Alabama to Beijing… and Back: The Search for a Pain Gene

By Stephen G. Waxman, M.D., Ph.D.

Editor's Note: Our author has adapted a chapter from his forthcoming book, Chasing Men on Fire: The Story of the Search for a Pain Gene, *to provide the fascinating tale of how he and his research team discovered a gene for pain. As he writes, "as exhilarating as it was to indict Nav1.7 as a major player in pain, that was just the beginning of a longer story. That story may change the way we treat pain."*

Pain—"it hurts"—is experienced at some time or another by almost every human being. When pain is transient, it can protect us, warning us to withdraw from a threatening situation. But pain is not always helpful: if it persists when a noxious stimulus is no longer there, it can invade and change a life. This occurs, for example, in people with neuropathic pain due to traumatic nerve injury, peripheral neuropathies associated with diabetes or chemotherapy, and post-herpetic neuralgia. Chronic pain affects more patients than cancer, heart disease, and diabetes combined.[1] And it is often unrelieved or only partially relieved by existing medications, whose side-effects may include double-vision, confusion, sleepiness, or gastrointestinal dysfunction. Some of these drugs can be addictive. There is a clear need for more effective pain medications without these drawbacks.

How can we find such medications? Might a clue lie in the human genome? Two patients with diabetes may both suffer from weakness and muscle atrophy due to peripheral neuropathy. In both, the neurologist's hammer cannot trigger a reflex. But one patient is debilitated by severe neuropathic pain that almost never abates, while the other notices numbness but does not seek medical attention, and goes dancing on the weekend. Might the human genome contain clues about molecules within our bodies that make the difference? Might there be a gene for a master-switch that turns pain on or off?

Such questions lead us to the search for families in pain. Rare familial diseases may direct researchers toward therapies effective for common illnesses in "the rest of us." Consider the statin medications, which control lipid levels within our blood, reducing the risk of heart attack. Crucial to their development was the discovery of rare families with inherited hypercholesterolemia. Mutations in specific genes in these families pointed to the culprit molecules, which became the target of new medications that have effectively lowered the incidence of heart disease in the broad general population.

Peripheral Generators of Pain

To cure chronic pain, we need to understand where it comes from. Throughout our bodies nerve fibers, issuing from pain-signaling nerve cells in ganglia next to the spinal cord serve as sentries and act as an early-warning system. Pain-signaling dorsal root ganglion (DRG) neurons send peripheral axons to the body surface, gut, bladder, and organs, where they can detect threatening mechanical stimuli such as a pinprick or a blow from a hammer, damaging levels of heat or cold, and irritants such as acid; they send central axons into the spinal cord (Figure 1). Trigeminal ganglion neurons serve the same function for the teeth, eyes, and face. These "first-order" pain-sensing neurons signal the presence of such threats by sending nerve impulses to the spinal cord. Within the spinal cord, these impulses excite "second-order" neurons that relay the signal to the brain, where it elicits the experience of pain. It is important to remember that the process of pain signaling begins in the periphery.

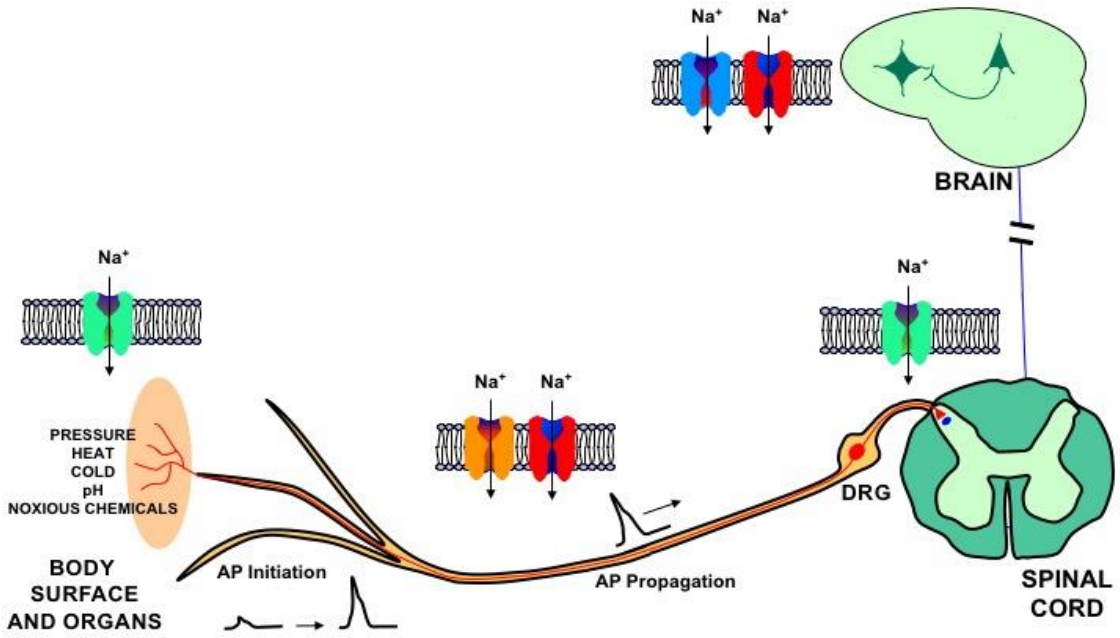

Figure 1. Dorsal root ganglion (DRG) neurons, with cell bodies within the dorsal root ganglia next to the spinal cord, extend an axon from the body surface and organs, all the way into the spinal cord. Sodium channels within the cell membrane of DRG neurons enable them to produce action potentials (AP). Pain-signaling DRG neurons are excited by injurious levels of pressure, heat, cold, acidity (pH), or irritating chemicals and, in response, send action potentials to the spinal cord which relays them to the brain. Multiple types of sodium channels, shown in orange, red, and green, participate in this signaling. $Na_V1.7$ channels (green) play a particularly crucial role, amplifying small stimuli in the periphery and thereby setting the gain on DRG neurons, and facilitate impulse transmission close to the spinal cord. Modified from Waxman and Zamponi (2014).

In the absence of threatening stimuli, pain-signaling peripheral neurons are normally quiet, and there is no pain. But in some disease states they generate too many impulses in response to a mild stimulus, or generate pain signals even when a noxious stimulus is not there. The result is a sensation of being burned when there is no hot object touching the body, or of sticking pain when there is no pointy object injuring the body. Figure 2 shows the abnormal repetitive nerve impulses generated by a hyper excitable axon within the sciatic nerve of a rat, one-year after a nerve injury. The staccato—machine-gun-like-firing— was evoked by a small stimulus that should have elicited only a single impulse.[2] Here, we see neuropathic pain in the making, as peripheral neurons scream while they should be silent. We see similar axon hyperactivity in human subjects with painful peripheral neuropathy.[3] What produces the abnormal firing?

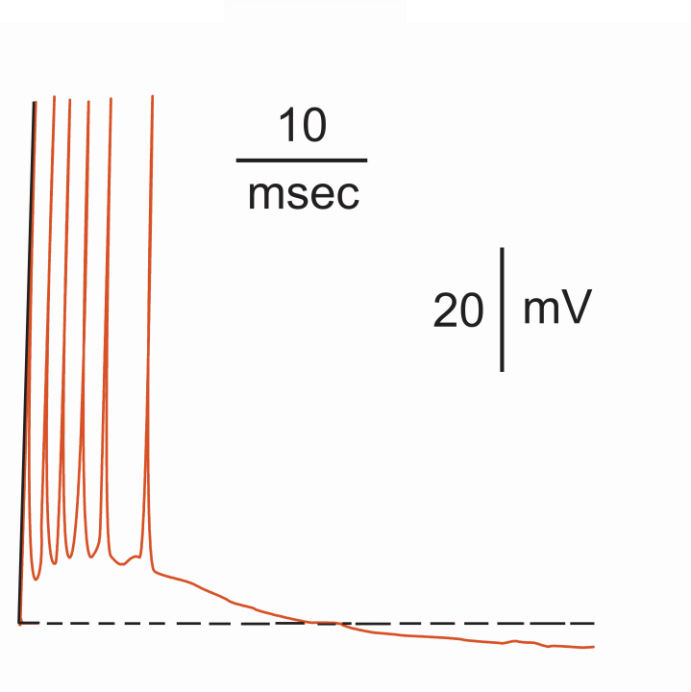

Figure 2. Inappropriate repetitive firing of action potentials, recorded with a microelectrode placed within a single axon within the sciatic nerve of a rat that had received a nerve injury one year previously. The aberrant repetitive action potentials sit upon an abnormal depolarization of the axon membrane which suggests abnormal sodium channel activity. From Kocsis and Waxman (1983).

Peripheral Sodium Channels – A Holy Grail

Anyone who has gone to a dentist knows that pain-signaling nerves can be silenced with certain medications—a nerve can be put to sleep so that pain within its territory will not be felt, after it is injected with a local anesthetic like Novocaine. These drugs block sodium channels, the specialized protein molecules within the cell membrane of neurons that produce nerve impulses. The blockade

of sodium channels prevents the generation of electrical impulses in nerve fibers that innervate the teeth and oral cavity.

Given the remarkable efficacy of sodium channel blockers when locally injected, it might have been hoped that they could be used more broadly to alleviate pain when taken by mouth. Indeed, some sodium channel blocking drugs can be taken orally. However, these drugs have limited effectiveness for the systemic treatment of pain because they can also block sodium channels within the brain. At doses sufficient to relieve pain, they produce side-effects that may include confusion, loss of balance, double-vision, and sleepiness. Thus, a major question in pain research was whether it might be possible to selectively block sodium channels in peripheral neurons, sparing those within the brain.

When I was a student, we learned about "the" sodium channel, and little was known about its biochemical makeup or structure. As a result of the molecular revolution, we now know that sodium channels are large protein molecules, strings of around 1800 amino acids folded into a barrel-like structure. In the mid-1980s, molecular cloning began to show that multiple genes encode multiple sodium channels, all sharing a similar overall molecular structure but with slightly different amino acid sequences and different physiological and pharmacological properties. Driven by the need for better pain medications, a hunt began for sodium channels that play important roles in peripheral nerve cells, particularly pain-signaling DRG and trigeminal ganglion neurons, but not in the brain. The logic was that, if such "peripheral" channels existed, it might be possible to develop focused medications that would selectively block them and mute the activity of pain-signaling neurons without a significant effect on neurons within the brain. This would provide pain relief without side-effects and without addiction potential. Peripheral sodium channel blockers became a "holy grail" of pain research.

Between 1996 and 1999 three different peripheral sodium channels, now called $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$, were identified in rodent DRG neurons.[4-9] In 1997, electrophysiologist Ted Cummins and I assessed the properties of sodium channel $Na_V1.7$ (at that time called hNE).[10] We showed that $Na_V1.7$ channels play a pivotal role in the response to small stimuli, bringing the DRG neuron closer to the potential needed to activate other types of sodium channels, which then produce the nerve

impulses that signal the presence of painful stimuli.[11,12] These early studies showed us that $Na_V1.7$ serves as a "volume knob" that regulates signal strength on peripheral nerve cells isolated from laboratory animals such as rats. We did not know, when we did these initial studies, that they would set the stage for a subsequent demonstration that $Na_V1.7$ is a master switch for *human* pain.

The Search for a Pain Gene

The search for the pain gene began in an Alabama neighborhood. There was something unusual here. Many of the people did not wear shoes. Some wore open-toed sandals, while others preferred to walk barefoot on a cool tile floor or in cold water that collected in puddles. The children avoided the playground and sometimes missed school. And if you spent time with them, you might see one of these people grimace. When asked, they would say that their feet, and sometimes their hands, felt as if they were on fire.

The pain in these people was excruciating. And it was unusual in another way: It was present in, and only in, members of one large, extended family. Five generations were known to have this mysterious disorder, about half of the individuals in each generation. Doctors were baffled; some even wondered whether it was a physical disorder at all, or was it "in the mind."

We now understand that the pain was not imagined; we know that this family suffers from the "man on fire" syndrome, a disorder whose medical name is erythromelalgia. The condition is incredibly uncommon, and inherited erythromelalgia is even more rare—just ten percent of cases occur on a familial basis. Most physicians will never see a case. But once a physician has seen one, it will remain in his or her memory.

Attacks of erythromelalgia, in which the limbs become severely painful and red, are triggered by mild warmth—putting on shoes or socks, entering a warm room, or mild exercise—and described with terms like "being on fire," or "feeling like hot lava has been poured into my body." Existing medications are at best only partially helpful. A picture prepared by a person with erythromelalgia (see illustration at beginning of article, submitted by Jennifer Beech to The Erythromelalgia Association for their 2012 art contest) depicts the pain better than any words.

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The human body is made up of diverse types of cells—skin cells, muscle cells, kidney cells, and nerve cells. Cells, in turn, contain protein molecules. Proteins are strings of amino acids, of which there are 20 varieties in humans. To form a functional protein molecule, the correct amino acids must line up, in the correct order, and the string must be folded into the right conformation. Imagine scrunching up a string of colored beads, so that bead number 101 lies next to bead number 148, and bead 160 touches bead 194, and so on. With one bead of the wrong color—one wrong amino acid—or one bead missing, the string may not fold into the needed three-dimensional structure and the protein may not work properly. That is what happens in sickle-cell anemia, where one of 146 amino acids is replaced by another, incorrect amino acid within the β-globin component of hemoglobin.

The blueprint for human proteins is contained in the 20,000 genes within the human genome. The gene for each protein encodes the amino acids, in precise sequence, that make it up. Genes are made of DNA, which consists of two strands composed of smaller units called nucleotides, coiled into a double helix. For their discovery of DNA's double helix configuration, James Watson, Francis Crick, and Maurice Wilkins were awarded the 1962 Nobel Prize. The four types of nucleotides within the DNA, labeled A, T, G, and C, are the letters of the genome's alphabet. Since there are 20 amino acids and only four nucleotides, a series of three nucleotides is needed to encode each amino acid. In 1961, Marshall Nirenbirg, in a first step toward breaking the code, showed that the triplet TTT encodes the amino acid phenylamine. Severo Ochoa went on to identify the DNA codes for all 20 amino acids. By the mid-1960s it was clear, for example, that ATG codes for the amino acid methionine, that CCA codes for proline, and that GC, followed by any of the four nucleotides, codes for alanine. And so forth for each amino acid. So a stretch of protein made of up methionine, then proline, and then alanine would be encoded:

ATGCCAGCT

This was the "Rosetta Stone" of genetics. By knowing the sequence of nucleotides within a gene, one could infer the sequence of amino acids within its protein.

Medical science moves forward in waves, and multiple waves can propel research on several fronts at the same time. This seems to have been true for the "man on fire syndrome." Just as these advances in understanding DNA were occurring, physicians were beginning to recognize that this disorder might be caused by an abnormality hidden within the genome.

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In 1965, a mother in the Alabama family took her young daughter, with burning feet, to the Mayo Clinic in Rochester Minnesota. It must have taken immense effort to arrange for a consultation so far away. The visit was worth the effort.

Mayo physician Mahlon Burbank recognized the girl's disorder and appreciated that, in this case, it was hereditary. So the Alabama family learned that their condition was very unusual and had a name, "familial erythromelalgia." In a brief paper, Burbank noted that he had assessed "a family in which 19 out of 51 family members, comprising five generations, have typical erythromelalgia. The disorder in this family is inherited as a dominant trait."[13] It thus became clear that this family's pain arose in the genes, not in the mind.

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More than 20 years later, in 1986, a pediatrician saw another young girl in the same Alabama family, and suggested that the child see Wayne Finley, M.D., a medical geneticist at the University of Alabama-Birmingham. Finley read the 1966 Burbank paper, contacted the author, and presciently decided to create a family tree.

The first full-length article on the Alabama family was published in 1992 by Finley, Burbank, and three other authors.[14] The paper described the pedigree of the Alabama family and posited that the disease "may be an autosomal dominant trait." Noting that "additional families must be studied," Finley concluded that "the mechanism for initiation of pain is not yet known." Indeed, the methods

were not yet available to reveal that mutations in one particular gene were the cause of inherited erythromelalgia, not just in this family but in families with the disease around the world.

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A large family, containing multiple individuals with a rare disorder, presents an opportunity for medical researchers. Which gene, out of the thousands in the human genome, might be responsible? And what has gone wrong within that gene to cause disease? There was a lot of territory to cover and a first step was to narrow the field using "linkage analysis."

Linkage analysis takes advantage of the presence within genes of "single nucleotide polymorphisms" or SNPs. Each SNP is a minor variation in DNA, the substitution of a nucleotide for the one present in the majority of the population. SNPs include relatively inconsequential "mis-weaves" that do not cause disease. However, they tend to be inherited, like mutations, and a SNP on the same gene as a mutation, especially if it is located close to it, tends to be inherited along with, or "linked to," the mutation. It is relatively straightforward to map the SNPs within a family; if a polymorphism is present in all affected members and none of the non-affected members, it suggests that the gene containing the SNP is the site of the mutation. Linkage analysis can even point to a specific region within a gene.

Linkage analysis depends on probabilities: If a polymorphism occurs in just one affected family member, its apparent association with the disease in question could be a random event, not indicative of causation. But when *multiple* affected family members—the more, the better—show the same pattern, the probability of random association goes down, and the probability of having found the culprit gene goes up.

The Alabama family was nearly ideal for linkage analysis: a disease with a dramatic, easily recognized clinical presentation, and a large number of affected family members in multiple generations. It is not surprising that researchers wanted to study that family.

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Well before erythromelalgia was recognized as a genetic disorder, Dutch medical student Joost Drenth, together with his professor, Jan Michiels,[15,16] had revised the diagnostic classification of its various forms. By the mid-1990s Drenth, trained in medical genetics in Paris and now a professor of internal medicine, was searching for a family with erythromelalgia, the larger the better. In 1999, he contacted Michiels who had located the Alabama family. Later that year, Drenth began a linkage analysis, which located the erythromelalgia susceptibility gene on chromosome 2, a large chromosome containing nearly 1500 genes.[17] Further, the results placed the gene between two markers within a small region of the gene, about three percent of its total size. Sodium channel genes had not yet been mapped to that region, so these investigators could not know that their findings pointed toward a sodium channel mutation. Nevertheless, their result was important: it limited the search to 50 of the 20,000 human genes. The search for the pain gene no longer resembled finding a needle in a haystack: it could focus on a small but still formidable tangle of hay.

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Sometime after 1999, the search moved to Beijing, where dermatologist Yong Yang was evaluating a Chinese family containing three generations of patients with burning pain and redness in their hands and feet, evoked by warmth and relieved by cooling.

As with the Alabama family, linkage analysis was a first step. And once again, it pointed to a small part of chromosome 2. By now, however, researchers knew that this region contained a cluster of sodium channel genes, including *SCN9A*, the gene that encoded sodium channel $Na_V1.7$. Every gene within this part of chromosome 2 was a potential culprit, but there was something special about *SCN9A*: its protein, $Na_V1.7$, is present at high levels within pain-signaling DRG neurons.

Together with his colleagues in Beijing, Yang began the task of sequencing the *SCN9A* gene from this family. The results showed that, within the mutant gene, one nucleotide had been changed:

AAC CTC ACC

had been changed to:

AAC CAC ACC

This change substitutes the amino acid histadine for leucine at position 858 within the mutant $Na_V1.7$ channel. The same leucine-to-histidine (L858H) change was found in other affected individuals within the family, but not in unaffected family members. And analysis of another patient with a similar but non-familial pain syndrome revealed another mutation, isoleucine-to-threonine (I848T) nearby in the same gene. These were major findings, an advance that pointed toward *SCN9A* as the culprit gene. But without knowing how the mutation affected the function of $Na_V1.7$, the analysis suggested, but did not prove that the mutations caused the disease.

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Yong Yang's paper[18] appeared in March 2004 in the *Journal of Medical Genetics*. My team and I at Yale University in New Haven had begun, in 2002, to search for families with inherited pain. When I saw the title of the article, I initially thought we had been scooped. I told my team that it was a bad day and retreated to my office.

It was only after a cup of coffee and a closer look at the article that I realized what had happened. Yong Yang and his colleagues had indeed identified mutations in the *SCN9A* gene in inherited erythromelalgia—an important step forward. But finding mutations did not prove that they caused these patients' pain. Appropriately, Yang ended his paper with the conclusion, "Mutations in *SCN9A* may cause primary erythermalgia [an alternative spelling of erythromelalgia]." More work was needed to move from "*may* cause" to "*do* cause."

Yang and his colleagues were dermatologists and not neuroscientists. Understandably, they did not ask the questions that a neuroscientist would have asked. Now it was time for our team to ask these questions. Some mutations produce amino acid substitutions that perturb channel function and cause disease. But other mutations do neither. When a neuroscientist encounters a mutation of a sodium channel, it triggers the questions: does the mutation change the way the channel works?

If so, how? If the mutation changes the channel's functional characteristics, do nerve cells carrying the mutation function in an abnormal way? And finally, if the mutation produces changes in nerve cell function, can these changes explain the disease? We were in a position to answer these questions—to generate a "functional profile" of the mutant channel that might link it to disease. What had appeared to be a bad day was, in fact, a good day. My colleagues reassembled in my office. There was a lot of work to do.

We rapidly planned the "must do" experiments. Each mutation replaced an amino acid in a part of the channel called the "S4-S5 linker"—a kind of hinge connecting the voltage-sensor with the channel pore, which must open to produce an electrical current. We needed, therefore, to assess the effects of the mutation on gating—opening and closing—of the channel. Serendipity now came into play. Five years earlier, we had carried out a detailed biophysical analysis of the "wild-type" or normal Nav1.7 channel.[10] So, we already had a high-fidelity understanding of the behavior of the normal Nav1.7 channel. We had the *SCN9A* gene in our freezer, and we had a powerful tool box for studying $Na_V1.7$. We could insert the gene for the normal channel into cells that do not normally contain any sodium channels, and study the gating of the channel in this electrically quiet background. And we could do the same thing with mutant $Na_V1.7$ channels.

Sulayman Dib-Hajj, a master molecular biologist with a unique knack for manipulating large genes, took only a few months to create mutant *SCN9A*. We then inserted the DNA into cells in tissue culture to determine the effect of the mutations on the function of Nav1.7 channels. The results were dramatic. Both mutations hyperpolarized channel activation, making it easier to turn the channels on. Both mutations also slowed the deactivation process whereby the channel closes. And, both mutations enhanced the amplifying effect of the channel on small depolarizing stimuli. This was like turning the volume up too high on a hearing aid.

We excitedly discussed the findings as they came in from our recording rigs, using terms like "trifecta." Our experiments established, at the channel level, three pro-excitatory effects of the L858H and I848T mutations, each of which made the channel hyperactive. Now we had some evidence for a role of the mutant Nav1.7 channels in producing pain. We published these findings in late 2004.[19]

Our knowledge that the mutant $Na_V1.7$ channels were hyperactive brought us close to proof that the mutations caused the man on fire syndrome. But to make a conclusive case we needed to ascertain the effect of the mutant channels on pain-signaling DRG neurons. To do this, we needed to insert the mutant gene into these cells, let the cells grow in tissue culture, and then record their electrical activity in response to precisely calibrated stimuli. Because this analysis would require a large number of measurements, we asked: "which mutation will teach us the most?" This brought us back to the Alabama family.

After getting approval from Yale's human studies committee, I sent a team to Alabama. We sequenced DNA from 17 affected family members, and found a new $Na_V1.7$ mutation in all of them. None of five unaffected members carried the mutation. The large number of DNA samples was in itself important: With 22 samples, the likelihood of a "false positive" was small. To be sure, however, we needed to show that mutant channels made pain-signaling neurons hyperactive.

This mutation, F1449V, replaced a phenylalanine with a valine in another functionally important part of the channel.[20] As with the L858H and I848T mutations, our experiments showed that F1449V hyperpolarized activation. We now needed to determine whether the mutation changed the firing properties of pain-signaling neurons and, if so, whether this shifted the activity of these nerve cells in the appropriate direction. Two electrophysiologists and two technicians worked in tandem as we moved toward an answer, assessing the effect of the mutant channels, cell-by-cell, until finally there was enough data. Our laboratory buzzed with conversation as they announced their findings: the mutant F1449V channels lowered the threshold for firing of DRG neurons, permitting a smaller stimulus to generate a nerve impulse. And at any given stimulation level, the frequency of firing was much higher in these neurons:[20] as result of the mutation, pain-signaling DRG neurons were more likely to fire. And when they fired, it was at abnormally high frequencies (Figure 3). Like traumatically injured DRG neurons that produce neuropathic pain, DRG neurons carrying the mutant Nav1.7 channel generated pain signals when they should have been silent. We now had a convincing link between Nav1.7 and pain.

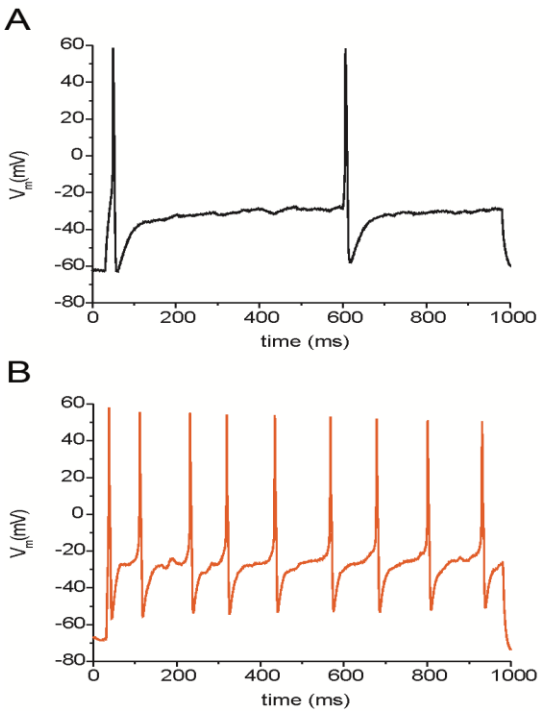

Figure 3. Current-clamp recordings from rat DRG neurons transfected with wild-type (normal) $Na_V1.7$ sodium channels (A) and with F1449V mutant $Na_V1.7$ channels. (B) The F1449V mutation is present in all affected individuals within the Alabama family. Note the abnormal high-frequency firing produced by the mutant channels. From Dib-Hajj et al., (2005)

From Burbank's 1966 observations on members of the Alabama family and the study by Finley in 1992, both of which suggested that the man on fire syndrome is a genetic disorder, it had taken until 2002 for Drenth to use linkage analysis to point to a particular part of one chromosome, containing about 50 genes. It took two more years for the story to move to Beijing, where the first erythromelalgia mutations were identified, and then to New Haven, where we showed how these mutations make the $Na_V1.7$ channel hyperactive. One year later, focusing again on the Alabama family, we showed how these mutations cause DRG neurons to scream when they should be whispering, closing the loop to pain. A gene for pain had been found and its role in disease uncovered.

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As exhilarating as it was to indict $Na_V1.7$ as a major player in pain, that was just the beginning of a longer story. That story may change the way we treat pain. Following the discovery that gain-of-function mutations of $Na_V1.7$ produce pain, mutations that abolish activity of $Na_V1.7$ were found, and were shown to produce total insensitivity to pain. Over the ensuing years, additional mutations

in $Na_V1.7$ taught us important lessons about pain and its molecular underpinnings. The biotech sector launched efforts to develop agents that block $Na_V1.7$, and these $Na_V1.7$ blockers have begun to enter clinical trials. While there is a lot more work to do, the initial clinical studies have provided hints that $Na_V1.7$ blockers may provide non-addictive pain relief.[21,22] And based on the early studies on $Na_V1.7$, attempts at "personalized," genomically-guided pharmacotherapy for pain[23,24] have moved forward. That longer story is told in my book, *Chasing Men on Fire: The Story of the Search for a Pain Gene.*[25]

Bio

Stephen Waxman, M.D., Ph.D. is the Flaherty Professor of Neurology, Neurobiology, and Pharmacology at Yale University, where he was chairman of neurology from 1986 to 2009. He founded Yale's Neuroscience & Regeneration Research Center in 1988 and is its director. He previously worked at Harvard, MIT, and Stanford, and is visiting professor at University College London. Waxman's research defined the ion channel architecture of axons and its importance for axonal conduction. He demonstrated sodium channel plasticity in demyelinated axons which supports remission in multiple sclerosis. His molecule-to-man studies combining molecular genetics, molecular biology, and biophysics have illuminated the contribution of ion channels to human pain. A new class of medications for pain, based partly on his work, has entered clinical trials. A member of the National Academy of Medicine, Waxman's honors include the Dystel Prize and Wartenberg Award, Middleton Award and Magnuson Award, and the Soriano Award. He was honored in Great Britain with The Physiological Society's Annual Prize, an accolade he shares with his heroes Andrew Huxley, John Eccles, and Alan Hodgkin.

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