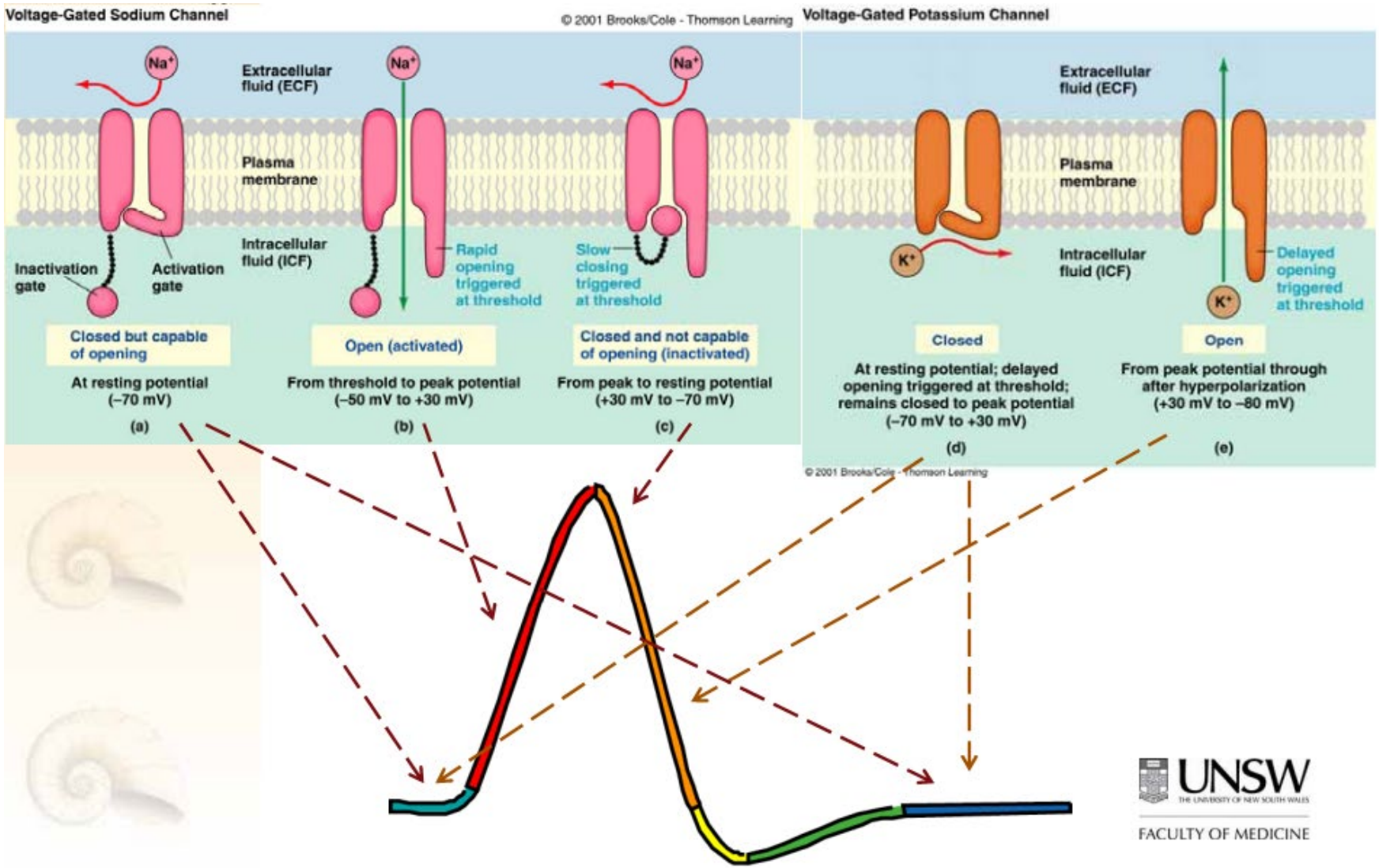
Modelling gain-of-function $Na_V1.7$ I228M mutation

Special series on Laboratory Animal Science

Regina Reimann

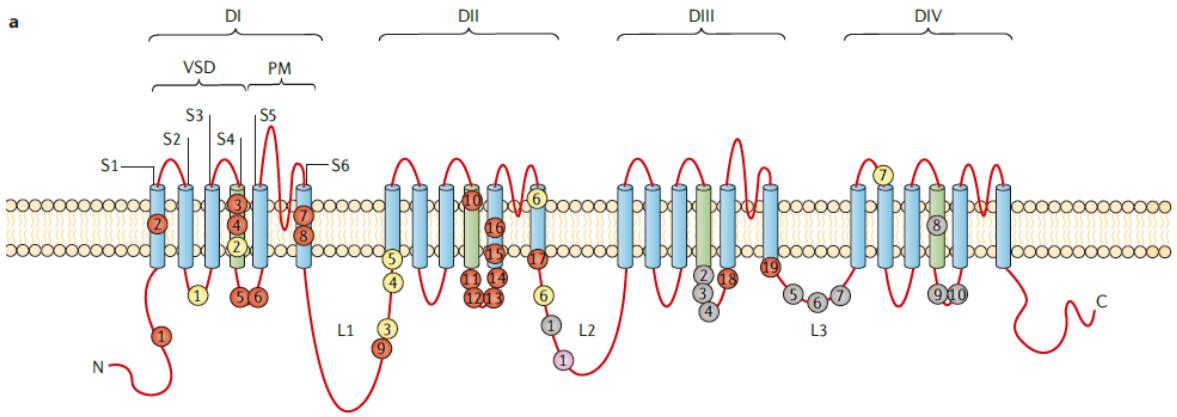
Voltage-Gated Sodium Channels in action potential

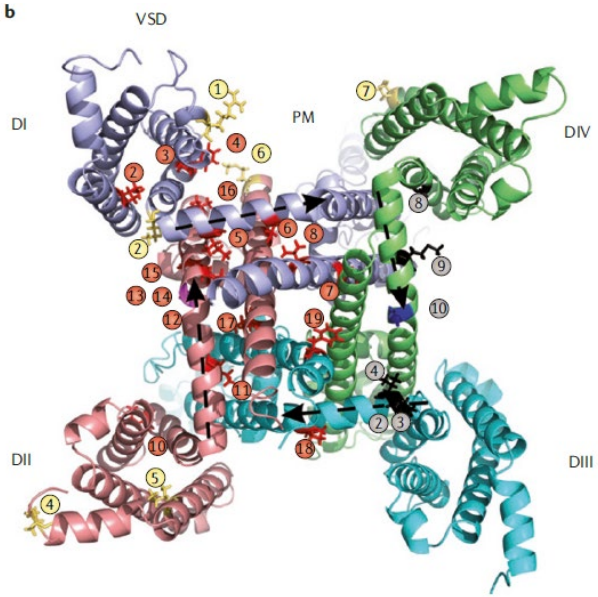


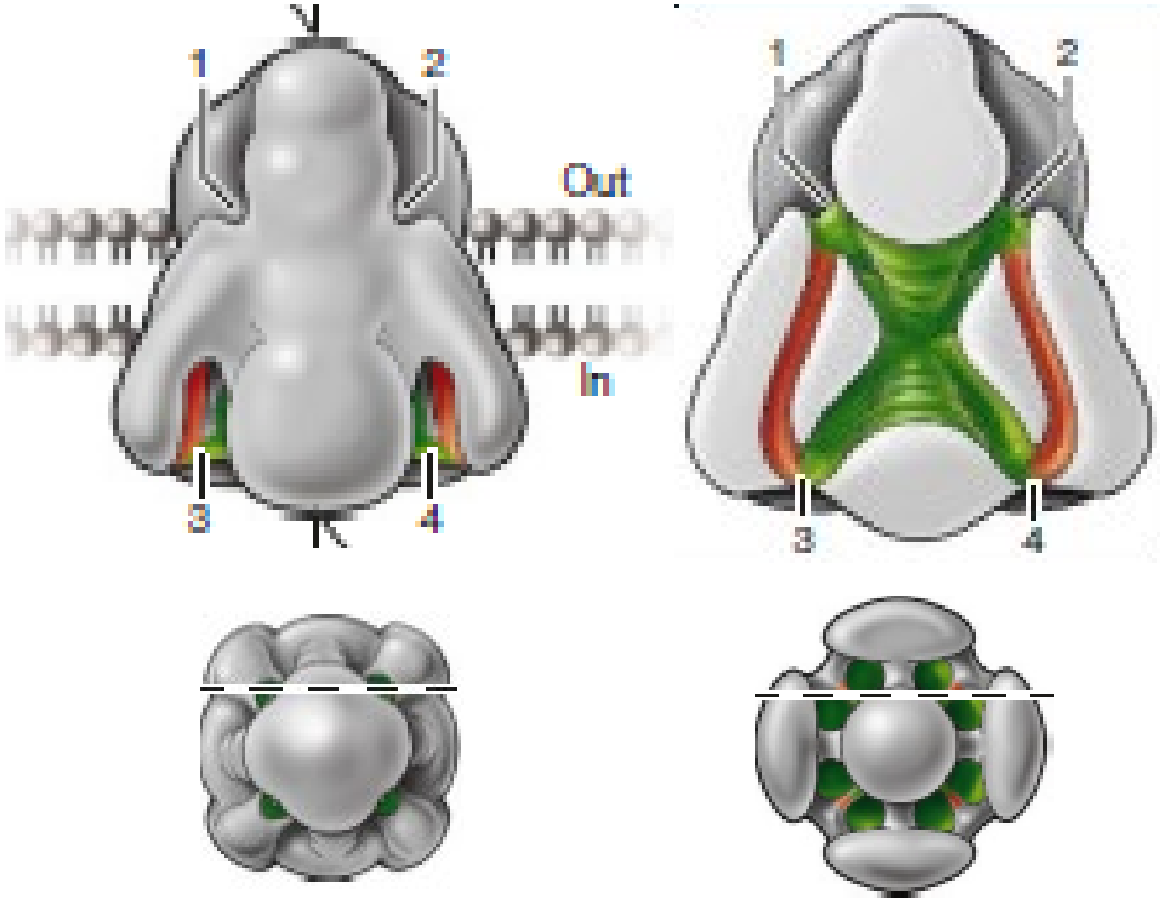
UNSW
THE UNIVERSITY OF NEW SOUTH WALES
FACULTY OF MEDICINE

http://studyingmed.com/wiki/index.php?title=AEA/Lectures/Action_potential_generation_and_propagation&mobileaction=toggle_view_mobile

Structure of voltage-Gated Sodium Channels



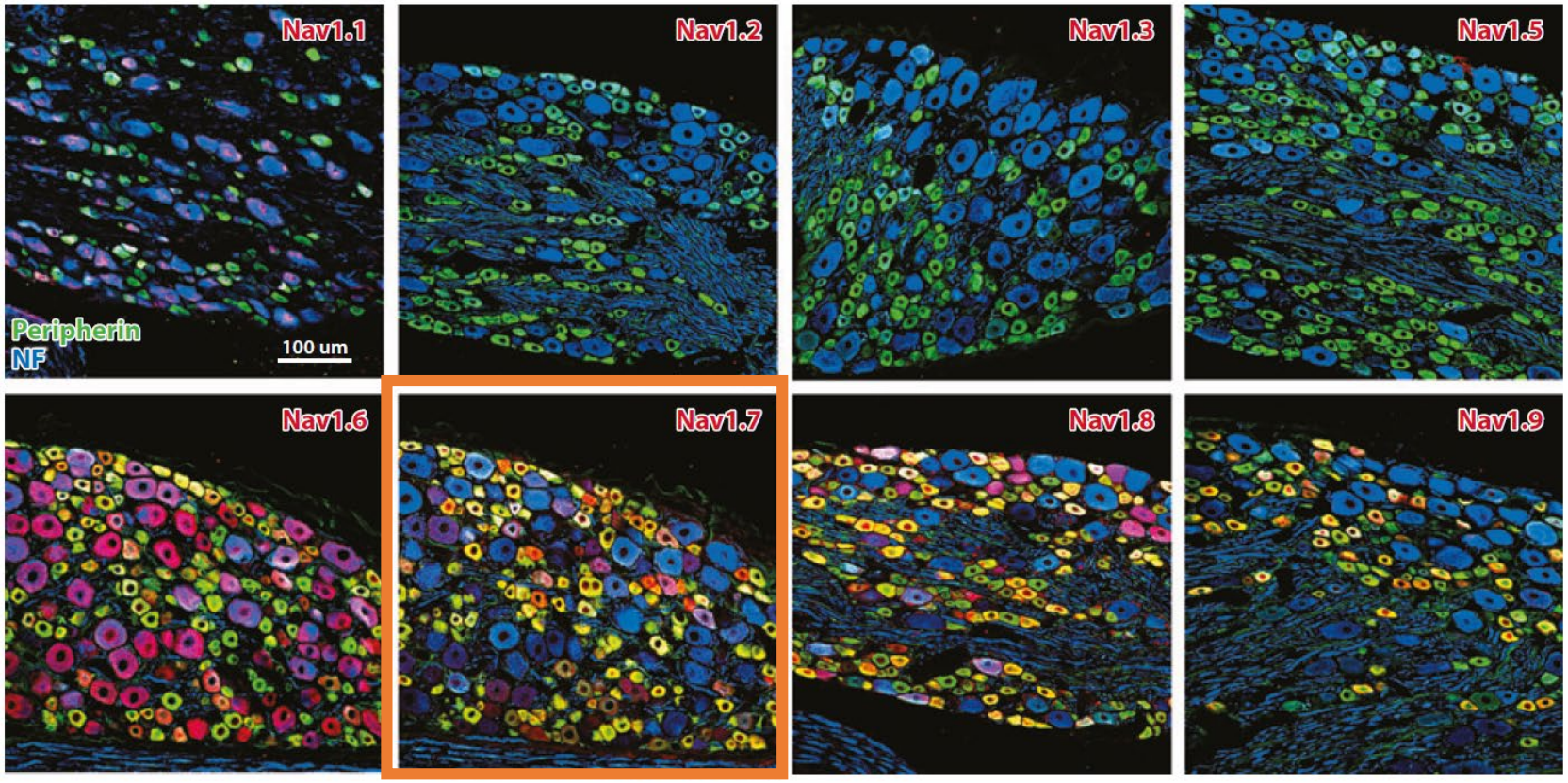




W. A. Catterall, nature, 2001

S. Dib-Hajj, nature reviews, 2012

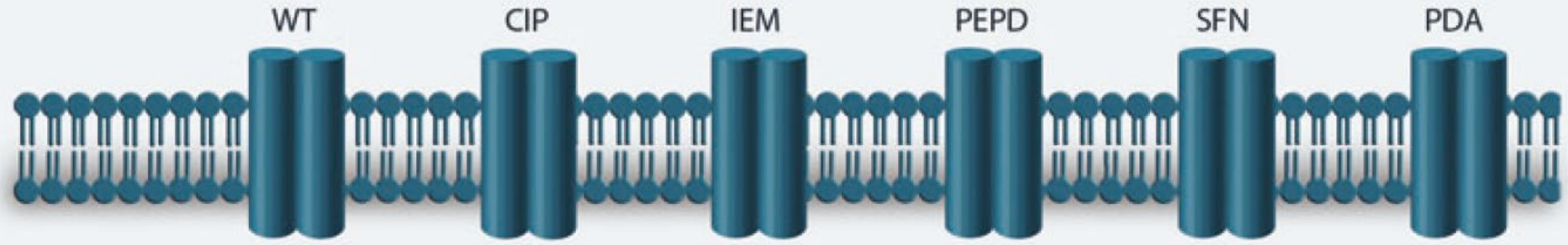
Expression of sodium channel isoforms in dorsal root ganglion (DRG) neurons



- red: Sodium channel isoform
- Green: Peripherin (small-diameter neuron marker) ; Yellow : Colocalization with sodium channel
- Blue : Neurofilament (medium and large neuron); Magenta : Colocalization with neurofilament

Sulayman et al, Annu Rev Neuroscienc, 2010

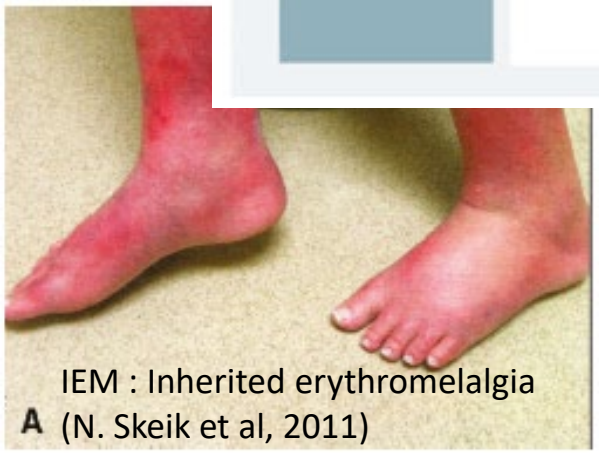
Nav1.7 is setting the gain in pain-signaling neurons



Mutation	Wild Type	Loss-of-function	Gain-of-function	Gain-of-function	Gain-of-function	Gain-of-function
Channel function	Normal	Absent	Hyperpolarized activation, slow deactivation, increased ramp response	Impaired fast-inactivation	Altered fast-inactivation, slow-inactivation or resurgent current	Enhanced activation, impaired fast-inactivation, enhanced persistent current

PEPD : Paroxysmal extreme pain

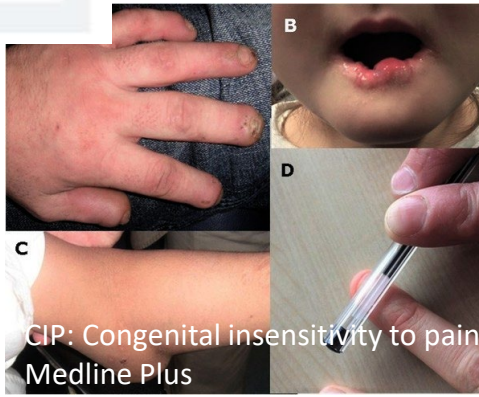
SFN: Small fiber neuropathy
PDA : Pain dysautonomia and acromesomelia



IEM : Inherited erythromelalgia
(N. Skeik et al, 2011)

Nav1.7:
- Fast activating and –inactivating
- Slow closed state inactivation / repriming (recovery from inactivation)

⟶ Setting the gain in pain-signaling neurons



CIP: Congenital insensitivity to pain
Medline Plus

Gain of Function $Na_V1.7$ Mutations in Idiopathic Small Fiber Neuropathy

Catharina G. Faber, MD, PhD,[1] Janneke G. J. Hoeijmakers, MD,[1] Hye-Sook Ahn, PhD,[2,3] Xiaoyang Cheng, PhD,[2,3] Chongyang Han, PhD,[2,3] Jin-Sung Choi, PhD,[2,3]* Mark Estacion, PhD,[2,3] Giuseppe Lauria, MD, PhD,[4] Els K. Vanhoutte, MD,[1] Monique M. Gerrits, PhD,[5] Sulayman Dib-Hajj, PhD,[2,3] Joost P. H. Drenth, MD, PhD,[6] Stephen G. Waxman, MD, PhD,[2,3] and Ingemar S. J. Merkies, MD, PhD[1,7]

Objective: Small nerve fiber neuropathy (SFN) often occurs without apparent cause, but no systematic genetic studies have been performed in patients with idiopathic SFN (I-SFN). We sought to identify a genetic basis for I-SFN by screening patients with biopsy-confirmed idiopathic SFN for mutations in the *SCN9A* gene, encoding voltage-gated sodium channel $Na_V1.7$, which is preferentially expressed in small diameter peripheral axons.
Methods: Patients referred with possible I-SFN, who met the criteria of ≥2 SFN-related symptoms, normal strength, tendon reflexes, vibration sense, and nerve conduction studies, and reduced intraepidermal nerve fiber density (IENFD) plus abnormal quantitative sensory testing (QST) and no underlying etiology for SFN, were assessed clinically and by screening of *SCN9A* for mutations and functional analyses.
Results: Twenty-eight patients who met stringent criteria for I-SFN including abnormal IENFD and QST underwent *SCN9A* gene analyses. Of these 28 patients with biopsy-confirmed I-SFN, 8 were found to carry novel mutations in *SCN9A*. Functional analysis revealed multiple gain of function changes in the mutant channels; each of the mutations rendered dorsal root ganglion neurons hyperexcitable.
Interpretation: We show for the first time that gain of function mutations in sodium channel $Na_V1.7$, which render dorsal root ganglion neurons hyperexcitable, are present in a substantial proportion (28.6%; 8 of 28) of patients meeting strict criteria for I-SFN. These results point to a broader role of $Na_V1.7$ mutations in neurological disease than previously considered from studies on rare genetic syndromes, and suggest an etiological basis for I-SFN, whereby expression of gain of function mutant sodium channels in small diameter peripheral axons may cause these fibers to degenerate.

ANN NEUROL 2012;71:26–39

Estacion *et al. Molecular Pain* 2011, **7**:92
http://www.molecularpain.com/content/7/1/92

MOLECULAR PAIN

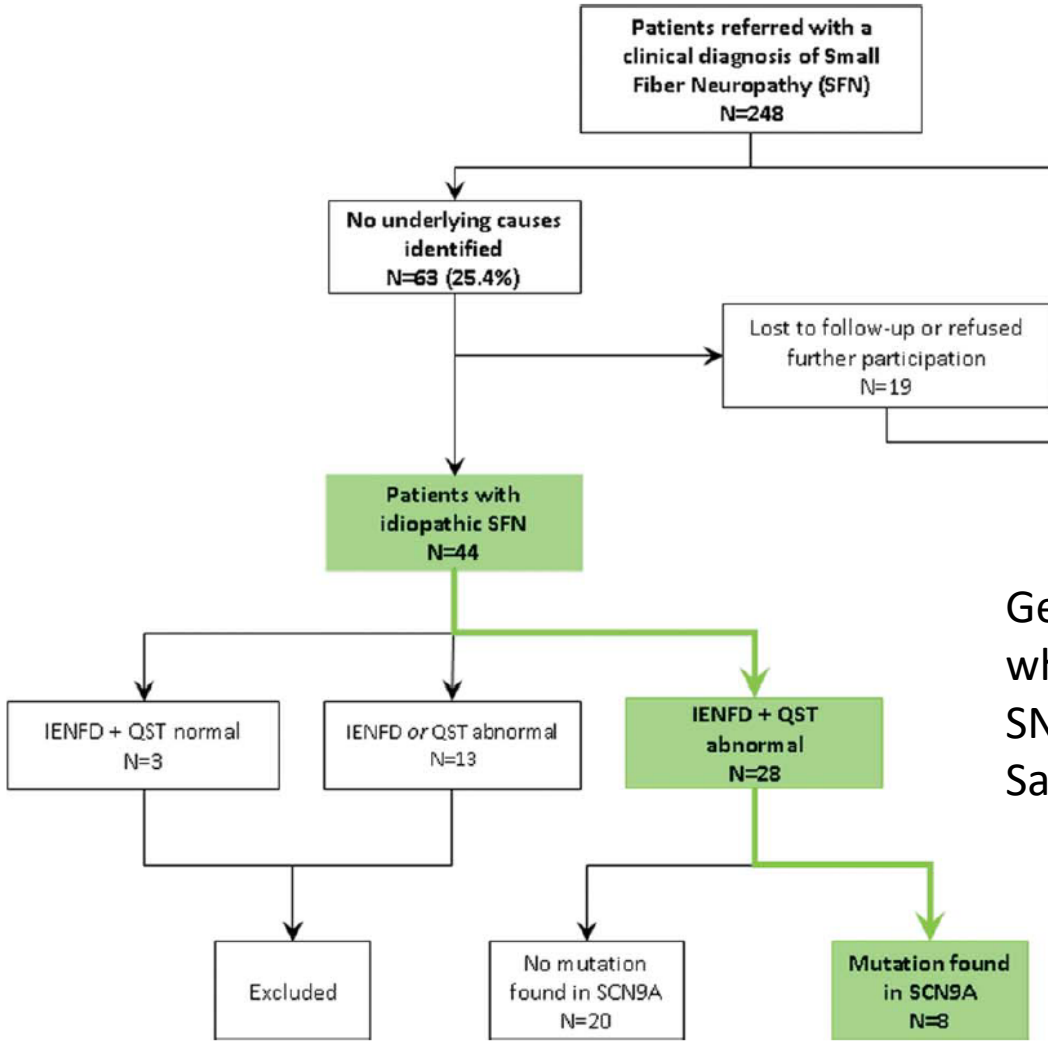
RESEARCH Open Access

Intra- and interfamily phenotypic diversity in pain syndromes associated with a gain-of-function variant of $Na_V1.7$

Mark Estacion[1†], Chongyang Han[1†], Jin-Sung Choi[1,7†], Janneke GJ Hoeijmakers[2], Giuseppe Lauria[3], Joost PH Drenth[4], Monique M Gerrits[5], Sulayman D Dib-Hajj[1], Catharina G Faber[2], Ingemar SJ Merkies[2,6] and Stephen G Waxman[1*]

Gain of function NaV1.7 mutations in idiopathic small fiber neuropathy

Goal: Can SCN9A mutations be found in a clinically well defined cohort of definitive SFN without known cause



Underlying causes:

- Sarcoidosis (150)
- Medication (9)
- Hemochromatosis (5)
- Diabetes mellitus (4)
- …

Genomic DNA from whole blood
SNC9A sequencing by Sanger sequencing.

TABLE. DIAGNOSTIC CRITERIA SETS FOR SMALL FIBER NEUROPATHY

NEURODIAB		Besta criteria	
Possible	Length-dependent symptoms and/or signs of small fiber damage	At least 2 of:	Clinical signs of small fiber neuropathy
Probable	Above with normal sural nerve conduction study		Abnormal QST thermal thresh-olds at foot
Definitive	Both of above and reduced IENFD at ankle		Reduced IENFD at distal leg
	AND/OR abnormal QST thermal thresh-olds at foot	Without:	Large fiber neuropathy signs or
			abnormal NCS

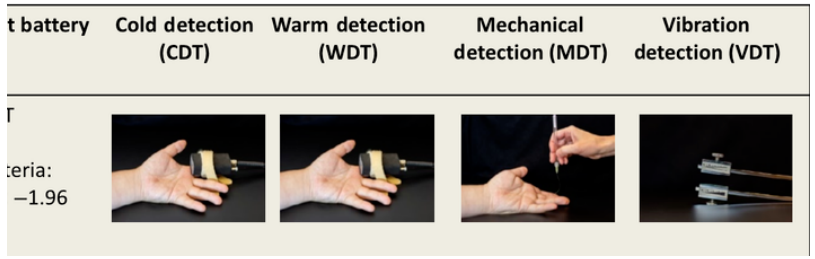
Abbreviations: IENFD, intraepidermal nerve fiber density; NCS, nerve conduction studies; NEURODIAB, Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes; QST, quantitative sensory testing.

Quantitative sensory testing (QST)

Table 1 Clinical signs, quantitative sensory testing, and possible underlying neurobiological mechanisms

Clinical signs	Definition	Quantitative sensory testing	Possible underlying neurobiological mechanisms		
		Testing for presence of plus or minus signs (tested peripheral fiber types)	Deafferentation	Peripheral sensitization	Central sensitization
Plus signs			Sensitivity to test stimuli		
Hyperalgesia	Increased pain sensitivity[a] of				
To heat	… the skin	Heat stimulation by means of thermotesting (C, Aδ)	↓	↑↑	→?
To cold	… the skin	Cold stimulation by means of thermotesting (C, Aδ)	↓	→	↑?
For pinprick stimuli	… the skin	Calibrated needle stimuli (pinprick) (C, Aδ)	↓	↑?	↑↑
For blunt pressure	… deeper tissues	Pressure algometer (C, Aδ)	↓	↑?	→?
Allodynia[b]	Pain in response to non-nociceptive stimuli[a]	Brush, cotton swab, Q-tip (Aβ) to skin brushing	→	→	↑
Minus signs					
Hypoesthesia (thermal/ mechanical/other)	Decreased sensitivity for nonpainful stimuli	Light cold stimulation by means of thermotesting (Aδ), light heat stimulation by means of thermotesting (C), von Frey filaments (Aβ), calibrated tuning fork (64 Hz, Rydel–Seiffer) (Aβ)	↓	→	→, ↓[c]
Hypoalgesia (thermal/ mechanical/other)	Decreased sensitivity for painful stimuli	To cold/heat stimulus by means of thermotesting (C, Aδ)Calibrated needle stimuli (pinprick) (C, Aδ)Pressure algometer (C, Aδ)	↓	→	→

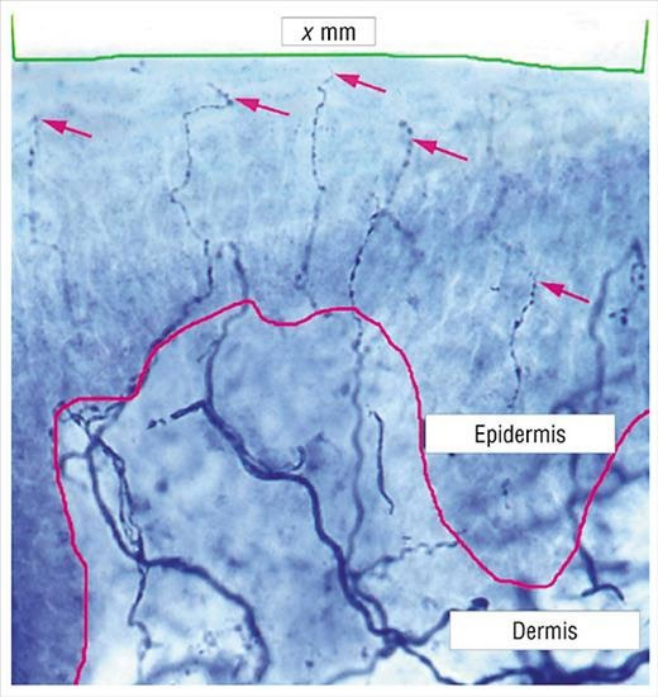
Table modified according to Woolf and Mannion [15], Hansson et al. [13], Rolke [21].
↑ increased sensitivity to test stimulus during a clinical neurological examination, ↓ decreased sensitivity, → sensitivity unchanged or phenomenon not examinable, ? has not been adequately studied or described in studies or is not yet generally accepted.
[a]IASP definition [22]; *IASP* International Association for the Study of Pain.
[b]This term should be used only when it is known that the test stimulus does not activate any nociceptors. What is meant here is the dynamic tactile allodynia for slightly moving tactile stimuli. A light brushing of the skin is the only established example (IASP 2008).
[c]A secondary tactile hypoesthesia was also observed in the context of central sensitization [23].



https://www.raynersmale.com/blog/2020/5/23/quantitative-sensory-testingin-literature-amp-the-clinic

M. Mücke, Schmerz, 2021

Intraepidermal Nerve Fiber Density (IENFD)



Mc Arthur et al, Arch Neurol 1998

Table 1. Intraepidermal nerve fiber density (IENFD) normative values for clinical use.

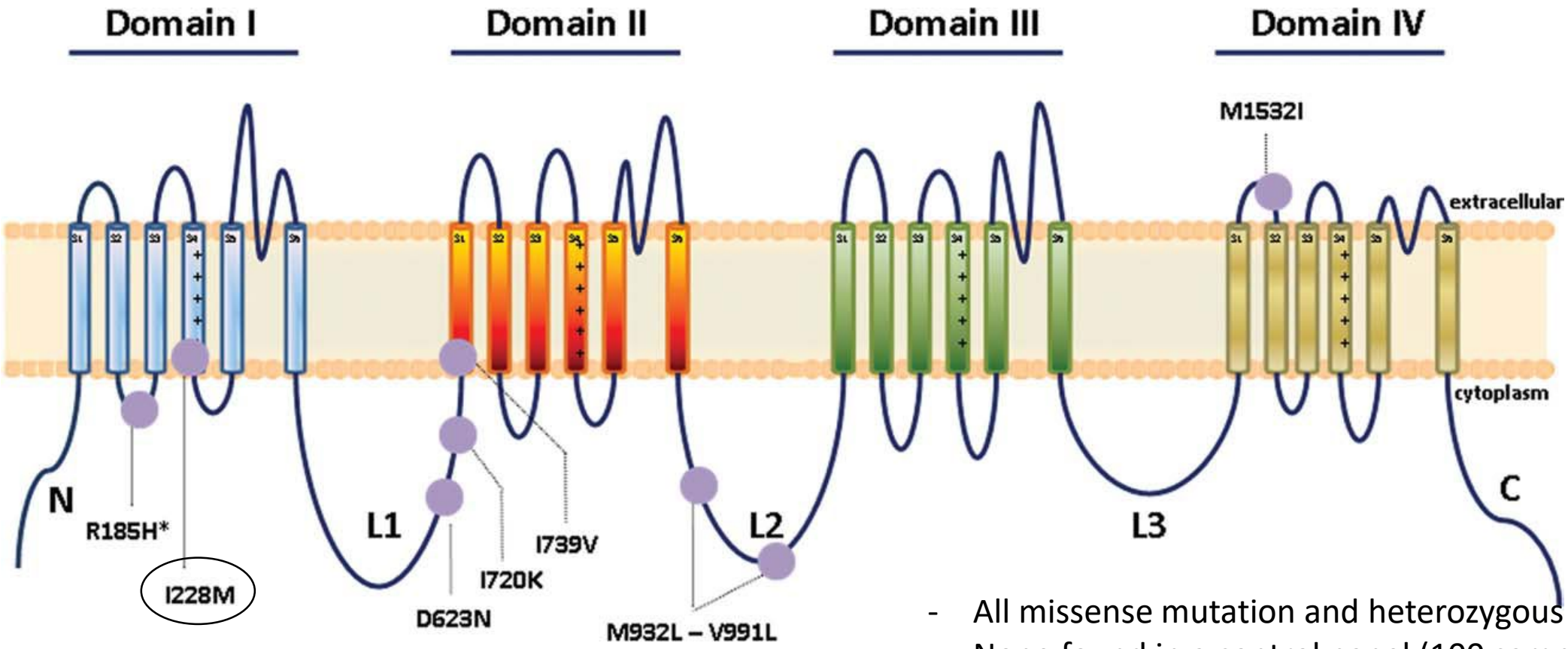
	Females (n = 285)			Males (n = 265)		
Age (years)	Number of subjects	0.05 Quantile IENFD values per age span	Median IENFD values per age span	Number of subjects	0.05 Quantile IENFD values per age span	Median IENFD values per age span
20–29	57	8.4	13.5	36	6.1	10.9
30–39	47	7.1	12.4	40	5.2	10.3
40–49	70	5.7	11.2	62	4.4	9.6
50–59	59	4.3	9.8	53	3.5	8.9
60–69	32	3.2	8.7	43	2.8	8.3
70–79	16	2.2	7.6	22	2.1	7.7
≥80	4	1.6	6.7	9	1.7	7.2

Lauria et al. J. Peripher. Nerv. Syt., 2010

Intraepidermal nerve fiber density (IENFD) measurement

- Fixation in 4% formalin and 20% sucrose
- Bleaching with potassium permanganate / oxalic acid
- 50 µm vertical section (cryostat)
- **Immunhistochemical staining with PGP9.5** free floating (over night incubation of antibodies)
- Hematoxylin counterstaining

Gain of function NaV1.7 mutations in idiopathic small fiber neuropathy



- All missense mutation and heterozygous
- None found in a control panel (100 samples from healthy donors)

Domain I – SV4 Segment

- Mutations affecting the charge (S211P, F216S) are linked to IEM, shift voltage-dependence of activation in a hyperpolarizing direction, easier to open the channel.
- I228M is not affecting the charge

Gain of function $Na_V1.7$ mutations in idiopathic small fiber neuropathy

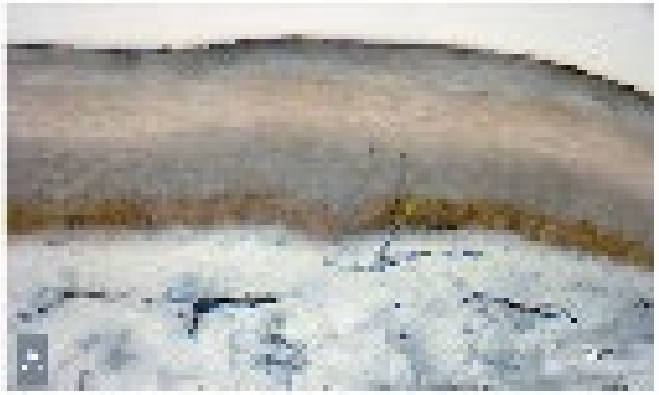
TABLE 2: SFN Symptoms Inventory Questionnaire Findings in Patients with *SCN9A* Novel Mutations

Patient	Mutation	Sweating	Diarrhea	Constipation	Micturation Problems	Dry Eyes	Dry Mouth	Orthostatic Dizziness	Palpitations	Hot Flashes	Skin Hyperesthesia	Burning Feet	Sheet Intolerance	Restless Legs
1	R185H	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	1[b]	2[b]	3[b]	3[b]
2	R185H	0[a]	0[a]	0[a]	0[a]	0[a]	1[b]	1[b]	0[a]	0[a]	3[b]	3[b]	2[b]	2[b]
3	D623N	0[a]	1[b]	1[b]	0[a]	1[b]	2[b]	2[b]	2[b]	0[a]	2[b]	2[b]	2[b]	2[b]
4	I739V	3[b]	2[b]	1[b]	2[b]	2[b]	3[b]	1[b]	1[b]	3[b]	2[b]	2[b]	2[b]	2[b]
5	I720K	3[b]	1[b]	0[a]	1[b]	1[b]	2[b]	0[a]	0[a]	1[b]	2[b]	1[b]	1[b]	1[b]
6	M1532I	0[a]	0[a]	0[a]	0[a]	1[b]	0[a]	1[b]	1[b]	0[a]	3[b]	3[b]	1[b]	3[b]
7	M932L + V991L	1[b]	0[a]	2[b]	1[b]	1[b]	1[b]	0[a]	1[b]	1[b]	1[b]	2[b]	0[a]	0[a]
8	I228M	1[b]	3[b]	1[b]	2[b]	2[b]	3[b]	1[b]	1[b]	2[b]	2[b]	2[b]	1[b]	1[b]

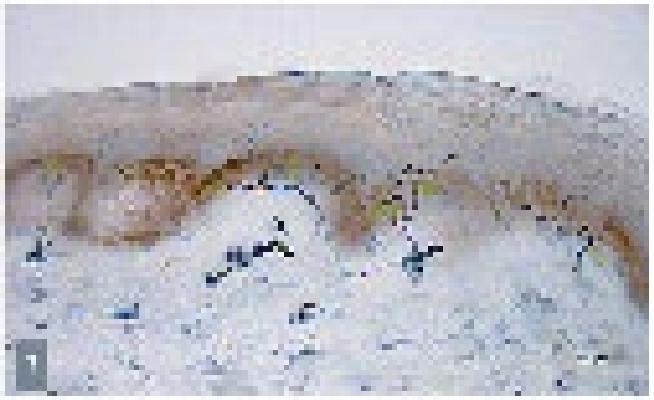
[a]Absence (score 0) of corresponding SFN-related symptom.
[b]Presence of SFN-related symptom, with variable intensity (score 1 = sometimes present; score 2 = often; score 3 = always present).SFN = small nerve fiber neuropathy.

Intra and interfamily phenotypic diversity in pain syndromes associated with $Na_V1.7$ I228M gain-of-function variant

	Symptoms	Age of onset	IENFD	QST
Patient 1	Pain of teeth, jaw, temporomandibular joint, behind eye	32	1.6/mm (cut off $\geq 3.5/\mathrm{mm}$)	Abnormal warm and cold thershold
Patient 2 (sister patient 1)	Burning pain and redness of hands and feet	36	8 / mm (cut off 5.7 /mm)	-
Patient 3	Occiput with red discoloration and tingling, burning and warm sensation; later also involvement of feet and hands	-	5.2 / mm (cut off $\geq$ 5.7)	-



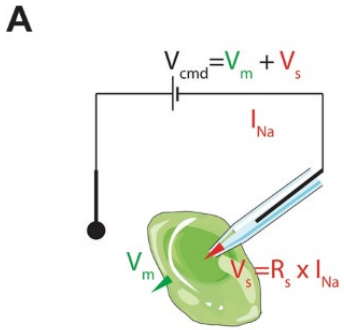
Patient 1



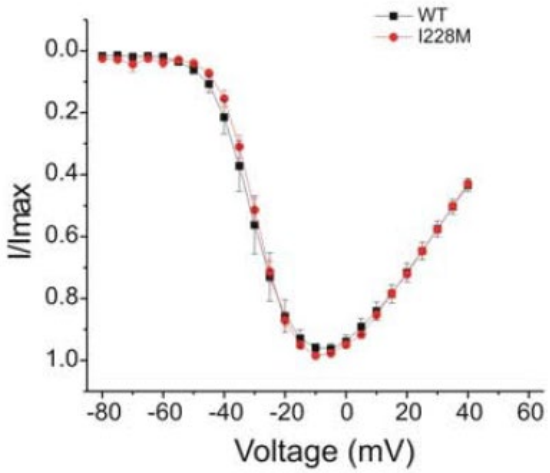
Age/ Gender matched control

M. Estacion et al, Molecular Pain 2011

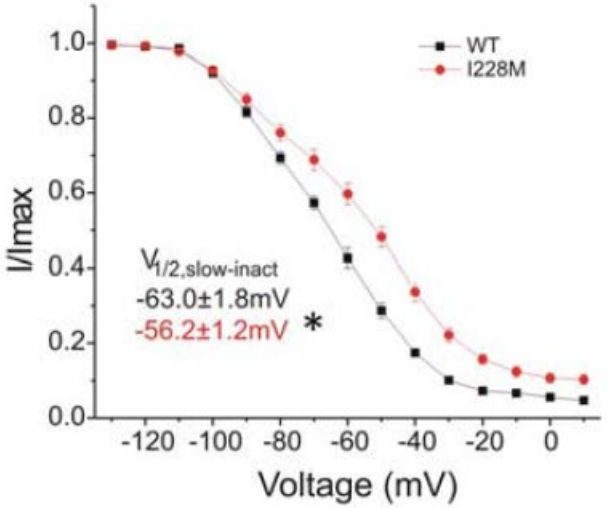
$Na_V1.7$ I228M gain-of-function impairs slow inactivation



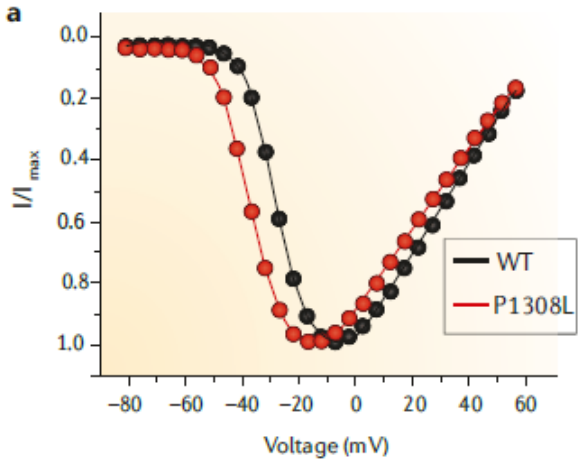
I-V curves



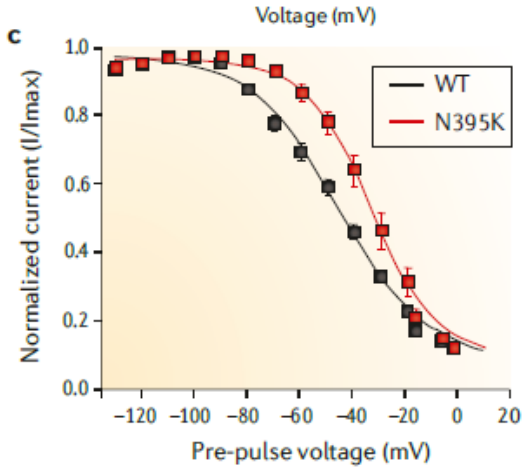
Slow inactivation



Comparison IEM



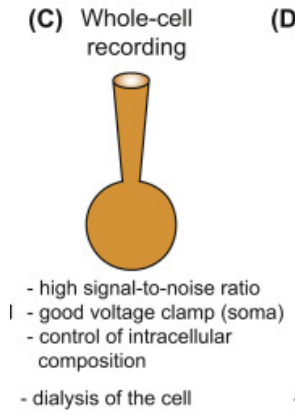
Hyperpolarization shift

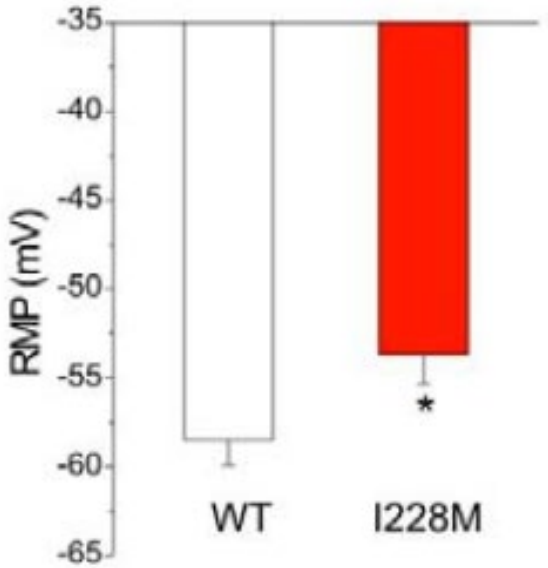


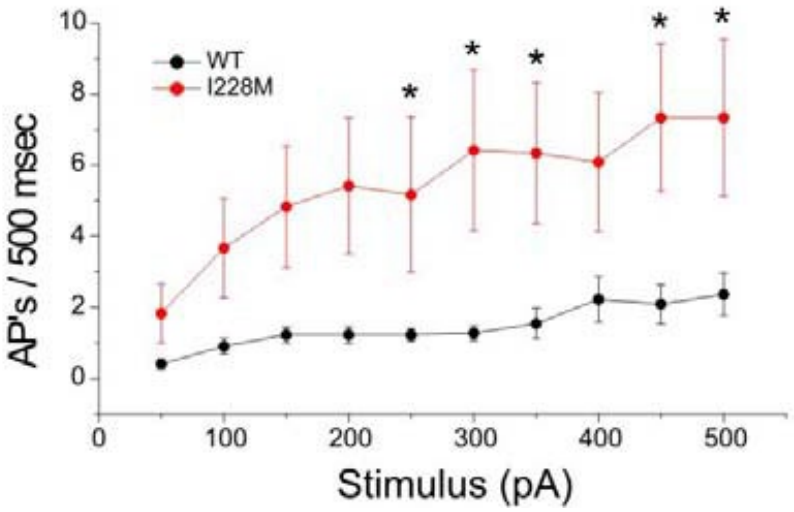
HEK293 cells, transient transfection, Voltage-clamp

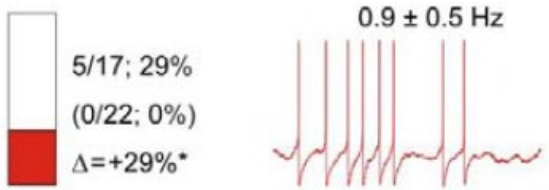
S. Dib-Hajj et al, Nature reviews 2013

I228M turns dorsal root ganglion (DRG) neurons hyperexcitable









Portion of spontaneous firing cells

Mean firing frequency across a range of current injections from 50 to 500 pA

- Depolarization shift in resting membrane potential
- Increasing firing rate
- Induction of spontaneous firing

M. Estacion et al, Journal of Neuroscience, 2008
M. Estacion et al, Molecular Pain 2011

DRG neurons (n=12) from rat pups, transient transfection, Current clamp whole cell recording

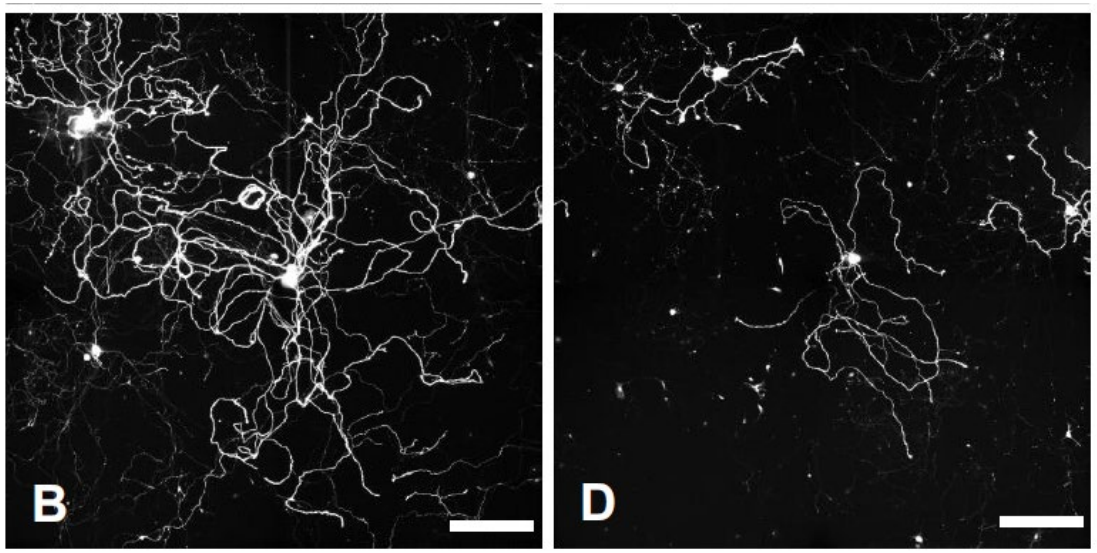
The small fiber neuropathy NaV1.7 I228M mutation: impaired neurite integrity via bioenergetic and mitotoxic mechanisms, and protection by dexpramipexole

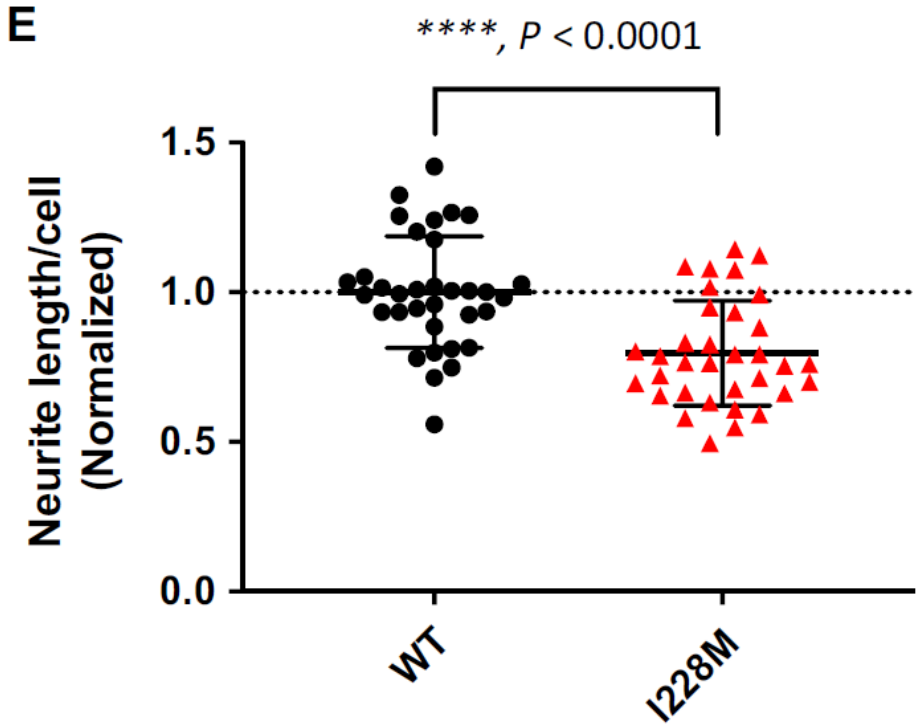
Seong-il Lee,[1,2] Janneke G. J. Hoeijmakers,[3] Catharina G. Faber,[3] Ingemar S. J. Merkies,[3,4] Giuseppe Lauria,[5,6] and Stephen G. Waxman[1,2]

[1]*Department of Neurology, Yale University School of Medicine, New Haven, Connecticut;* [2]*Center for Neuroscience and Regeneration Research, Veterans Affairs Connecticut Healthcare System, West Haven, Connecticut;* [3]*Department of Neurology, School of Mental Health and Neuroscience, Maastricht University Medical Center+, Maastricht, The Netherlands;* [4]*Department of Neurology, Curaçao Medical Center, Willemstad, Curaçao;* [5]*Neuroalgology Unit, Foundazione IRCCS Istituto Neurologico "Carlo Besta," Milan, Italy; and* [6]*Department of Biomedical and Clinical Sciences "Luigi Sacco," University of Milan, Milan, Italy*

Submitted 10 June 2019; accepted in final form 17 December 2019

Transient expression of $Na_V1.7$ I228M is inducing reduced neurite length

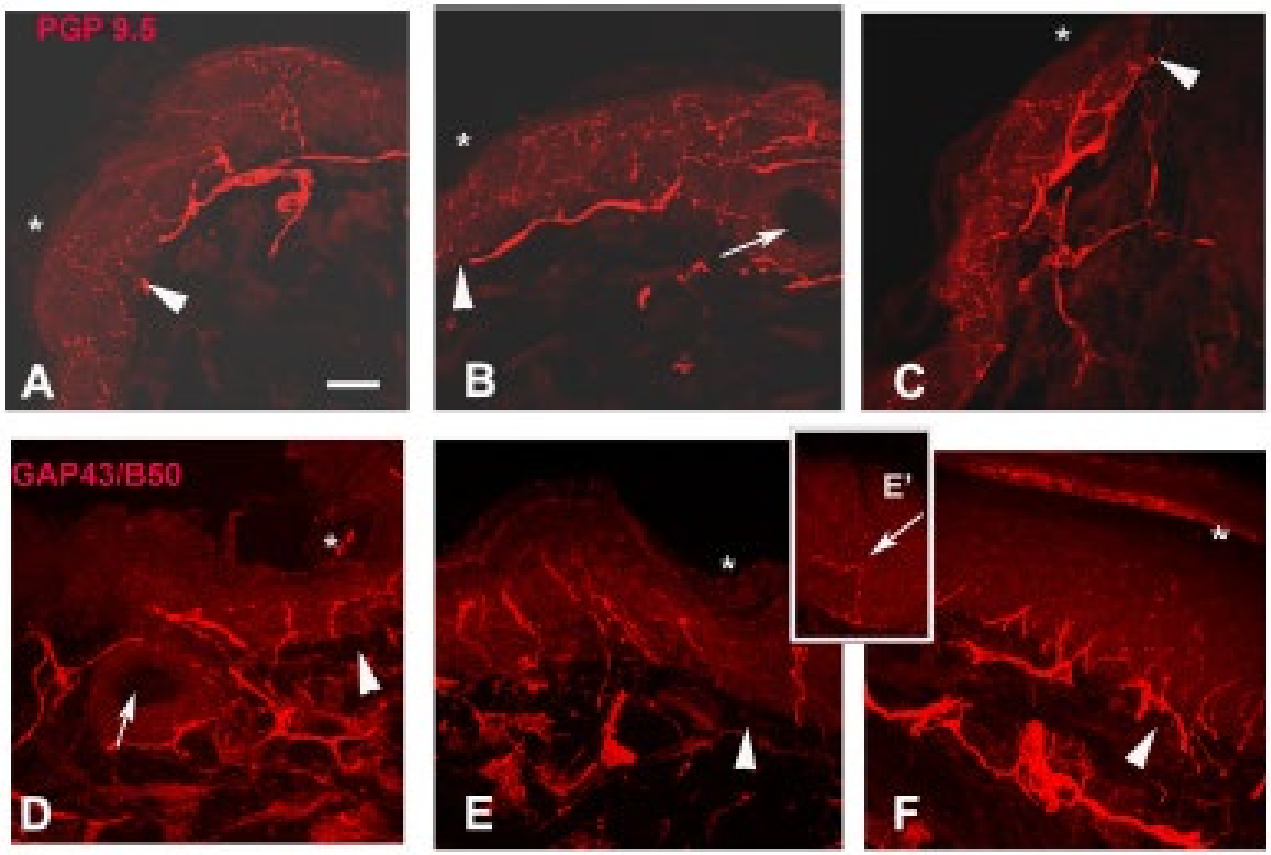




→ Conclusion: Reduced neurite length is a potential model for IENFD loss in SFN

- DRG neurons isolated from 6- / 8- week old mice
- Electroporation (WT/I228M + RFP)
- Culturing for 7 days

Excurse: IENF are in a dynamic process involving repeated regeneration and degeneration, as the epidermis continuously remodels itself



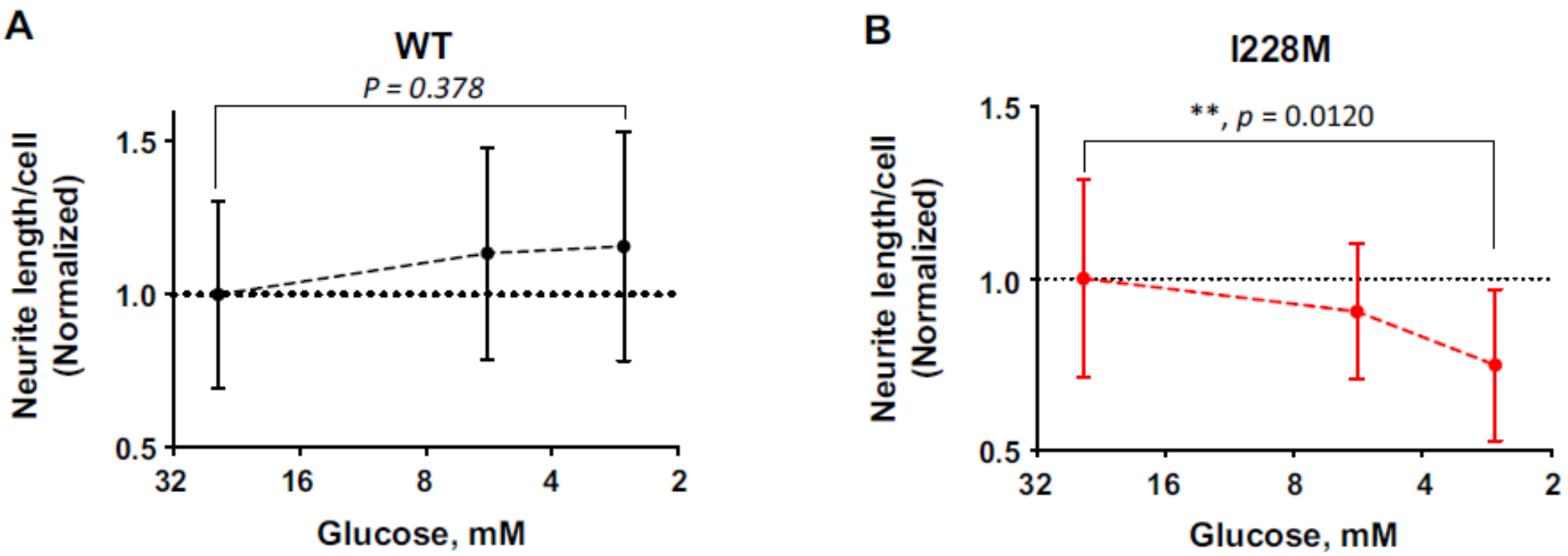
GAP43/B50 : regeneration-related Growth molecule

Dorsal trunk skin of mouse

Cheng et al, Journal of neuroscience 2010

Glucose restriction is enhancing the phenotype

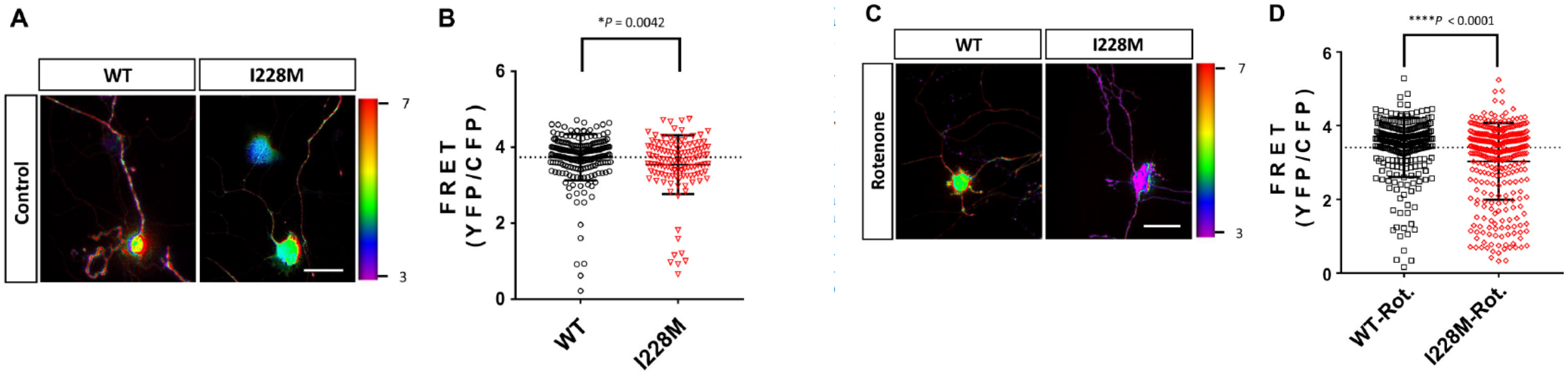
Neurite growth and maintenance is high-energy demanding →Shortage of ATP is associated with neurite degeneration (Presson et al. 2016) ⟶ Glucose is the major substrate of ATP production



→ Conclusion: Presence of I228M mutated channels imposes an energetic burden on sensory fibers, rendering them more vulnerable to damage under conditions where the glucose level is low

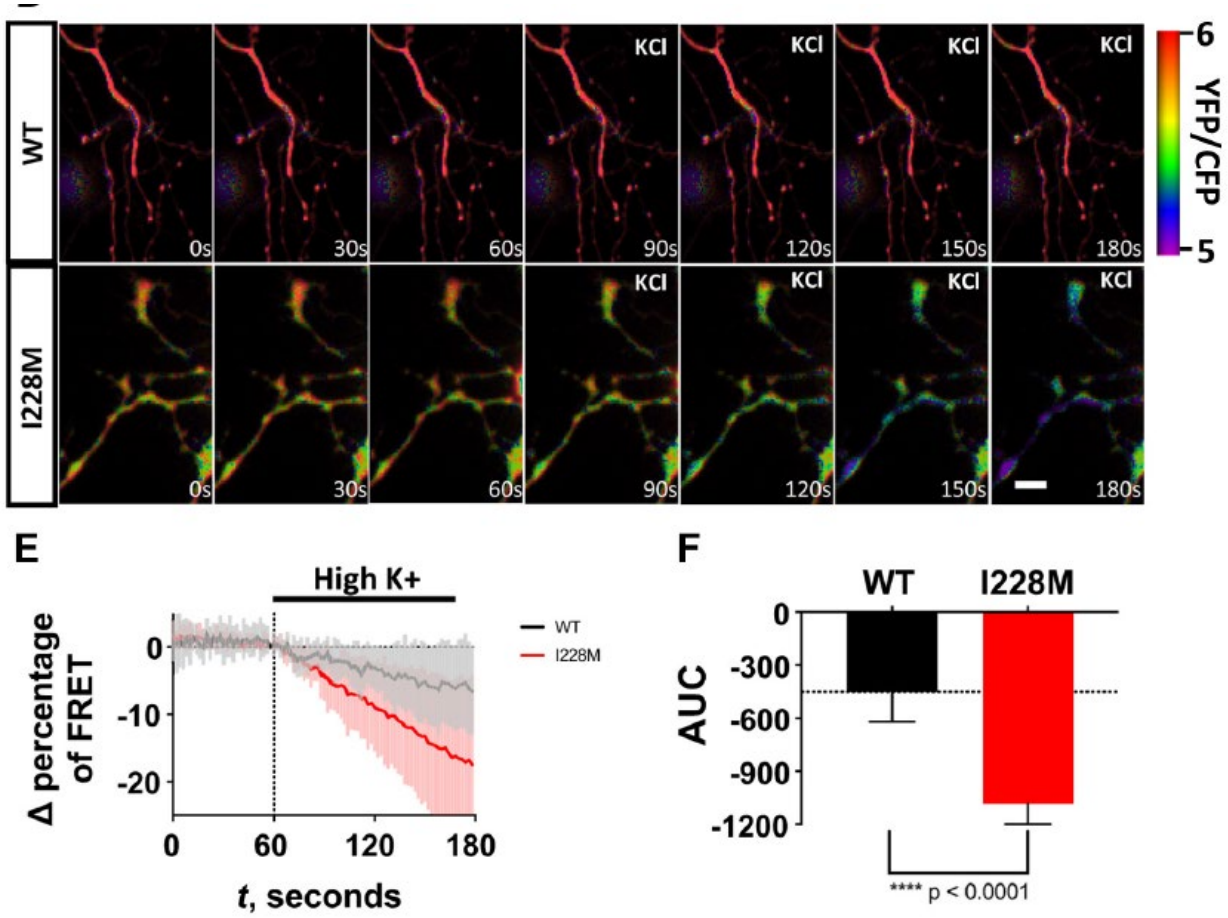
DRG neurons isolated from 6- / 8- week of mice, Electroporation (WT/I228M + RFP), Culturing for 7 days

Modest demonstration of decreased intracellular ATP levels in DRG expressing I228M



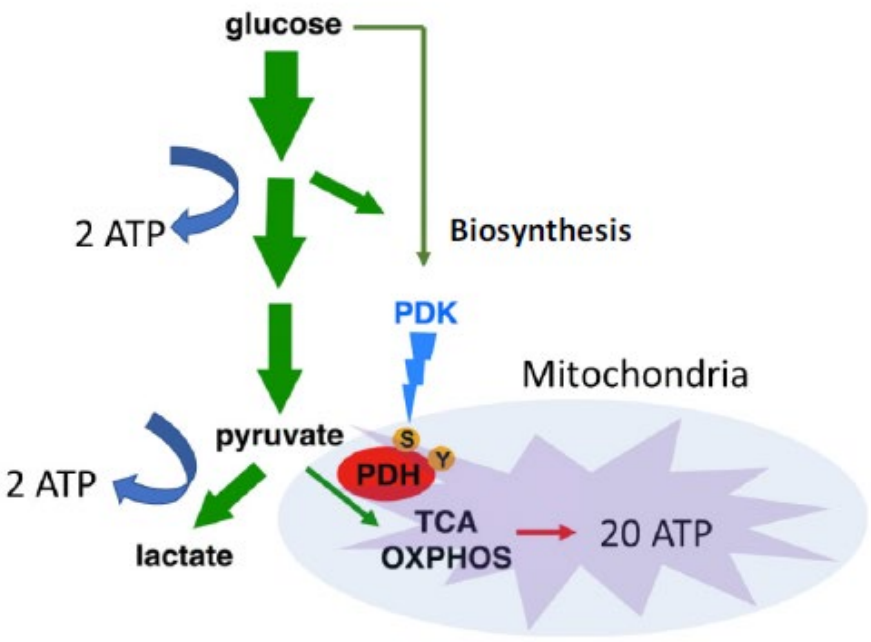
- DRG neurons isolated from 6- / 8- week old mice
- Electroporation (WT/I228M + RFP)
- Culturing for 7 days
- Fluorescence resonance energy transfer (FRET) based ATP indicator

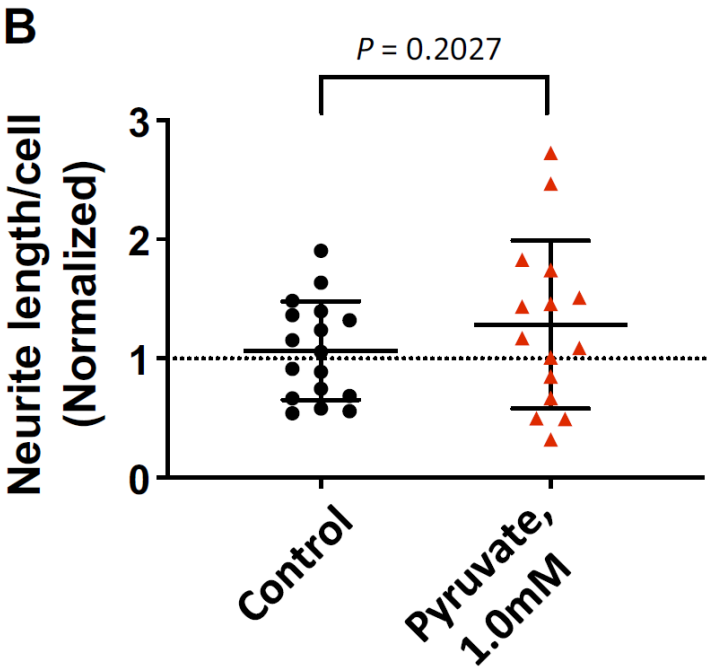
Accelerate ATP reduction after depolarization (DRG neurites)

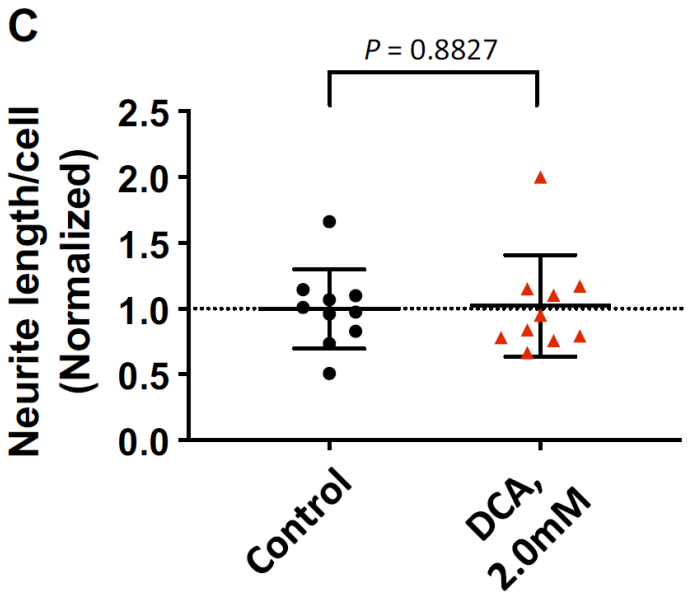


- Depolarization with 50 mM KCL
- Fluorescence resonance energy transfer (FRET) based ATP indicator

Increased Pyruvate availability failed to increase neurite length of I228M neurite length of I228M transfected DRG neurons

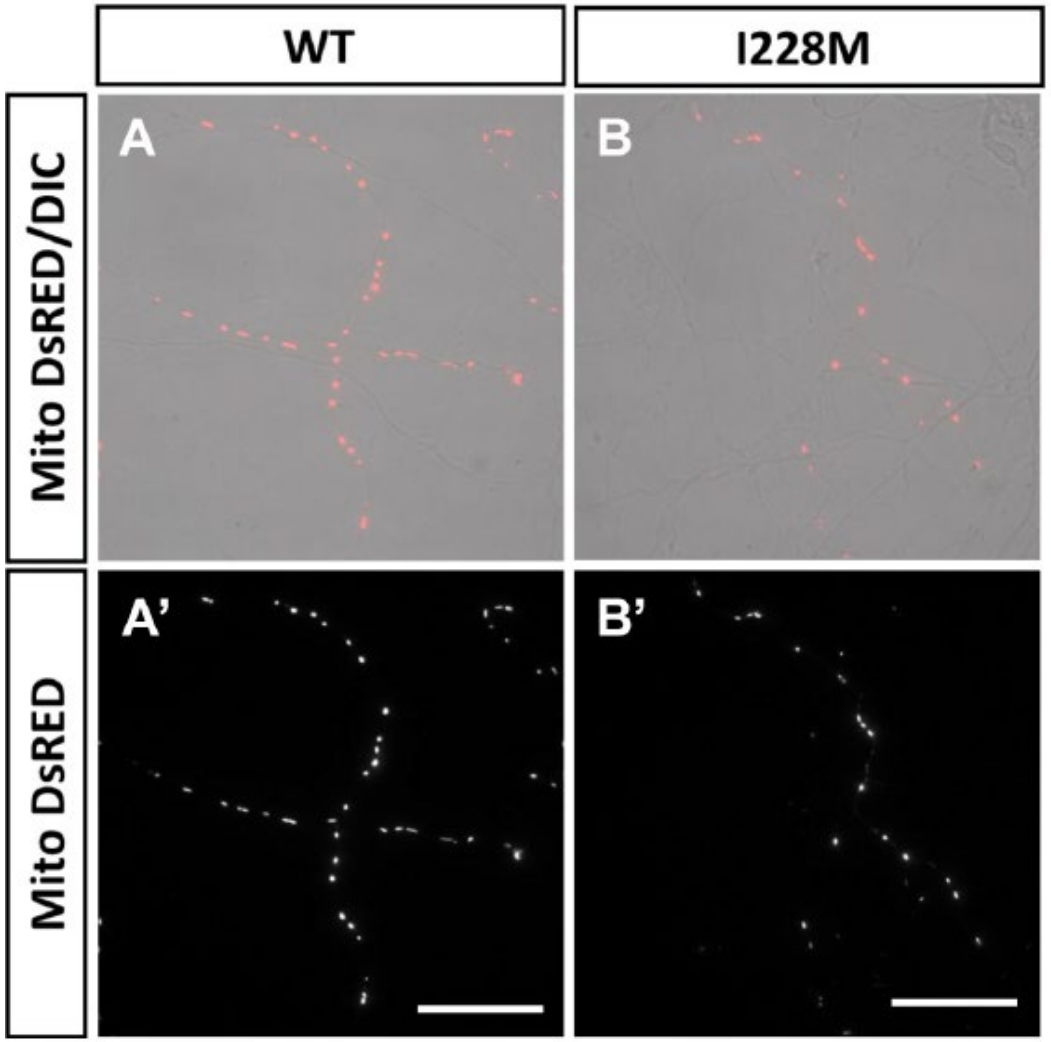


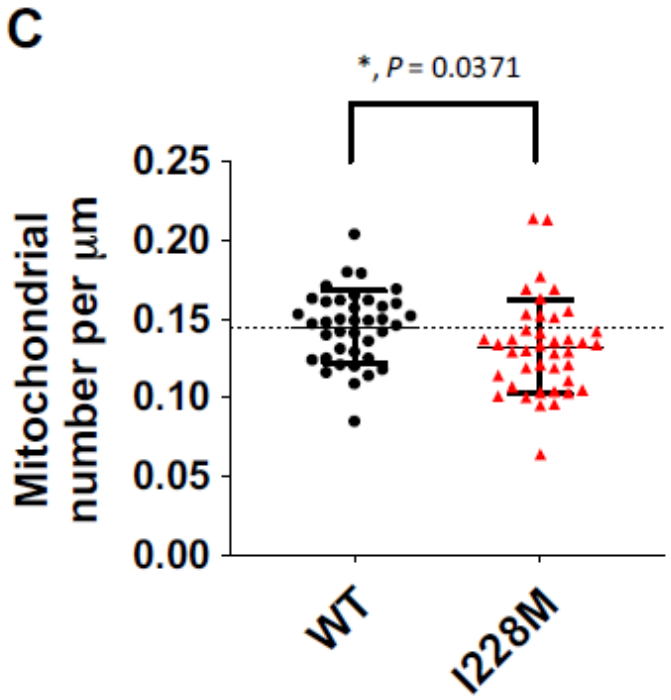


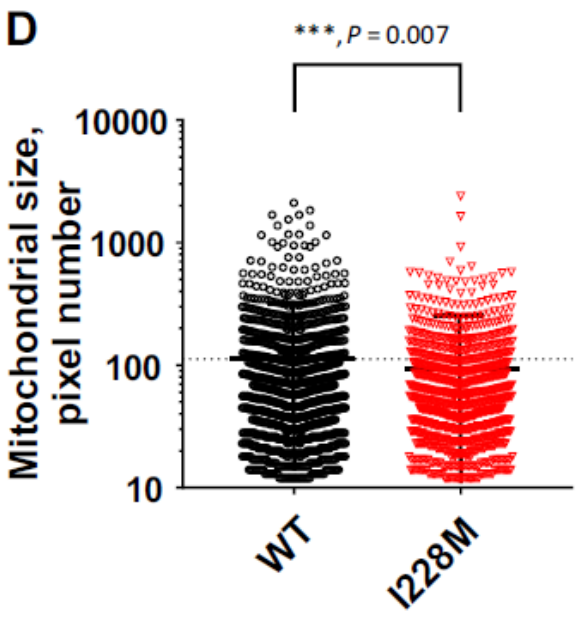


Dichloroacetate (DCA) is a PDK (pyruvate dehydrogenase kinase) inhibitor; here used to exclude a negative feedback regulation

Alteration in mitochondrial distribution and morphology







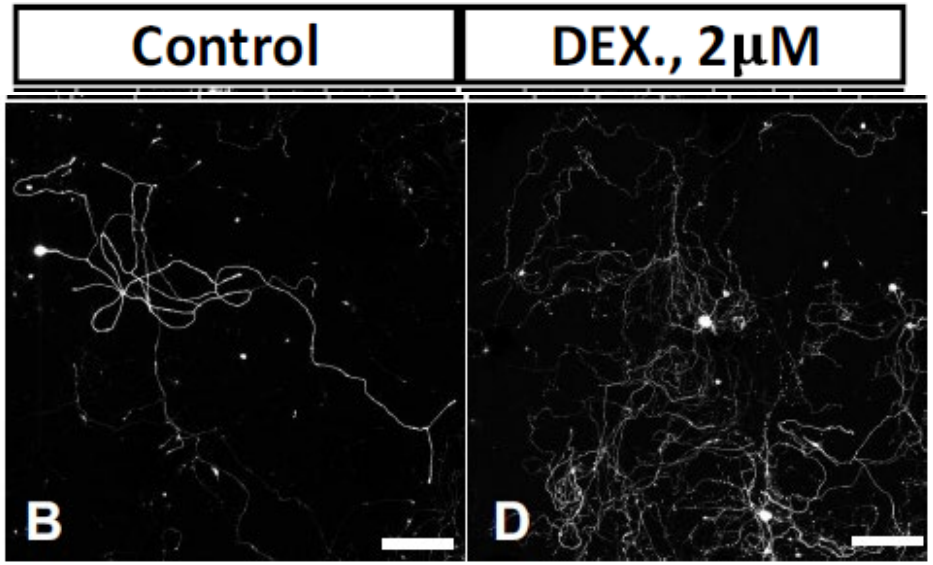
Labelling of mitochondria by co-transfection with mito-DsRed

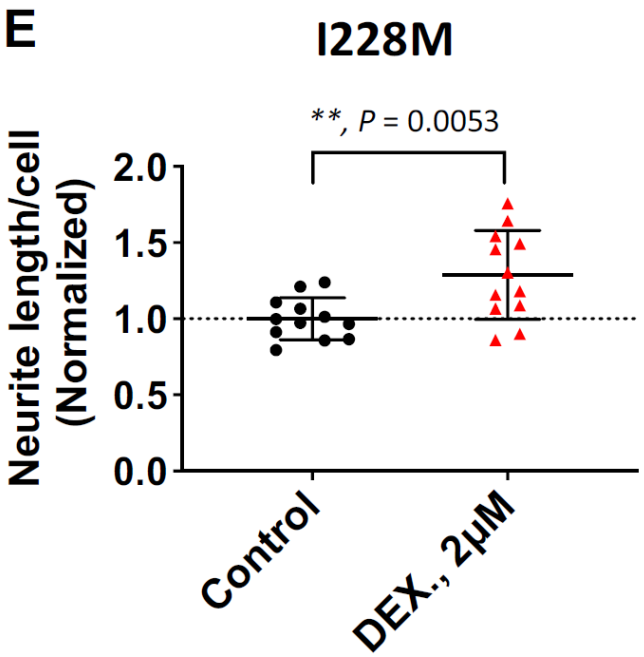
Dexpramipexole (DEX) promotes neurite growth of I228M expressing neurons

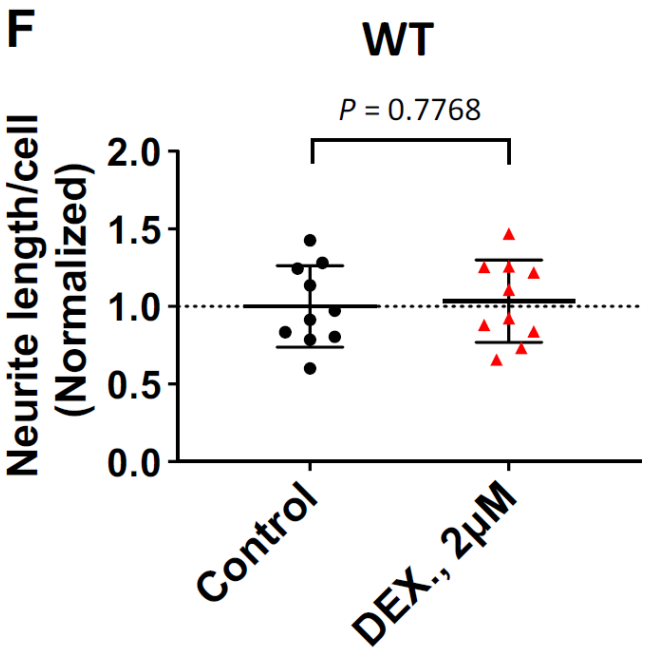
Maintenance of a proton gradient over mitochondrial membrane is critical for ATP synthesis → The mitochondrial permeability transition pore (mPTP) is known to regulate ionic gradient in mitochondrial matrix

⟶ Pathologic conditions related to energetic stress can cause a prolonged opening of mPTP, DEX blocks mPTP

⟶ Improvement of mitochondrial energy metabolism







→ Conclusion: Mitochondrial mechanism are involved in neuritic impairment of I228M neurons

Two independent mouse lines carrying the $Na_V1.7$ I228M gain-of-function variant display dorsal root ganglion neuron hyperexcitability but a minimal pain phenotype

Lubin Chen[a,b,c], Nivanthika K. Wimalasena[d,e], Jaehoon Shim[d,e], Chongyang Han[a,b,c], Seong-Il Lee[a,b,c], Rafael Gonzalez-Cano[d,e], Mark Estacion[a,b,c], Catharina G. Faber[f], Giuseppe Lauria[g,h], Sulayman D. Dib-Hajj[a,b,c], Clifford J. Woolf[d,e], Stephen G. Waxman[a,b,c,*]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

L. Chen and N.K. Wimalasena contributed equally to this work.

C.J. Woolf and S.G. Waxman contributed equally to this work.

[a] Department of Neurology, Yale University School of Medicine, New Haven, CT, United States, [b] Center for Neuroscience and Regeneration Research, Yale University School of Medicine, New Haven, CT, United States, [c] Center for Rehabilitation Research, VA Connecticut Healthcare System, West Haven, CT, United States, [d] FM Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA, United States, [e] Department of Neurobiology, Harvard Medical School, Boston, MA, United States, [f] Department of Neurology, School of Mental Health and Neuroscience, Maastricht University Medical Center, Maastricht, the Netherlands, [g] Neuroalgology Unit, IRCCS Foundation, "Carlo Besta" Neurological Institute, Milan, Italy, [h] Department of Biomedical and Clinical Sciences, "Luigi Sacco", University of Milan, Milan, Italy

**Corresponding author. Address: Neuroscience and Regeneration Research Center, VA Connecticut Healthcare System, 950 Campbell Ave, Bldg 34, West Haven, CT 06516, United States. Tel.: (203) 937-3802; fax: (203) 937-3801. E-mail address: stephen.waxman@yale.edu (S.G. Waxman).*

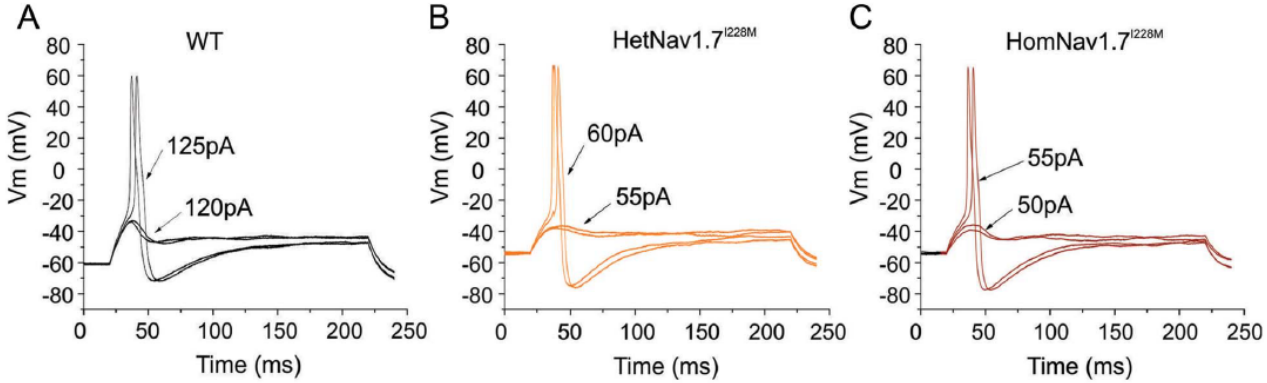
PAIN 162 (2021) 1758–1770

http://dx.doi.org/10.1097/j.pain.0000000000002171

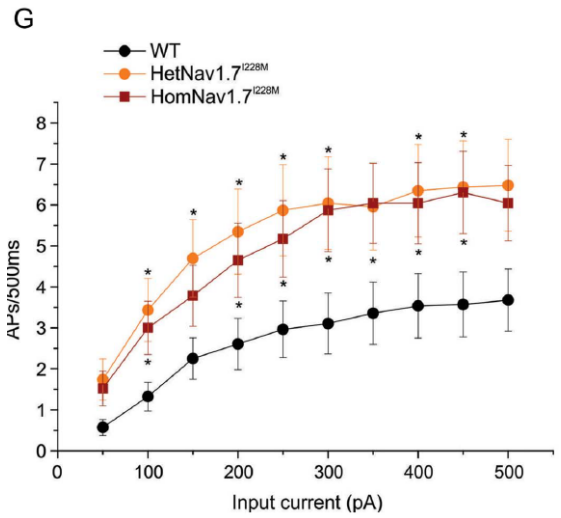
Generation of $Na_V1.7$ I228M knock-in mice

- One line generated by targeted homologous recombination
- Second line generated by CRISPR editing

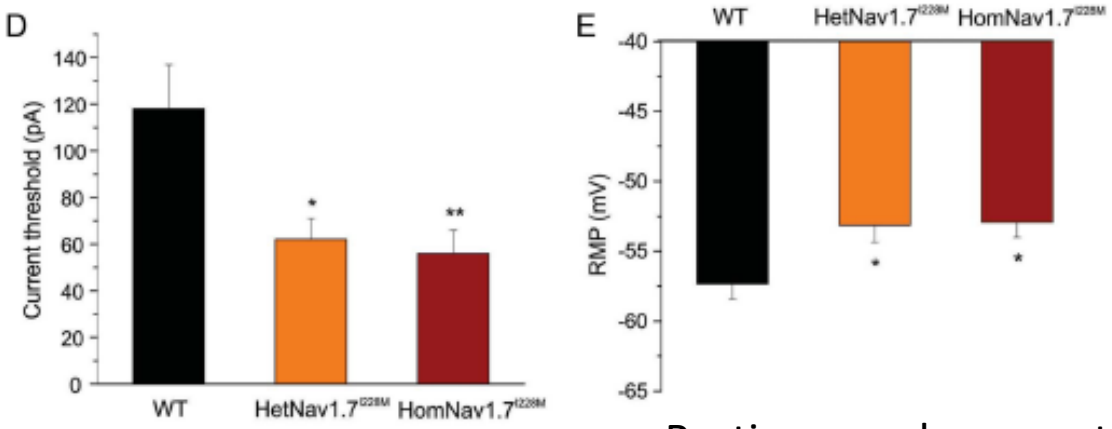
Ganglion neurons from I228M knocking mouse line is hyperexcitable and demonstrate a increased firing rate



Representative traces of DRG neurons



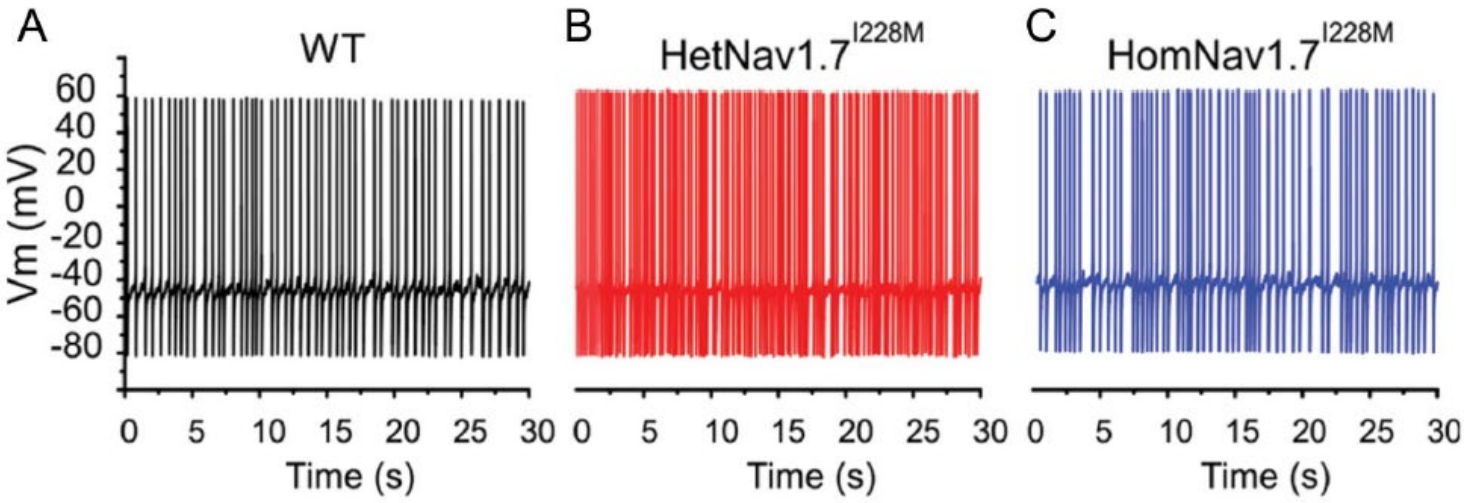
Mean firing frequency across a range of current injections from 50 to 500 pA

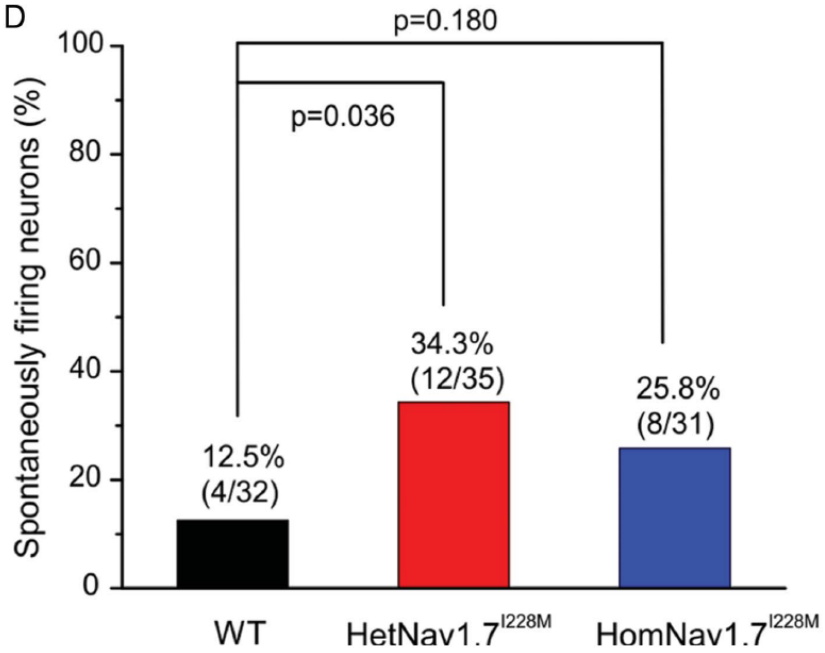


Resting membrane potential

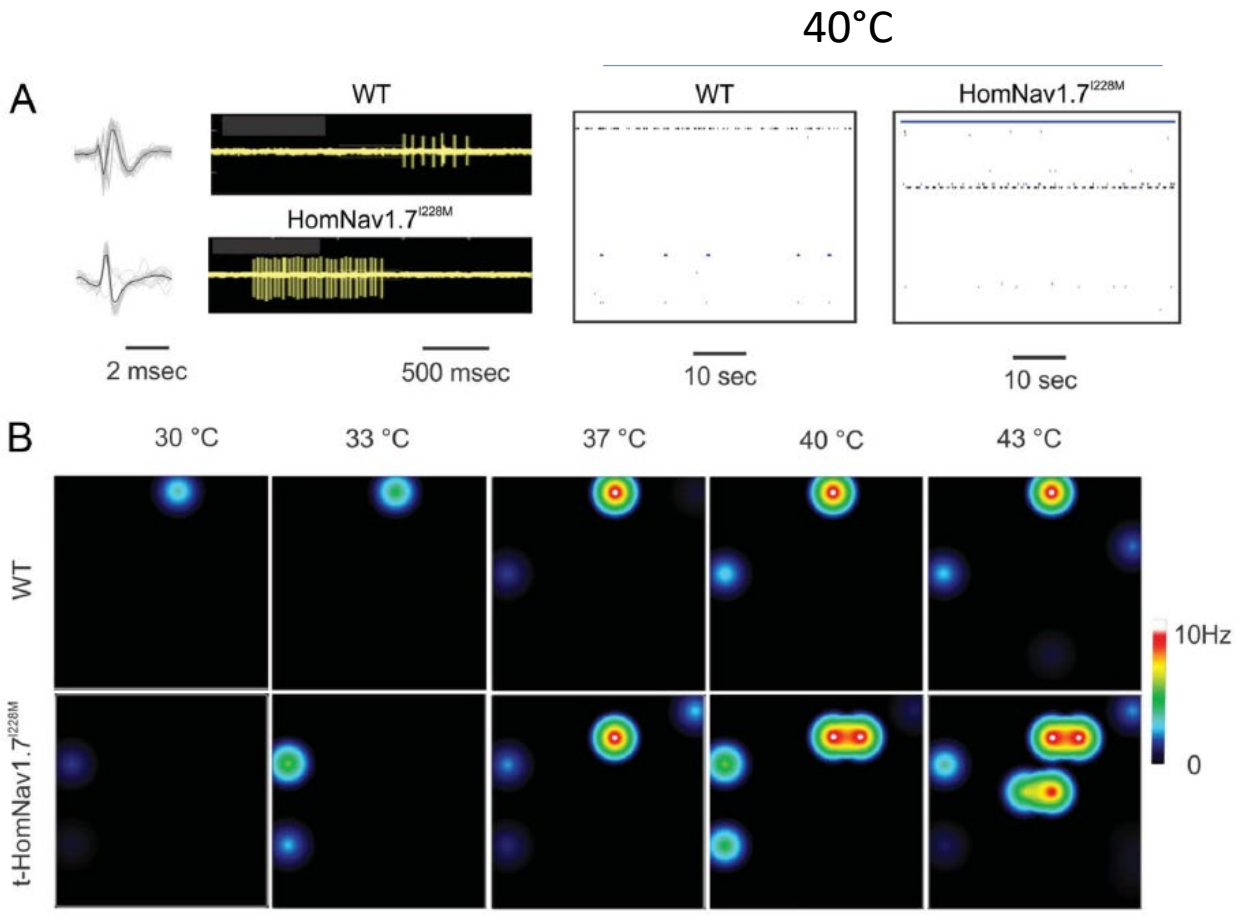
- DRG neurons isolated from 4-8 week old mice (targeted homologous recombination)
- Whole-cell current-clamp recoding

Increased portion of spontaneously firing





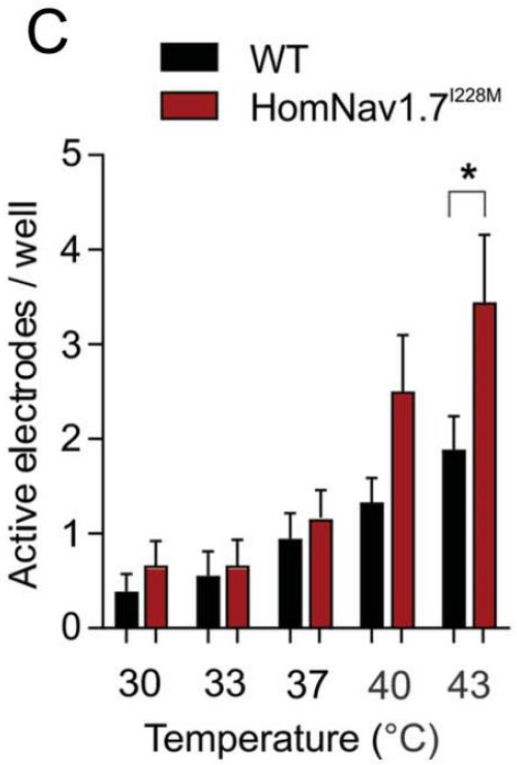
DRG neurons from one I228M knocking mouse lines demonstrate an enhanced response to heat -1

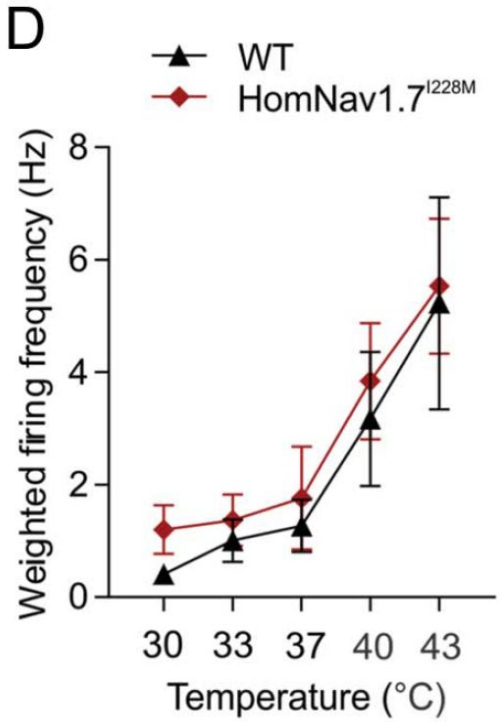


Maestro, Axion Biosystem

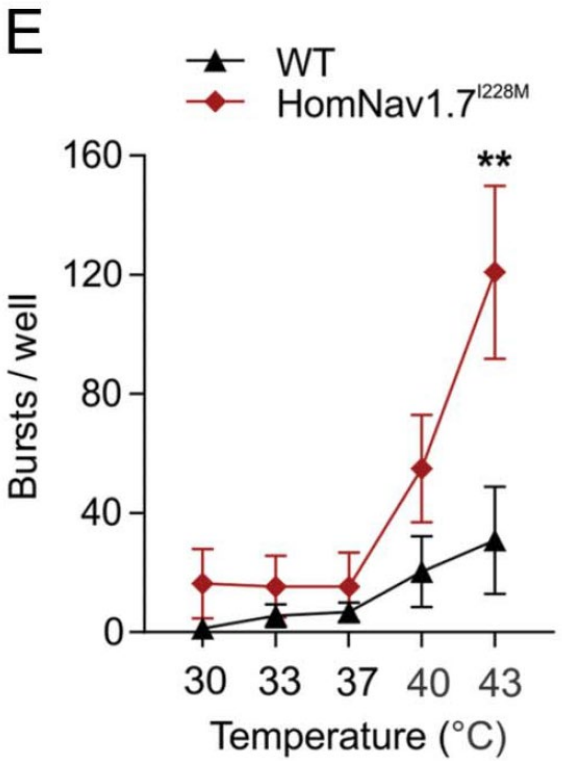
- DRG neurons isolated from 4-8 week old mice
- Multielectrode array (MEA) recording (12-well recording plate with 768 electrodes)
- Precise temperature control to create temperature ramps and maintain temperature
- Noxious warmth (40°C) ; noxious heat (43°C)

DRG neurons from one I228M knocking mouse lines demonstrate an enhanced response to heat -2

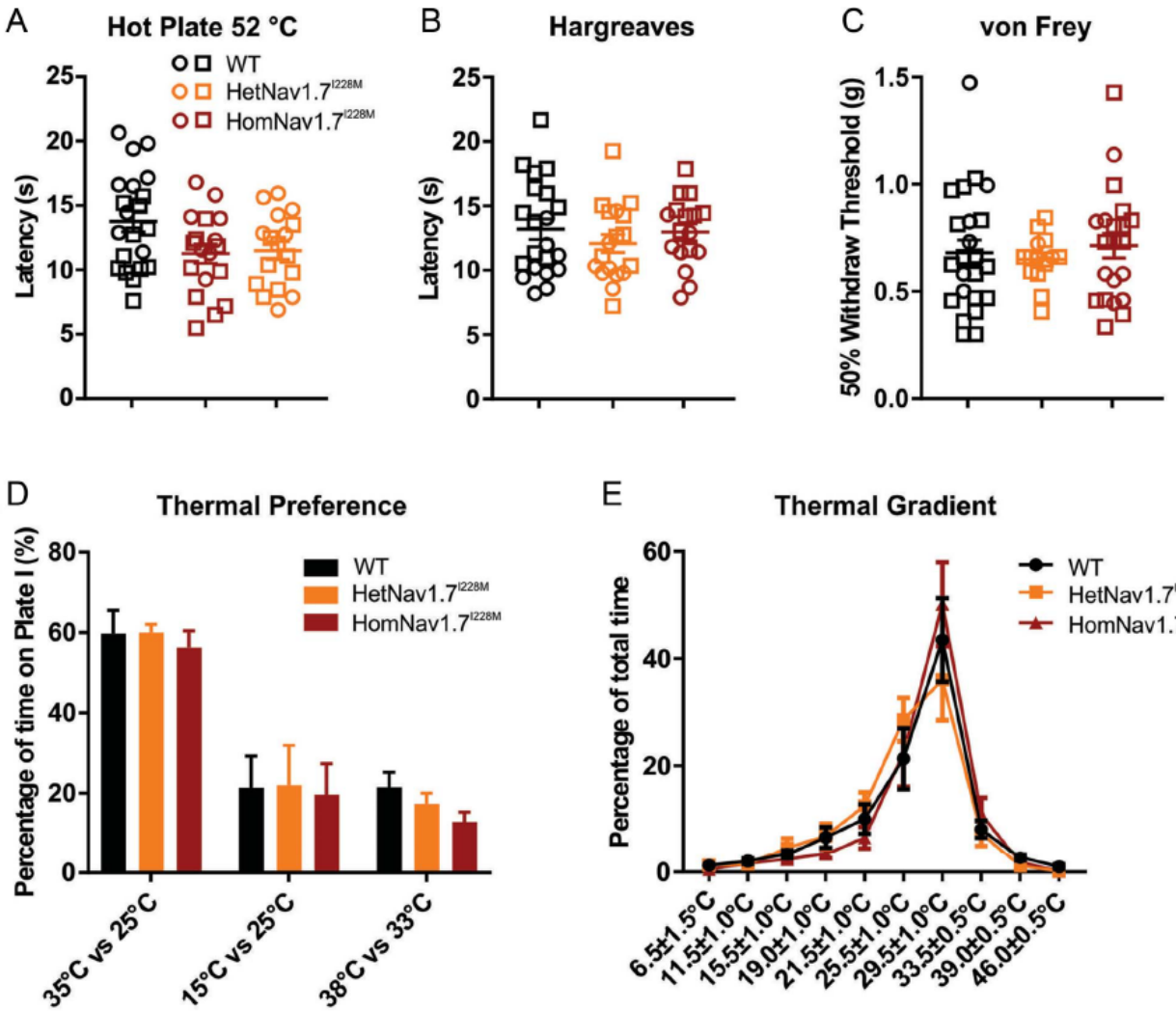


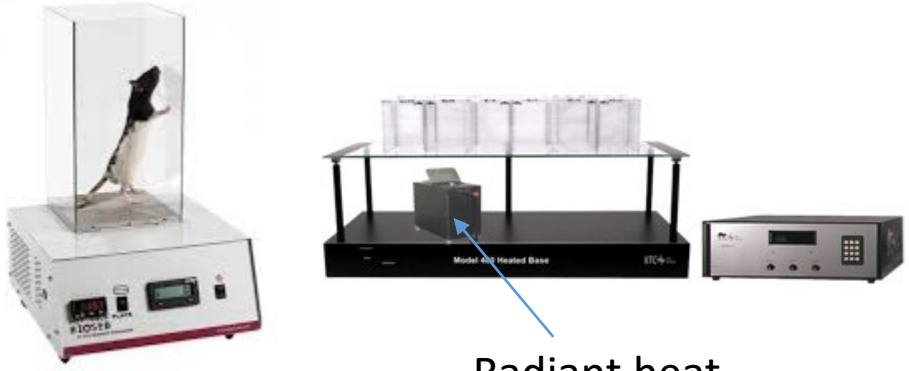


Mean action potentials



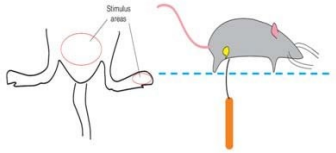
I228M knocking mouse line (homologous recombination) do not show thermal or mechanical hypersensitivity

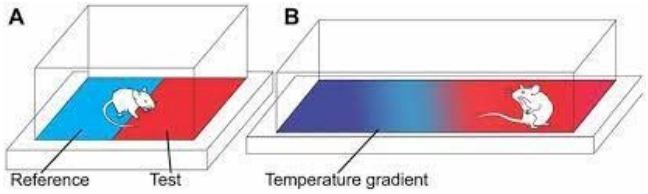




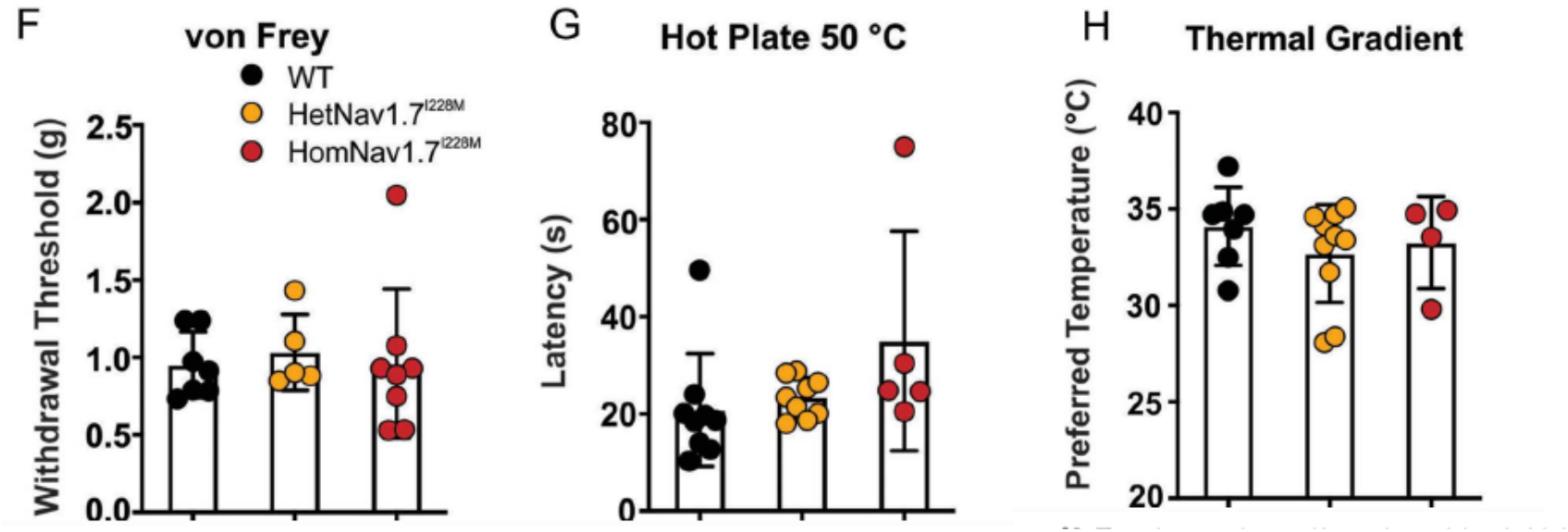
Radiant heat

Latency:
- flicking, licking
- Paw withdrawal

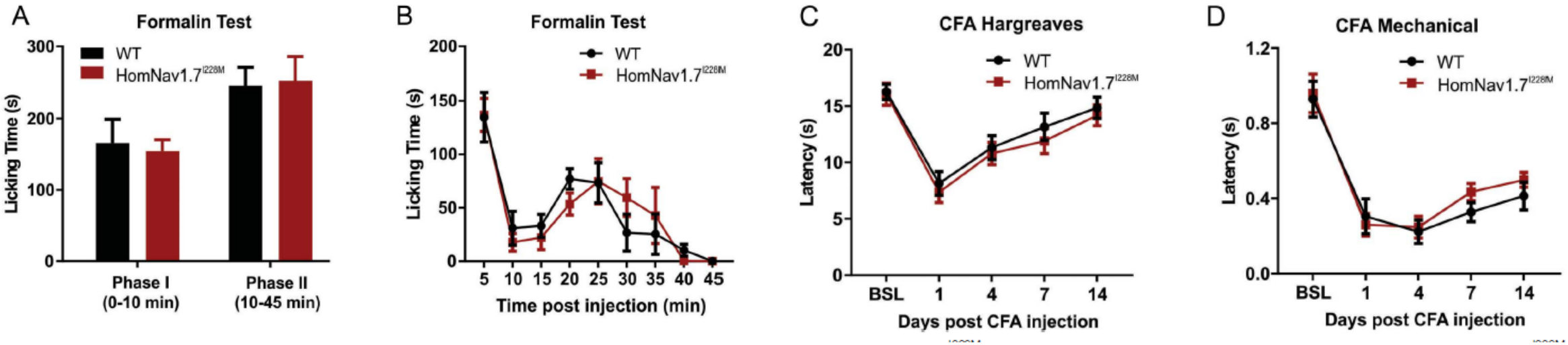




I228M knocking mouse line (CRISPR) do not show thermal or mechanical hypersensitivity



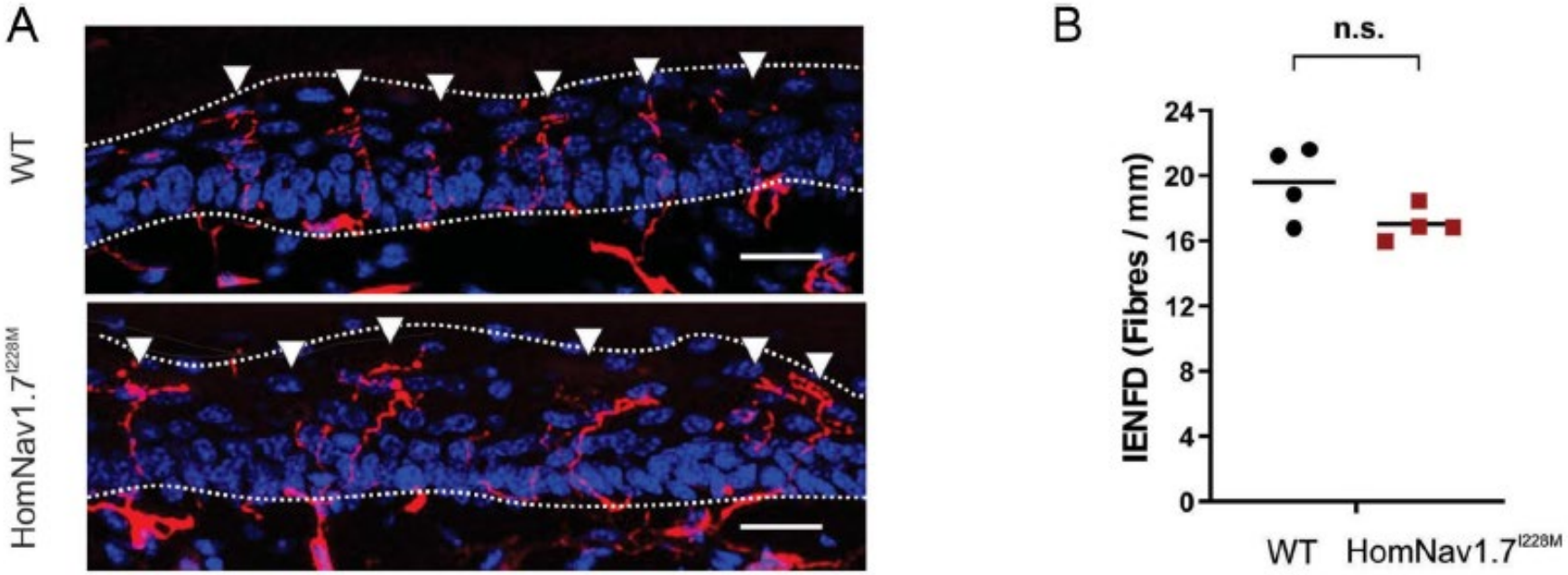
I228M knocking mouse line do not show hypersensitivity inflammation pain



Formalin test:
Subcutaneous injection of 5% formalin into the plantar surface of the left hind paw for induction of **short-term inflammation**

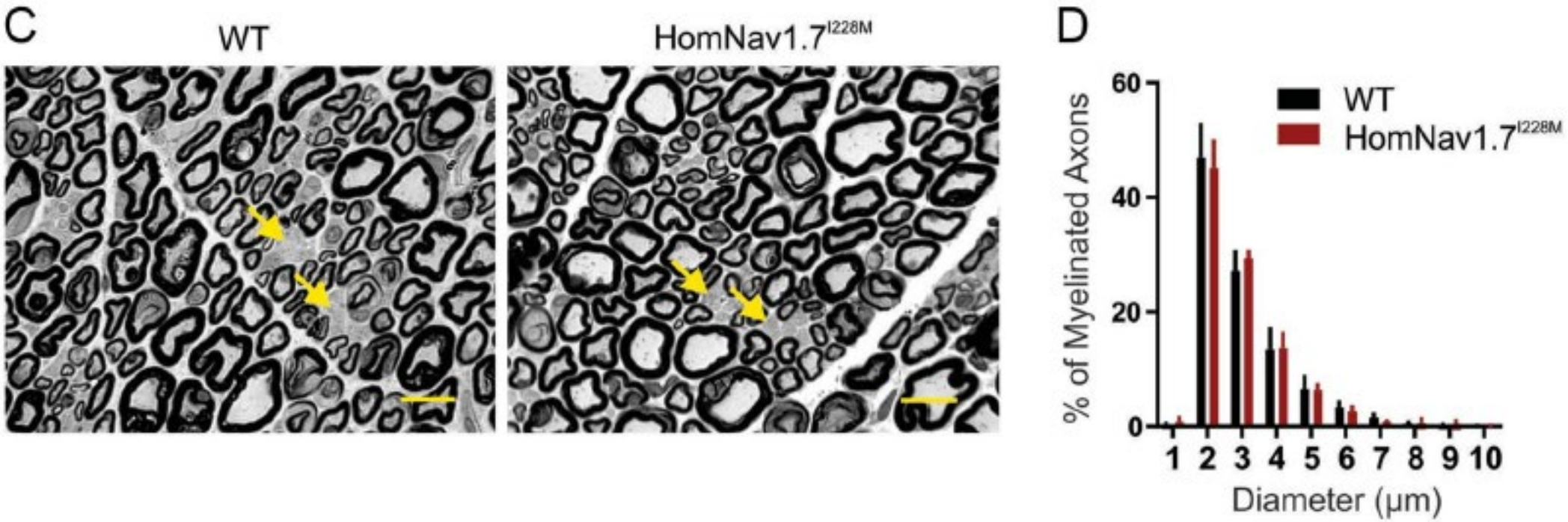
Freund's adjuvant
Injection into the left hind paw for **long-term inflammation**

I228M knocking mouse line (homologous recombination) has a normal IENFD



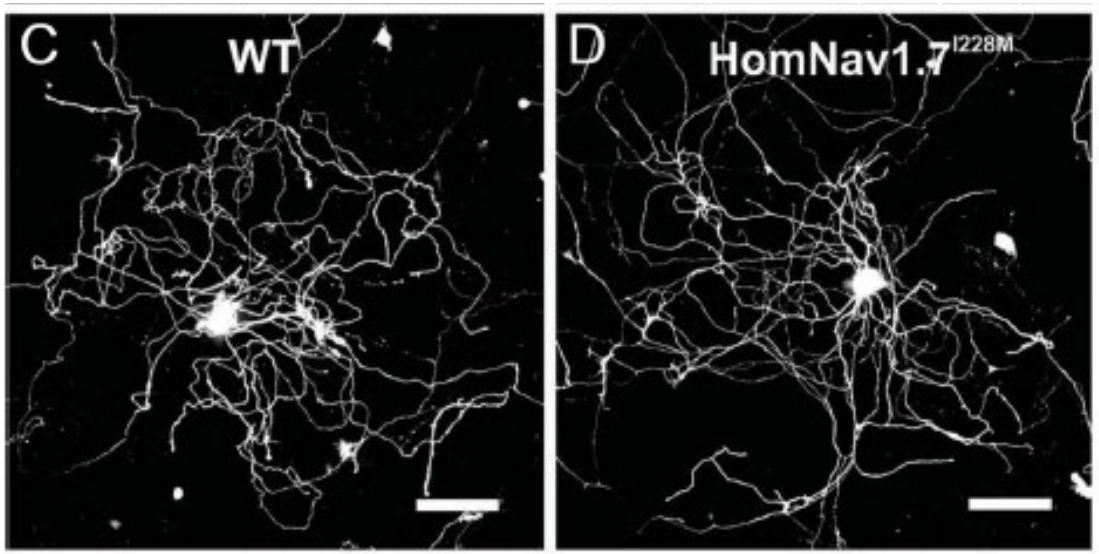
- 12 week old mice
- 3mm Punch biopsy from mice (after trancardial perfusion) from ventral hind paw
- Standard protocol like used for humans (anti-PGP 9.5)

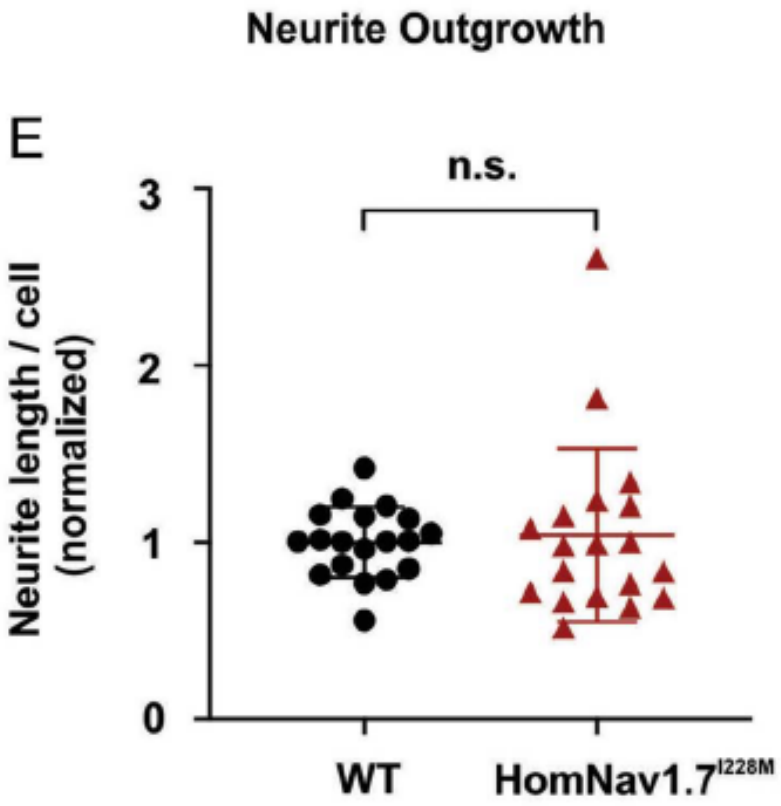
I228M knocking mouse line has a normal nerve fiber morphology



- 12 week old mice
- Micrographs of semithin cross-sections of tibial branch of the sciatic nerve (Epon embedding)
- Arrow: Remak bundles (group of unmyelinated axons)

DRG neurons from I228M knocking mouse (homologous recombination) line demonstrate normal neurite length in vitro





Summary & Conclusions

- DRG neurons from I228M knock-in mice demonstrate an in-vitro phenotype
  (hyper excitable, enhanced response to heat)
- This in-vitro phenotype could not be reproduced by in-vivo tests
  (difficulties in the assessment in mice?)
- No epidermal fiber degeneration (IENFD) was found in the knock-in mice
  (differences in axon length between mice and humans?, age of mice?)
- DRG neurons from I228M knock-in mice demonstrate normal neurite length in-vitro

⟶ Degeneration of DRG neurons seen in in-vitro studies by transient expression was likely caused by overexpression of the mutant channel

⟶Proposal that I228M is an important risk factor for SFN and that a secondary insult or aging is may required to cause a phenotype (including IENFD degeneration) and is may an explanation for the diverse phenotype seen in humans

Thank you for your attention!