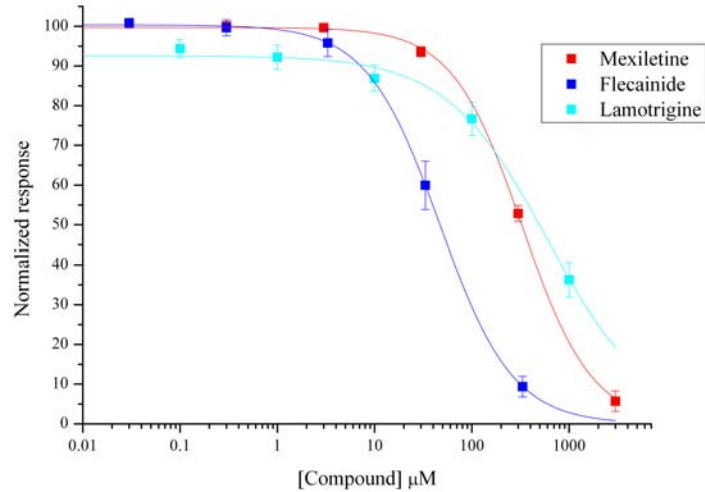
Profiling Na_v subtypes with standard blockers using Ionworks - summary



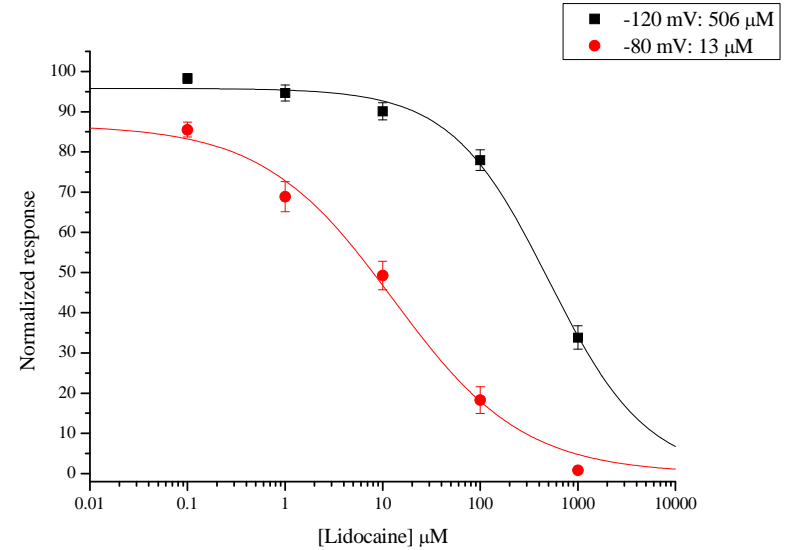
- Ionworks can be used to detect tonic and use-dependent block by different classes of Na_v inhibitor with good resolution and reproducibility
- Standard Na_v channel inhibitors have little or no subtype-selectivity

Na_v 1.7 PatchXpress assay

A) Concentration response curves for standard blockers

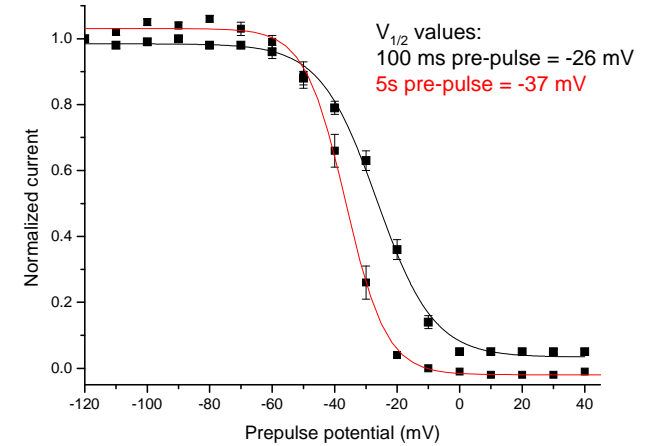
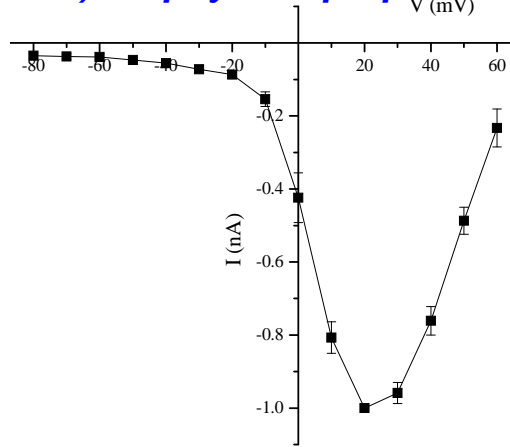
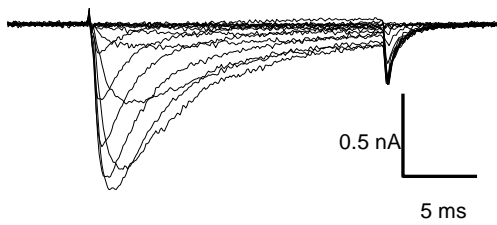


B) Voltage-dependent block by lidocaine

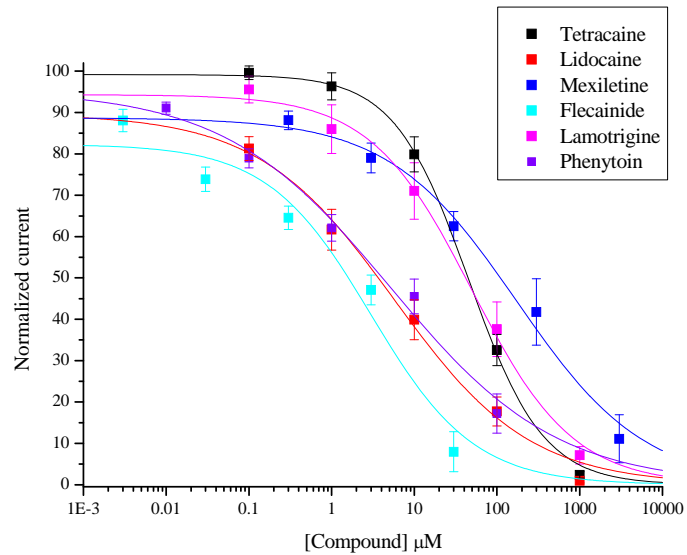


rat Na_v 1.8 PatchXpress assay

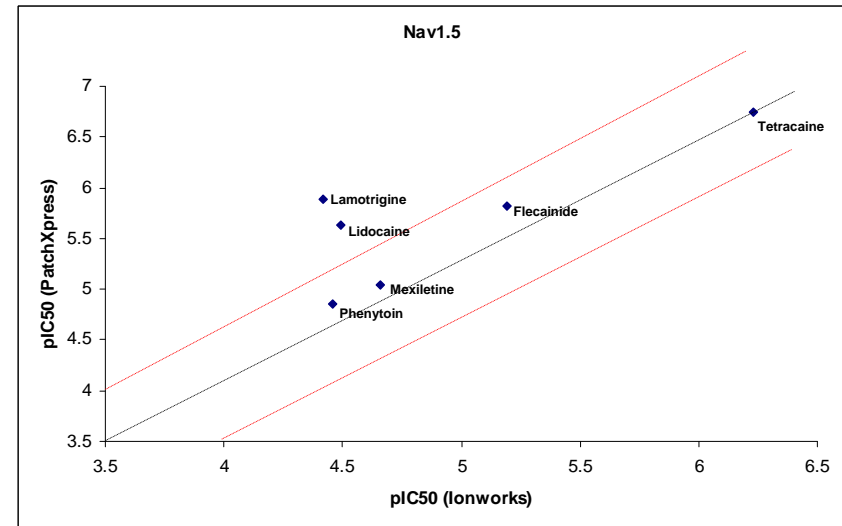
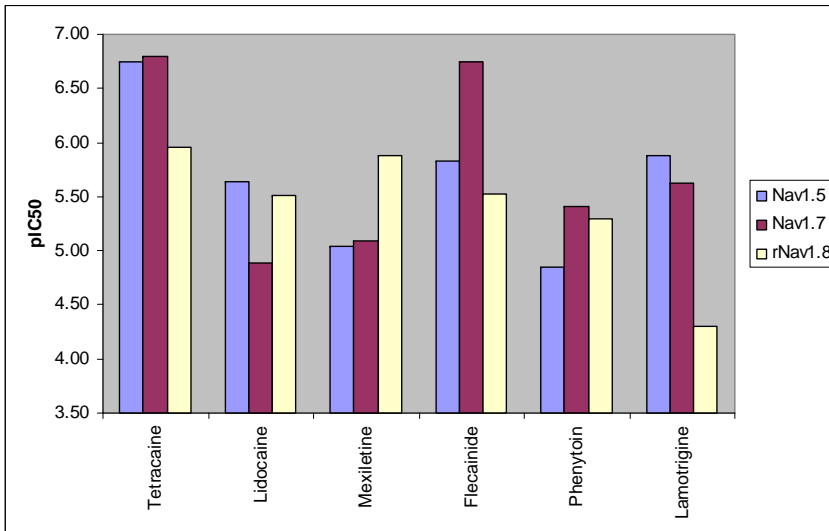
A) Biophysical properties



B) Pharmacology



Profiling Na_v subtypes with standard blockers using PatchXpress - summary



- PatchXpress can be used to analyse effects of different classes of Na_v inhibitor
- PatchXpress data further validates Ionworks assays for the detection Na_v inhibitors

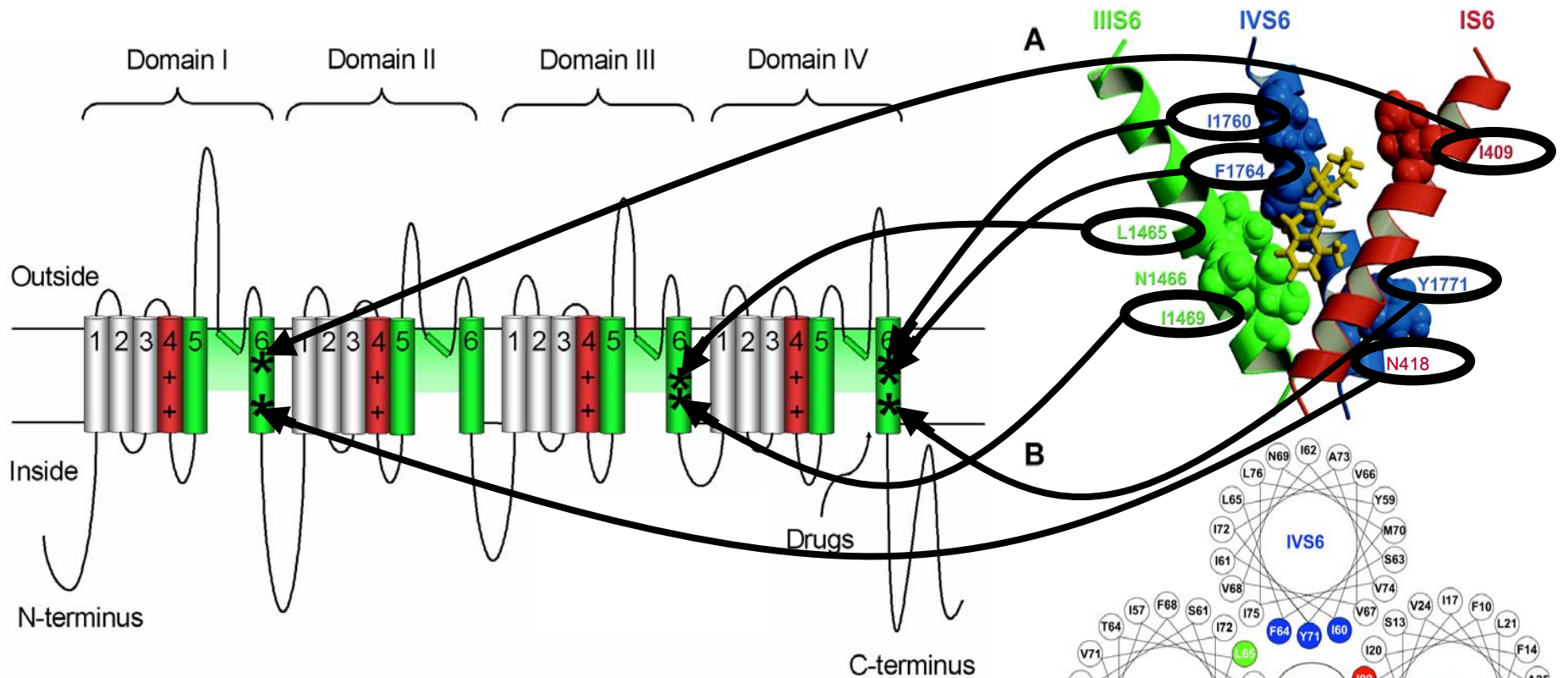
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- Generation and characterisation of Na_v channels with mutations within the drug binding site

Characterisation of Na_v1.8 drug binding site mutants

Na_v1.8

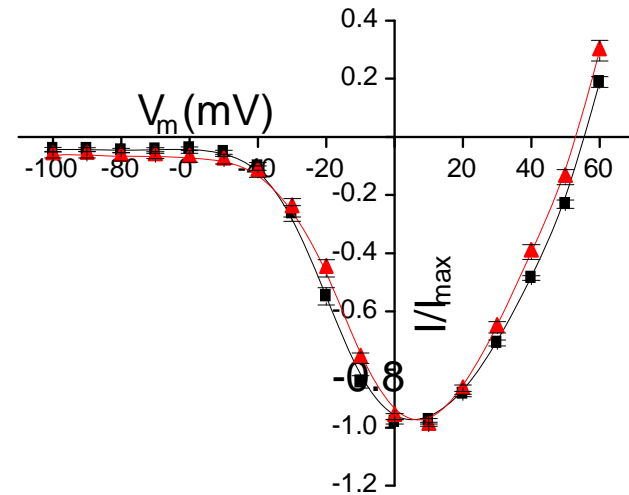
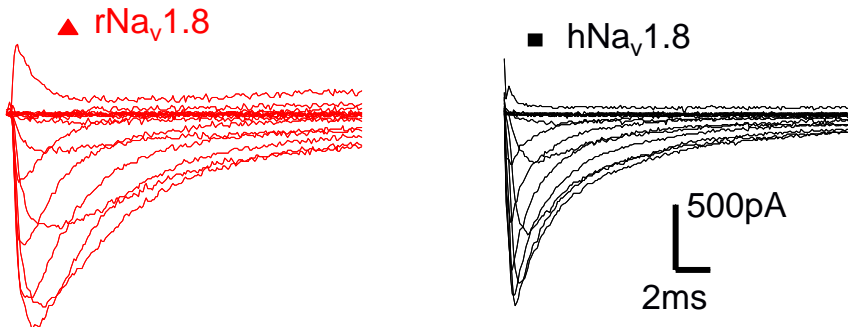
Na_v1.2



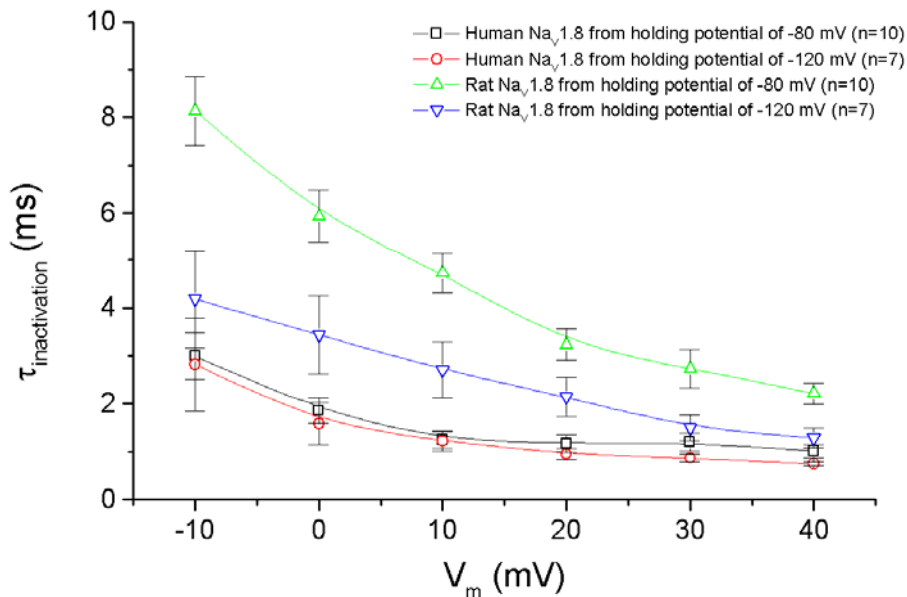
1. I381A or I380A
2. N390A or N389A
3. L1410A or L1411A
4. V1414A or V1415A

5. I1706A or I1707A
6. F1710A or F1711A
7. Y1717A or Y1718A

Comparison of wild type rat vs human $\text{Na}_v1.8$



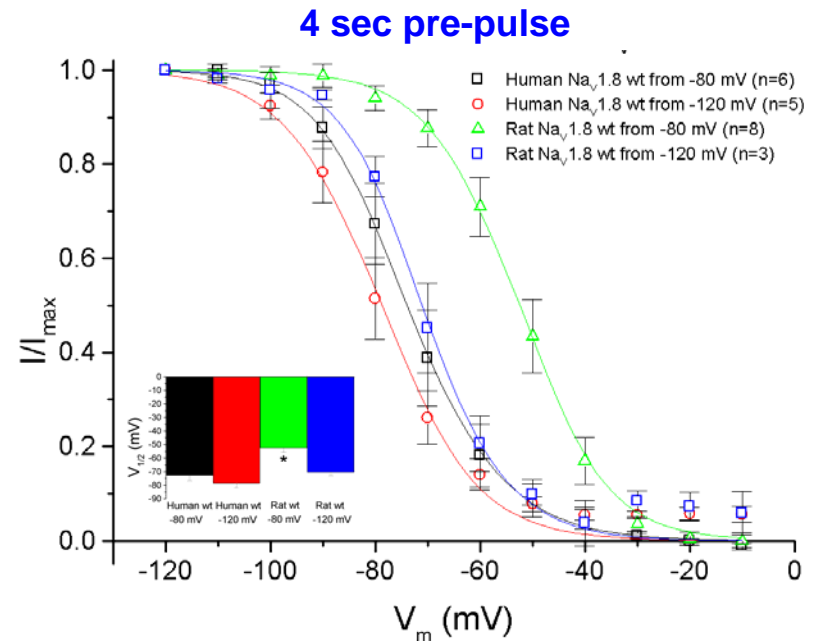
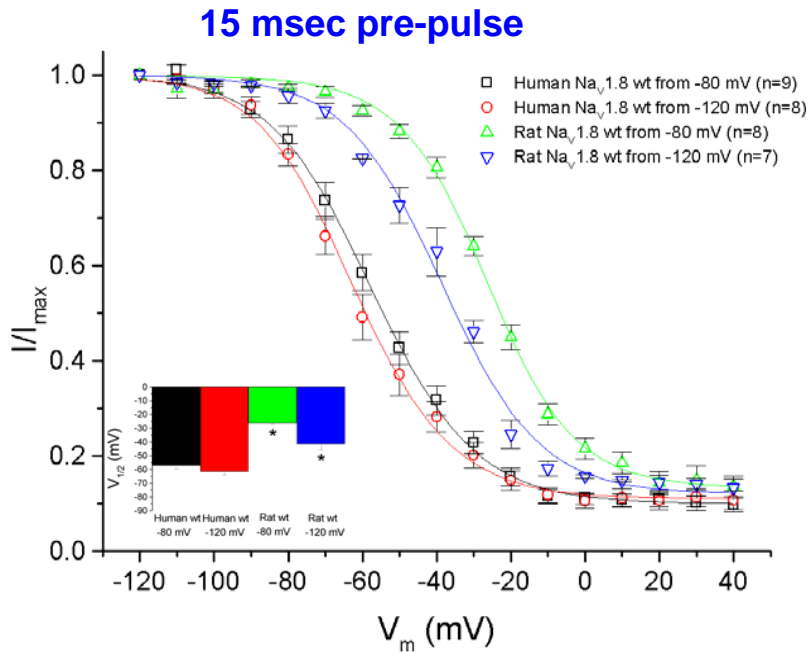
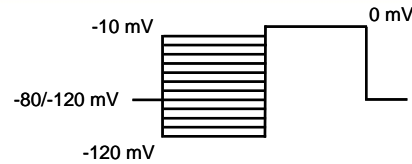
Voltage-dependence of τ in wildtype human and rat $\text{Na}_v1.8$



- Compared using transient expression in ND7-23 cells
- Human $\text{Na}_v1.8$ has faster inactivation kinetics than rat

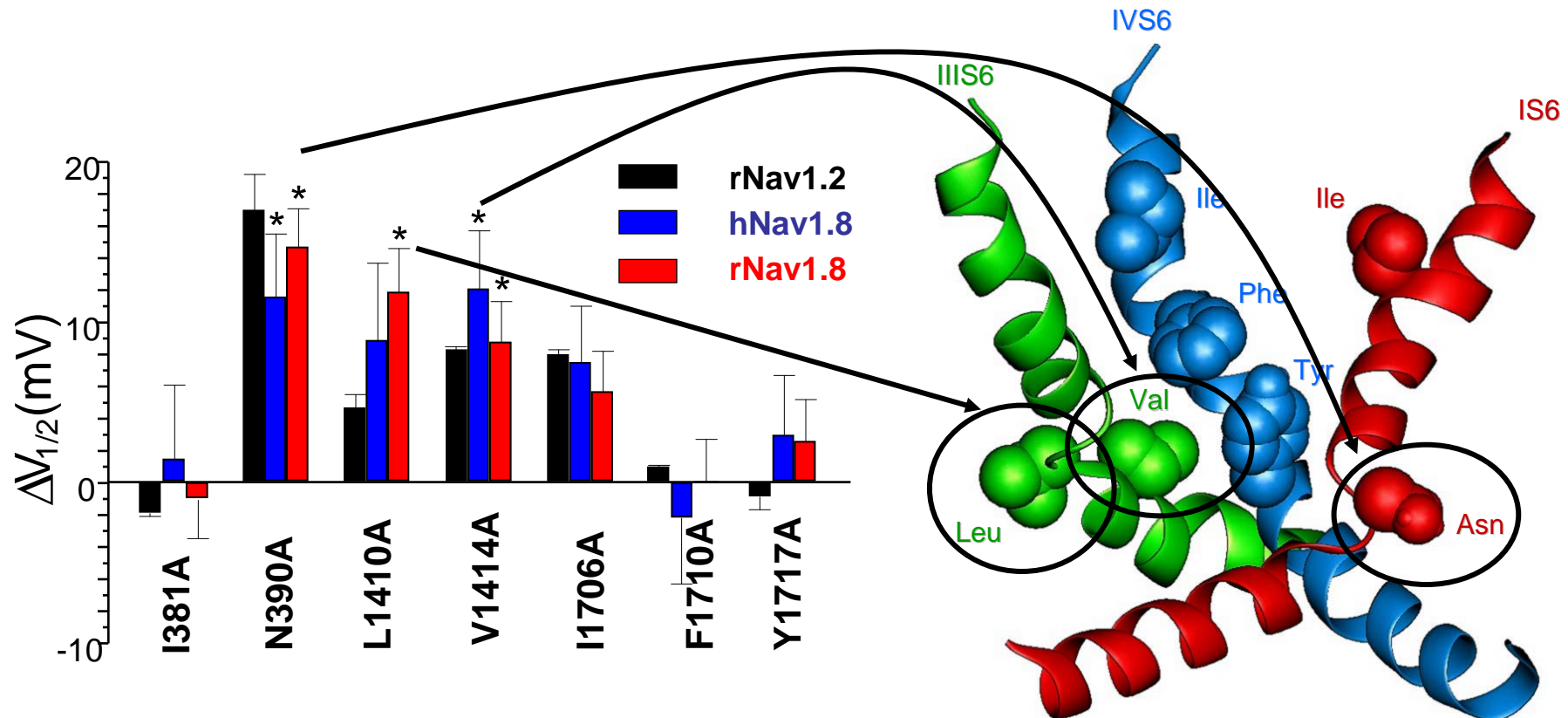
Liam Browne, Univ Leeds
(in collaboration with Dennis Wray)

Wild type rat vs human $\text{Na}_v1.8$ - voltage-dependence of inactivation



- Rat $\text{Na}_v1.8$ inactivates at more depolarised voltages than human
- Possible implications for *in vivo* channel function?
- Suggests inactivated state blockers may be less potent at rat vs human for a given membrane potential
 - e.g. A-803467 is 5.5-fold selective for human vs rat $\text{Nav}1.8$ (Jarvis *et al.*, 2007)
 - Tetracaine is 16-fold selective (Dekkar *et al.*, 2005)

Effects of mutations on voltage-dependence of activation



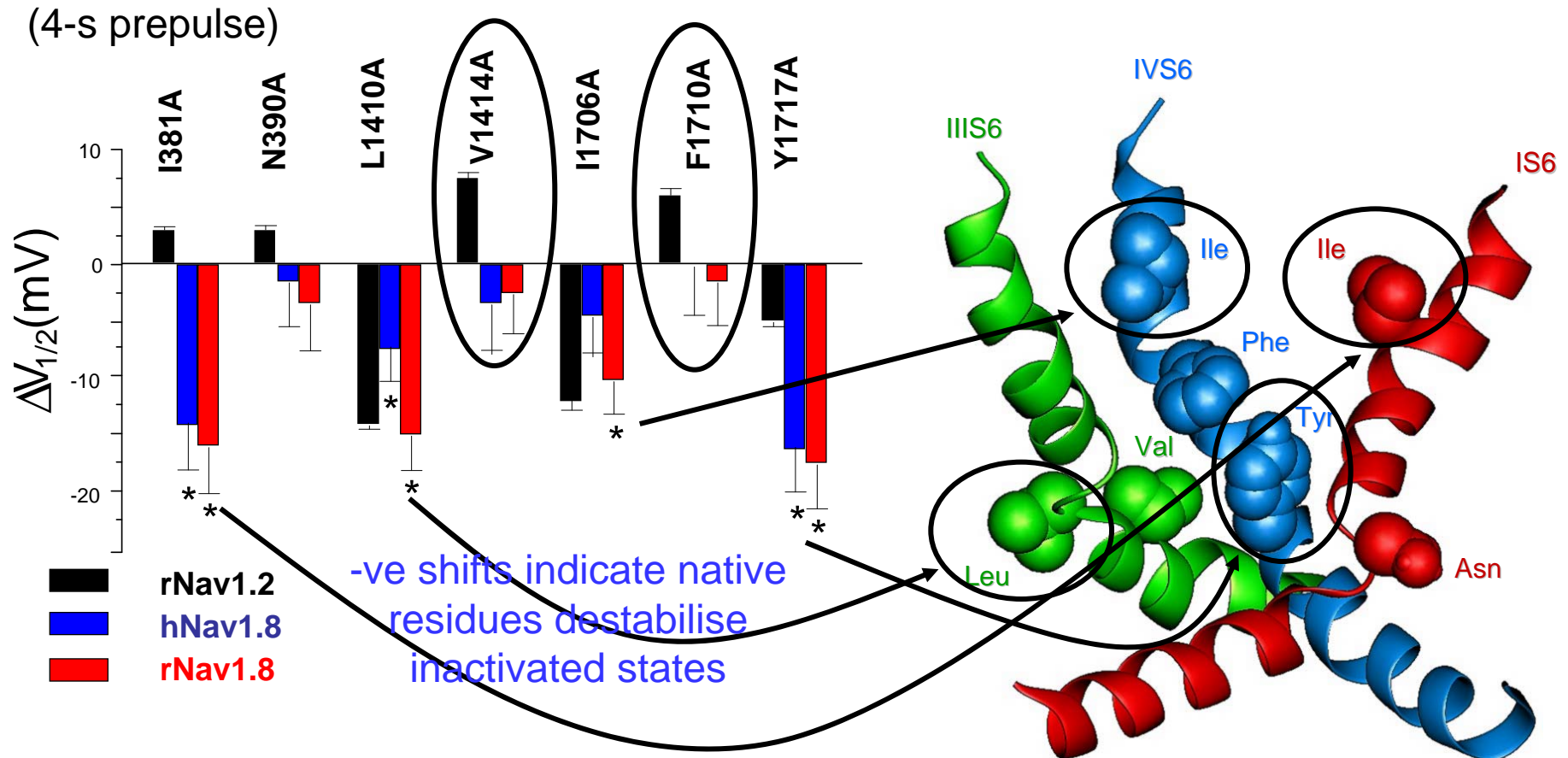
Mutation of leu (IIIS6) causes a shift for rat but not for human $Na_V1.8$

+ve shifts indicate native residues stabilise open states

h $Na_V1.8$ mutations generally mirror shifts for r $Na_V1.8$ mutations

These shifts are consistent with corresponding mutations of $Na_V1.2$ and $Na_V1.4$

Effects of mutations on voltage-dependence of inactivation

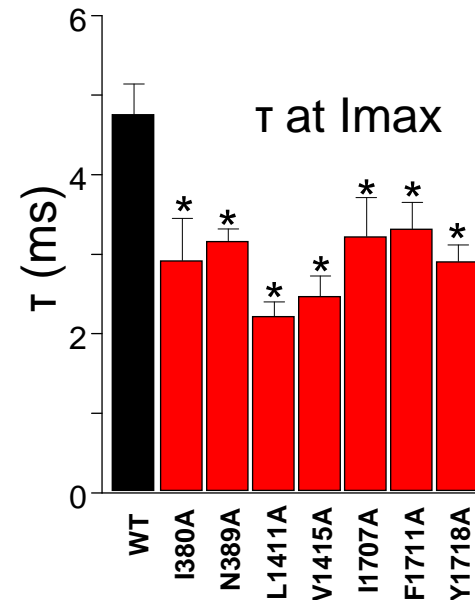
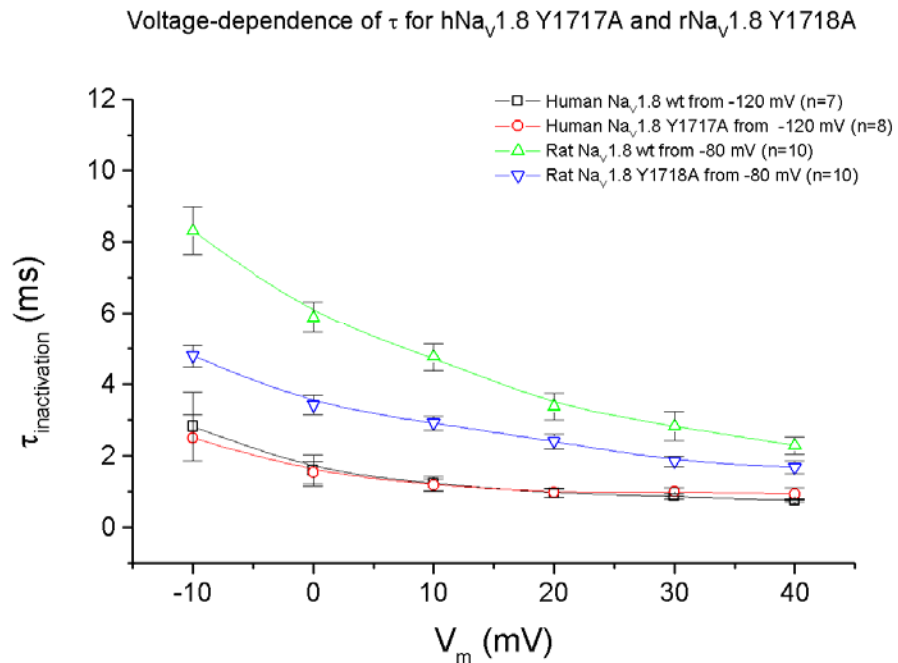


Mutation of Ile (IS6) and Tyr (IVS6) gave greater shifts than described for $Na_v1.2$.

Mutation of Ile (IVS6) causes a shift for rat but not for human $Na_v1.8$

In contrast to $Na_v1.2$, mutation of Val (IIIS6) and Phe (IVS6) did not cause a shift

Effects of mutations on kinetics of fast inactivation



- Mutations in rat Na_v1.8 generally speed up fast inactivation
- Contrasts with human Na_v1.8 (and rat Na_v1.2) – mutations have no effect

Summary and Conclusions

- Using two automated platforms (Ionworks and PatchXpress) robust, high throughput electrophysiology assays have been developed for profiling compounds against the family of Na_v channels (1.1-1.8)
- Standardised protocols have been configured to assess both tonic and use dependent block
- These assays have been validated using different classes of standard Na_v inhibitors and correlate well with published data
- A panel of mutants carrying alanine substitutions at key drug binding determinants of both rat and human Na_v1.8 have been generated and characterised
- These mutations define S6 residues involved in both activation and inactivation (generally stabilising open states and destabilising inactivated states)
- Although conserved in Na_v1.2, mutation of some residues has differing effects in Na_v1.8 suggesting other (nearby?) residues are also important
- This panel of mutants should be useful in exploring the molecular basis of selectivity of recently emerging Na_v1.8-selective inhibitors

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Louise Webdale

*Cell line generation partly carried out in
collaboration with GSK, Stevenage, UK*

Na_{v1.8} mutants :-

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