S-palmitoylation of the sodium channel Nav1.6 regulates its activity and neuronal excitability

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Abstract

S-Palmitoylation is a reversible post-translational lipid modification that dynamically regulates protein functions. Voltage-gated sodium channels are subjected to S-palmitoylation and exhibit altered functions in different S-palmitoylation states. Our aim was to investigate whether and how S-palmitoylation regulates Nav1.6 channel function, and to identify S-palmitoylation sites that can potentially be pharmacologically targeted. Acyl-biotin exchange assay showed that Nav1.6 is modified by S-palmitoylation in the mouse brain and in a Nav1.6 stable HEK 293 cell line. Using whole-cell voltage clamp, we discovered that enhancing S-palmitoylation with palmitic acid increases Nav1.6 current, while blocking S-palmitoylation with 2-Br-palmitate reduces Nav1.6 current and shifts the steady state inactivation in the hyperpolarizing direction. Three S-palmitoylation sites (C1169, C1170, C1978) were identified. These differentially modulate distinct Nav1.6 properties. Interestingly, C1978 is exclusive to Nav1.6 among all Nav isoforms and is evolutionally conserved in Nav1.6 among most species. C1978 S-palmitoylation regulates current amplitude uniquely in Nav1.6. Furthermore, we showed that eliminating S-palmitoylation at specific sites alters Nav1.6-mediated excitability in dorsal root ganglion neurons. Therefore, our study reveals S-palmitoylation as a potential isoform-specific mechanism to modulate Nav activity and neuronal excitability in physiological and diseased conditions.

Introduction

Excitability of a neuron is determined by the composition, distribution, and properties of ion channels in its plasma membrane. It can be profoundly changed by slight alteration in the biophysics of a channel. The voltage-gated sodium channel (Nav) family plays crucial roles in the initiation and propagation of action potentials. The 9 Nav isoforms (Nav1.1 - Nav1.9) have distinct biophysical properties and differential cellular and subcellular distribution [1]. They are responsible for the electrical signal transduction in a wide range of physiological processes. Abnormal or dysregulated Nav activity can lead to varied diseased conditions including epilepsy, pain, autism, cardiac arrhythmia, and skeletal muscle disorders. Therefore, Navs have long been therapeutically desirable targets in the drug discovery industry. However, the high sequence and structural conservation shared among the Navs have posed a significant challenge to the development of isoform-specific therapeutics and modulators with minimum side effects.

Navs consist of four domains (DI-DIV), with each containing six transmembrane segments (S1-S6) (Fig. 1). S1-S4 comprise the voltage sensors, while the S5-S6 regions combine to form the sodium conducting pore. These regions are critical for normal voltage sensing and sodium ion selectivity. They are highly conserved among Nav isoforms. The relatively less conserved extracellular loops are frequent binding sites for peptidic gating...
modifier toxins like conotoxins, protoxins and huwentoxins [2, 3]. These sites present some degree of isoform specificity, and thus have attracted tremendous pharmacological interest over the past two decades [1, 4]. The intracellular loops and cytosolic N- and C-terminal domains are heavily involved in protein-protein interactions and are subject to extensive post-translational modifications [5]. Importantly, they contain highly variable regions. Therefore, understanding the important roles of the Nav intracellular loops and their post-translational modifications may identify new strategies to help target Nav’s with improved specificity.

In recent years, S-palmitoylation has emerged as an important mechanism that regulates protein functions. It is a reversible post-translational modification that covalently attaches palmitate, a 16 carbon saturated fatty acid, to cysteine thiols in a protein via thioester bonds. Since S-palmitoylation alters protein hydrophobicity, it has the potential to impose significant structural and thus functional changes on proteins. It can regulate membrane association, trafficking, conformation, and protein-protein interactions of a diverse range of proteins [6-8]. It also plays crucial roles in ion channel regulation [5, 9-14]. It has been shown that S-palmitoylation substantially modulates cardiac excitability by modifying the cardiac sodium channel Nav1.5 [9], and that manipulating S-palmitoylation status of Nav1.2 can alter its voltage dependence and toxin sensitivity [10]. However, it is unclear whether Nav1.6 is modified by S-palmitoylation. To test this, we performed acyl-biotin exchange (ABE) assay on proteins extracted from the mouse cerebellum due to its enriched expression of Nav1.6 as well as the human embryonic kidney (HEK) 293 cell line stably expressing Nav1.6. Fig. 2A shows that Nav1.6 is S-palmitoylated in the mouse brain and the Nav1.6 cell line.

2) S-palmitoylation modulates Nav1.6 current amplitude and voltage-dependence of inactivation.

Next we explored how S-palmitoylation regulates Nav1.6 channel function. Due to the difficulty of achieving satisfactory level of Nav1.6 expression in the HEK 293 cell line with heterologous cDNA transfection, we used the ND7/23 cells, a hybrid cell line of mouse neuroblastoma fused with rat dorsal root ganglia neurons [21], as our expression system for Nav1.6 transient transfection and voltage clamp recordings. Since ND7/23 cells do not express tetrodotoxin resistant (TTXr) sodium channels [22], the Nav1.6 construct was rendered resistant to TTX (Nav1.6r) by a Y371S substitution [23] and 500nM TTX was added to the extracellular solution for patch clamp recordings to isolate Nav1.6r currents. To manipulate the S-palmitoylation status of the Nav1.6r channel, we incubated the transfected cells for overnight with either 25 μM 2-Br-palmitate (2BP) to block S-palmitoylation or 10 μM palmitic acid (PA) to enhance S-
palmitoylation. These concentrations of 2BP and PA were determined previously for optimal S-palmitoylation blockage and enhancement [9] and has been verified that these treatments manipulate protein S-palmitoylation status in our expression system as expected (Fig. 3H).

Fig. 2B shows the representative traces of Nav1.6 currents from each treatment group. We found that blocking S-palmitoylation with 2BP reduces Nav1.6 current by 67% (2BP: -26.30 ± 3.29 pA/pF vs. DMSO: -80.16 ± 8.30 pA/pF; P < 0.0001), while increasing S-palmitoylation substrate availability with PA treatment enhances Nav1.6 current by 78% (PA: -142.4 ± 17.27 pA/pF; P = 0.0021 PA vs. DMSO) (Fig. 2C). Notably, the current density in the PA group is more than five-fold of the 2BP group. To eliminate the possibility that these bidirectional effects were mediated by different mechanisms, transfected cells were simultaneously treated with 25 μM 2BP and 10 μM PA. The current density of the co-treated cells (-32.12 ± 7.43 pA/pF) is similar to that of the 2BP group (P = 0.3365 2BP + PA vs. 2BP) (Fig. 2C), suggesting that the current density effects produced by the separate 2BP and PA treatments are very likely mediated by S-palmitoylation. These data support the contention that 2BP effectively blocks S-palmitoylation in our expression system, although excessive S-palmitoylation substrate was provided to bias the palmitoylation – depalmitoylation equilibrium.

We next examined whether S-palmitoylation affects the voltage dependence of Nav1.6. We observed that blocking S-palmitoylation with 2BP causes an 8mV hyperpolarizing shift of steady-state inactivation (2BP: V1/2 = -72.34 ± 0.28 mV vs. DMSO: V1/2 = -64.46 ± 0.21 mV; P < 0.0001), similar to the modulation observed with Nav1.2 [10] and Nav1.5 [9]. Moreover, blocking S-palmitoylation resulted in a slower recovery from inactivation (Fig. 2E, 2BP: τ = 13.18 ± 1.28 ms vs. DMSO: τ = 9.84 ± 0.76 ms; P = 0.0246).

On the other hand, increasing S-palmitoylation substrate availability with PA treatment does not alter Nav1.6 voltage dependence of activation, steady-state inactivation, or recovery from inactivation. The lack of modulation by PA treatment may be due to saturation of S-palmitoylation at the site(s) regulating voltage dependence of Nav1.6 in our expression system. Thus, providing excessive S-palmitoylation substrate does not achieve appreciable alteration in channel function. In contrast, S-palmitoylation at the site(s) that regulate Nav1.6 current amplitude (Fig. 2B,C) are likely not saturated, demonstrated by the opposing effects of 2BP and PA treatments. Therefore, our data suggest that S-palmitoylation regulates distinct functional properties of Nav1.6, potentially by modifying different S-palmitoylation sites in the channel.

3) S-palmitoylation of Nav1.6 at C1169, C1170 regulates voltage-dependence of steady state inactivation.

To identify S-palmitoylation sites responsible for the observed functional modulations in Nav1.6, we used CSS-Palm [24] for potential site prediction. In Nav1.6, two cysteines (C1169, C1170) located near the AnkG binding motif (1094-1102) in loop2 were predicted to be S-palmitoylated (Fig. 1). These two cysteines are highly conserved among human Nav isoforms (Fig. 3A), yet their functions vary: depalmitoylation of the first cysteine in Nav1.2 (C1182) was reported to enhance Nav1.2 inactivation (Bosmans, 2011), while the homologous cysteine in Nav1.5 (C1178) was not involved in such modulation (Pei, 2016). This indicates potential isoform-specific functionality of S-palmitoylation. We were interested to test whether S-palmitoylation of the homologous cysteines in Nav1.6 regulate channel function and how it might differ from Nav1.2 and Nav1.5. For this purpose, we eliminated S-palmitoylation at C1169 and C1170 by mutating both to non-palmitoylatable alanines (Nav1.6-CCAA) and evaluated the functional consequences. Fig. 3B shows the representative traces of TTXr Nav1.6-CCAA current from each treatment group. Compared to Nav1.6-WT, Nav1.6-CCAA displays an 11mV hyperpolarizing shift in voltage dependence of steady-state inactivation (V1/2 = -75.5 ± 0.39 mV; P < 0.0001) (Fig. 3D). This replicates the voltage dependence modulation observed in Nav1.6-WT with S-palmitoylation blockage, suggesting that S-palmitoylation at these two cysteines is critical for voltage dependence regulation of Nav1.6. However, the Nav1.6-CCAA channel does not completely lose its sensitivity to 2BP treatment and has a minor hyperpolarizing shift in steady-state inactivation (V1/2 = -80.48 ± 0.30 mV; P = 0.0046) (Fig. 3E). Consistent with the WT channel, increasing S-palmitoylation substrate availability with PA treatment does not alter the voltage dependence of activation, steady-state inactivation (Fig. 3E), or recovery from inactivation (Fig. 3F) of Nav1.6-CCAA, while blocking S-palmitoylation with 2BP
treatment results in slower recovery from inactivation (2BP: $\tau = 12.69 \pm 0.69$ ms; DMSO: $\tau = 10.70 \pm 0.38$ ms; $P = 0.0114$). Interestingly, Nav1.6-CCAA exhibits the same bidirectional current density response to 2BP and PA treatments as the WT channel (Fig. 3C), indicating that C1169 and C1170 do not contribute to regulating Nav1.6 current amplitude.

To biochemically confirm S-palmitoylation occurs at C1169 and C1170, we designed a CD4-Nav1.6-Loop2 fusion protein for use in the ABE assay due to low expression level of Nav1.6 in the heterologous expression system (Fig. 3G). Using this construct, we observed that the S-palmitoylation signal of the CD4-Loop2 fusion protein can be enhanced by PA treatment and reduced by 2BP treatment in our expression system (Fig. 3H) and the signals are within the linear range of detection (Fig. S1A). Moreover, eliminating S-palmitoylation at the cysteines corresponding to C1169 and C1170 in Nav1.6 (Loop2-CCAA) greatly reduces the S-palmitoylation signal compared to the WT fusion protein, suggesting that C1169 and C1170 are major S-palmitoylation sites in Nav1.6-Loop2. However, S-palmitoylation is not completely abolished in Loop2-CCAA. This may indicate additional S-palmitoylation sites in the fusion protein. Intriguingly, C1163, a cysteine near the double cysteines 1169 and 1170 (Fig. 1), becomes a predicted S-palmitoylation site in CSS-Palm in both Nav1.6-CCCAA and Loop2-CCCAA, while it is not a predicted site in the WT proteins. This suggests the possibility of unmasking and favoring S-palmitoylation at non-canonical residues when primary S-palmitoylation sites are eliminated in a protein. Indeed, the ABE assay showed that Loop2-C1163A displays similar S-palmitoylation signal as Loop2-WT, suggesting that C1163 is not a major S-palmitoylation site, although it might become one when neighboring S-palmitoylation sites are removed (e.g. Loop2-CCCAA). This may also explain the extra hyperpolarizing shift of steady-state inactivation observed in the Nav1.6-CCCAA channel with 2BP treatment (Fig. 3E), even though the CCAA mutant itself could account for a comparable inactivation shift in the WT channel with 2BP treatment.

4) **Nav1.6 C1978 confers the regulation of current amplitude in response to S-palmitoylation enhancement.** Since S-palmitoylation at C1169 and C1170 is only involved in regulating Nav1.6 voltage dependence, we set out to identify the S-palmitoylation site(s) responsible for Nav1.6 current amplitude regulation. C1978 is another predicted S-palmitoylation site. It is the last residue of Nav1.6, located at the very end of the cytoplasmic c-terminal domain (CTD) (Fig. 1). This site is of tremendous interest, because it is exclusive to Nav1.6, and is not found in any other isoform in the Nav family (Fig. 4A). More importantly, C1978 is evolutionally conserved among Nav1.6 among most species (Fig. S1B). These observations indicate that C1978 may convey crucial isoform-specific function for Nav1.6. Moreover, there is evidence that S-palmitoylation in the CTD of ion channels plays a role in surface targeting [13, 25, 26]. Therefore, we hypothesized that Nav1.6-C1978 S-palmitoylation regulates Nav1.6 surface expression, and thus produces the opposite current density effects observed with 2BP and PA treatments. To test this hypothesis, we eliminated S-palmitoylation at C1978 in Nav1.6 and examined the functional properties of this mutant channel. Fig. 4B shows the representative traces of TTXr Nav1.6-C1978A current from each treatment group. We found that increasing S-palmitoylation substrate availability with PA treatment no longer increases Nav1.6 current (PA: -120.50 ± 16.18 pA/pF, vs. DMSO: 113.2 ± 13.68 pA/pF; $P = 0.8123$) (Fig. 4C). However, blocking S-palmitoylation with 2BP still slightly decreases Nav1.6-C1978A current (-74.20 ± 12.70 pA/pF), although the reduction does not reach statistic significance ($P = 0.0544$) (Fig. 4C). We speculated that this decrease of Nav1.6-C1978A current by 2BP treatment may be accounted for by secondary S-palmitoylation site(s) or other unknown mechanisms, similar to the additional hyperpolarization of steady state inactivation in Nav1.6-CCCAA observed with 2BP treatment. Importantly, Nav1.6-C1978A demonstrates the same voltage dependence response to 2BP and PA treatments as Nav1.6-WT (Fig. 4DE), suggesting that S-palmitoylation at C1978 does not regulate voltage dependence of the channel. Using the same fusion protein strategy as in Fig3G, we confirmed C1978 as a major S-palmitoylation site in the Nav1.6-CTD (Fig. 4GH). It is noteworthy that there is no functional interaction or competition between S-palmitoylation at C1169, C1170 and that at C1978, because the triple cysteine mutant (Nav1.6-CCCAA) with all three cysteines mutated to alanines, replicates the...
voltage-dependent response of Nav1.6-CCAA and the current density response of Nav1.6-C1978A (Fig. 4I-K). Together, our data demonstrate that S-palmitoylation at different residues of Nav1.6 differentially regulates distinct channel functions.

5) Nav1.2 current is not increased by S-palmitoylation enhancement, but an exogenous cysteine renders it sensitive to the regulation.

Since c-terminus cysteine (C1978) is exclusive to Nav1.6, we predicted other Nav isoforms would lack similar current amplitude regulation by S-palmitoylation. To address this question, we evaluated how S-palmitoylation affects the functional properties of Nav1.2. Nav1.2 has a high degree of sequence homology and a similar expression pattern in the central nervous system as Nav1.6. However, it has a lysine (K2005) at the homologous position to Nav1.6-C1978. Fig. 5A shows the representative traces of Nav1.2-WT currents. We found that increasing S-palmitoylation substrate availability with PA treatment does not increase Nav1.2 current density (PA: -641.2 ± 92.95 pA/pF vs. DMSO: -648.7 ± 69.50 pA/pF; P = 0.8517) (Fig. 5B), although blocking S-palmitoylation with 2BP decreases Nav1.2 current (-397.3 ± 47.31 pA/pF; P = 0.0074) (Fig. 5B). This resembles the pattern of current density response to 2BP and PA treatments observed in the Nav1.6-C1978A channel. This suggested that Navs lacking S-palmitoylation at the c-terminus are not subject to current amplitude increased by S-palmitoylation. On the contrary, the voltage dependence of steady-state inactivation of Nav1.2 is altered by its S-palmitoylation states (Fig. 5C): blocking S-palmitoylation with 2BP causes a 4mV hyperpolarizing shift (2BP: V1/2 = -61.95 ± 0.21 mV vs. DMSO: V1/2 = -57.79 ± 0.18 mV; P < 0.0001), while increasing S-palmitoylation substrate availability results in a 4mV depolarizing shift (V1/2 = -54.01 ± 0.10 mV; P = 0.0003) (Fig. 5C), similar to previous results obtained from Xenopus oocytes expressing Nav1.2 [10]. With these data, we demonstrate that Nav1.2 fails to display sensitivity to current enhanced by S-palmitoylation, but preserves the voltage dependence modulation conveyed by the conserved double cysteines in loop2.

To further explore the role of S-palmitoylation at the c-terminus of a voltage-gated sodium channel other than Nav1.6, we introduced an exogenous cysteine to Nav1.2 at its c-terminus end point (K2005). Fig. 5A shows the representative traces of TTXr Nav1.2-K2005C currents. We observed that Nav1.2-K2005C displays the bidirectional current density modulation produced by 2BP and PA treatments (Fig. 5E) similar to Nav1.6-WT (Fig. 2C), suggesting that S-palmitoylation at the c-terminal exogenous cysteine in Nav1.2 alters the Nav1.2 current density response to S-palmitoylation modulation treatments. This indicates that the functional importance of S-palmitoylation at this position has the potential to apply to multiple Nav isoforms. However, the bidirectional modulation of Nav1.2 current is not as dramatic as that observed in Nav1.6. This might be due to a lower S-palmitoylation efficiency in Nav1.2, possibly due to difference in sequence environment, protein conformation and/or interacting partners associated with the channel. Unexpectedly, the pattern of voltage dependence response of Nav1.2-K2005C is more similar to Nav1.6-WT, rather than Nav1.2-WT (Fig. 5F): increasing S-palmitoylation substrate availability does not depolarize the voltage dependence of steady-state inactivation in Nav1.2-K2005C (PA: V1/2 = -55.57 ± 0.22 mV vs. DMSO: V1/2 = -57.20 ± 0.19 mV; P = 0.2877), while blocking S-palmitoylation with 2BP causes a hyperpolarizing shift in the voltage dependence of steady-state inactivation (V1/2 = -62.91 ± 0.26 mV; P < 0.0001). The loss of voltage dependence modulation in response to PA supplementation could be caused by the introduction of an extra S-palmitoylation site. This is in contrast to Nav1.6, where the S-palmitoylation status of the two sites seem to be independent, further supporting our hypothesis that S-palmitoylation modifies Nav functions in an isoform-dependent manner. Moreover, our data also suggest that whether 2BP and PA individual treatments can lead to appreciable effects also depends on the saturation level of S-palmitoylation at specific sites: if saturated, possibly as in the case of C1169 and C1170 in Nav1.6, increasing S-palmitoylation substrate availability would not show an appreciable effect on channel properties; while if a S-palmitoylation site is non-saturated, likely the case with the double cysteines in Nav1.2 loop-2 and C1978 in Nav1.6, manipulating the S-palmitoylation status of the channels with 2BP and PA treatments results in bidirectional effects.

6) Loss of S-palmitoylation at C1169, C1170 and C1978 in Nav1.6 reduces channel activity and dampens Nav1.6-mediated excitability in DRG neuron.
To investigate how loss of S-palmitoylation at C1169, C1170 and C1978 impacts Nav1.6 activity in neurons, we compared the properties of transfected Nav1.6r-WT and Nav1.6r-CCCAA channels in DRG neurons. The use of adult DRG neurons was specifically advantageous for our study due to the ease of transient transfection and high expression level of recombinant Nav1.6 we could achieve with this method. Since Nav1.8 is the major TTXr sodium channel in DRG neurons, Nav1.6r-WT and Nav1.6r-CCCAA cDNA constructs were co-transfected with Nav1.8-targeting shRNA to knock down the endogenous Nav1.8 channels [27]. DRG neurons express multiple TTX-sensitive sodium channels (including Nav1.6 [28]) and therefore transfected Nav1.6r current was isolated by applying 500nM TTX in the extracellular solution in patch clamp recordings.

We found that DRG neurons expressing Nav1.6-CCCAA conduct smaller TTXr sodium currents (Fig. 6AB) and these currents have a hyperpolarizing shift of steady state inactivation compared to the Nav1.6-WT transfected neurons (Fig. 6C).

Next we examined the Nav1.6-mediated excitability in DRG neurons. We observed a higher current threshold (Fig. 6F, CCCAAA: 459.5 ± 59.82 pA vs. WT: 250.0 ± 46.17 pA; P = 0.0089) as well as a higher voltage threshold (Fig. 6G, CCCAAA: -10.42 ± 2.136 mV vs. WT: -29.27 ± 4.123 mV; P = 0.0007) for evoked action potential in the Nav1.6-CCCAA transfected neurons with 500ms stimulations compared to Nav1.6-WT transfected neurons. The maximum number of action potentials fired during the 500ms stimulation are not different (Fig. 6H). This is because in our experiments most of the DRG neurons fire only one action potential with endogenous Nav1.8 knocked down. However, we found that fewer Nav1.6-CCCAA transfected neurons are able to fire an action potential upon a 1ms stimulation up to 1nA compared to WT transfected neurons (Fig. 6I AP/total cell count, WT: 11/22 vs. CCCAAA: 2/21, P = 0.0039 in Chi-square test). Collectively, these data suggest that loss of S-palmitoylation at C1169, C1170 and C1978 reduces Nav1.6 activity and dampens Nav1.6-mediated excitability in DRG neurons. Importantly, this was not due to any difference in intrinsic properties (resting membrane potential, input resistance, and cell capacitance) between WT- and CCCAAA-transfected neurons (Fig. 6J).

Discussion

We show here that Nav1.6 is modified by S-palmitoylation. 3 major S-palmitoylation sites are identified: the two adjacent cysteines in loop 2, C1169 and C1170, and the c-terminus C1978. The two cysteines in loop 2 are highly conserved among Nav isoforms (Fig. 3A), but their functional significance can vary: in Nav1.6 (Fig. 3 and Nav1.2 (Fig. 5 and [10]), S-palmitoylation at the conserved cysteines modulates channel steady-state inactivation; in Nav1.5, similar modulation is mediated by S-palmitoylation at a non-conserved residue (C981) without involving the conserved cysteines [9]. This suggests that the function of a S-palmitoylation site in a voltage-gated sodium channel depends on the specific isoform. It is also possible that S-palmitoylation may not ubiquitously occur at conserved sites in all Nav isoforms.

We also demonstrate that S-palmitoylation regulates distinct Nav1.6 functions by modifying different cysteine residues in the channel. Without affecting steady-state inactivation, the S-palmitoylation status of C1978 substantially modulates the current amplitude of Nav1.6 (Fig. 4). This uncovers the possibility to fine tune specific channel properties by modulating S-palmitoylation at respective residues. The underlying mechanism of current amplitude regulation might be that S-palmitoylation at Nav1.6-C1978 promotes the surface expression of the channel. However, we were not able to directly test this hypothesis due to the extremely low expression level of Nav1.6 in our heterologous system.

Intriguingly, the c-terminus cysteine is unique to Nav1.6 and absent from all other Nav isoforms (Fig. 4A). More importantly, this residue is invariant in Nav1.6 among most mammalian and vertebrate species with only a few exceptions in lower vertebrates (Fig. S1B). From an evolutionary perspective, this conservation indicates that C1978 is a more recent adaptation for Navs and probably confers critical regulation that is specific to Nav1.6 and to its special physiological role. Indeed, we observed current enhancement by S-palmitoylation only in the presence of the c-terminus cysteine in Nav1.6. Nav1.2, lacking the homologous S-palmitoylation site, only obtained such modulation when an exogenous cysteine was introduced (K2005C)
It is important to point out that the effect size of current enhancement in Nav1.2-K2005C is far smaller than that in Nav1.6, possibly due to lower S-palmitoylation efficiency at an exogenous S-palmitoylation site or to other c-terminal isoform specific difference. There is growing evidence that there is enzyme-substrate specificity for S-palmitoylation [29-34]. Palmitoyl-acyl transferases (PATs), the enzymes that catalyze the S-palmitoylation reaction, may recognize their substrates based on protein secondary structure [35] and/or protein-protein interacting domains [30, 31, 36-38], although these PATs were once deemed stochastic because a consensus sequence motif has yet to emerge [39]. The existence of PAT-substrate specificity is further supported by our observation of sub-optimal effect of current enhancement by S-palmitoylation on an exogenous site in Nav1.2-K2005C (Fig. 5) along with the possible lack of S-palmitoylation at the conserved double cysteines in Nav1.5 [9].

So far, 23 human PATs have been identified. It has been shown that these PATs differ in tissue, cell type, and subcellular distribution [16, 37, 40-43], creating a spatial, and thus functional segregation among these enzymes. Additionally, PAT distributions are subject to dynamic regulation [7], adding another layer of complexity for the regulation of S-palmitoylation. Although more work is needed to clarify the mechanisms of S-palmitoylation and its regulation, the intricacy of PAT specificity and the variety of S-palmitoylation functionalities substantiate its potential of being exploited to target Navs with isoform specificity.

Our discovery of Nav1.6 modification by S-palmitoylation provides a novel strategy to modulate neuronal excitability, considering the crucial role of Nav1.6 in action potential initiation and propagation in both CNS and PNS. We demonstrated, as a proof of concept, that altering S-palmitoylation of Nav1.6 exerts significant impact on the excitability of adult DRG neurons (Fig. 6). The DRG neurons provide important advantages for the purpose of our study. Adult DRG neurons can express high level of recombinant Nav1.6 via transient transfection [44] and adult neurons can be used for both voltage-clamp characterization of isolated ionic currents and current clamp analysis of excitability [45]. On the contrary, in our experience, the vast majority of cultured CNS neurons are either too immature to support sufficient level of Nav1.6 expression (as Nav1.6 is an adult brain isoform that starts its expression relatively late in development) or too difficult to be transfected. Moreover, the need for transient transfection of Nav1.6 and its S-palmitoylation deficient variants is imposed by the fact that pharmacological treatments (2BP and PA) affect global S-palmitoylation of a wide range of neuronal proteins, and thus it is impossible to isolate the effect of altering Nav1.6 S-palmitoylation using a pharmacological approach to manipulate palmitoylation in neurons. DRG neurons are not a perfect neuronal environment to demonstrate the functional significance of Nav1.6 S-palmitoylation on excitability. Although endogenous Nav1.6 expression is detected in DRG neurons [28], Nav1.6 has a more prominent role in setting the excitatory tone of central neurons. Thus, it may be highly desirable to further evaluate the impact of Nav1.6 S-palmitoylation on central neuron activity in future studies, possibly using transgenic mice with specific palmitoylation site knocked out. Additionally, the requirement for knocking down the endogenous TTXr Nav1.8 to isolate the effect of Nav1.6 rendered the majority of DRG neurons unable to fire multiple action potentials under our experimental conditions. The DRG neurons endogenously express a combination of TTX-sensitive (Nav1.6, Nav1.7) and TTXr (mainly Nav1.8) sodium currents [46]. It is well established that Nav1.6 and Nav1.7 mainly conducts sub-threshold sodium currents and set the threshold for action potentials, while Nav1.8 is the major contributor to action potential upstroke and repetitive firing in the DRG neurons [46]. Although Nav1.6 supports high frequency, repetitive firing in a wide range of CNS neurons [47-53], the cellular background of DRG neurons, including the subset of K+ channels, the leaky soma membrane property, the post-translational modifications and possibly distinct combination of Nav1.6 interacting proteins presented, may limit the ability of this channel to generate multiple action potentials on its own. Finally, while the use of DRG neurons for Nav1.6 expression provided a clear picture of the role of S-palmitoylation in modulating Nav1.6-mediated excitability, it does
not reflect the firing behavior of DRG neuron in vivo.

Nav1.6 has long been an attractive target for excitability modulation due to its unique properties that support high frequency, repetitive firing in a wide range of neurons [47-52, 54]. Moreover, Nav1.6 is distinguished by its ability to generate high levels of persistent current [47, 55] and resurgent current [56, 57], which are often perturbed (usually augmented) in channelopathies. Over 200 disease mutations are reported in ClinVar [58] for Nav1.6, implicating its direct involvement in the etiology of diverse neurological diseases including epilepsy, cognitive deficit, and movement disorders. Although none of them directly removes or introduces a Nav1.6 S-palmitoylation site, disease mutations that are seemingly distant from a S-palmitoylation site may influence S-palmitoylation by altering the posttranslational code [59], changing the secondary structure or disrupting protein-protein interaction. Additionally, there can be complex interplays between different post-translational interactions [5, 60-64]. Thus, mutations that interfere with, for example, phosphorylation or its related signaling pathways may also affect S-palmitoylation and indirectly alter channel maturation, trafficking and functional properties. Conversely, altering S-palmitoylation status of specific sites offers an opportunity to attenuate abnormal Nav1.6 activity imposed by disease mutations. Collectively, we present S-palmitoylation as a novel mechanism for isoform specific regulation for voltage-gated sodium channels and propose a new therapeutic strategy to modulate excitability disorders.

Experimental procedures

DNA constructs

pcDNA3-Nav1.6r was modified from a pcDNA3-Nav1.6r-EGFP construct [23] with a stop codon inserted before the EGFP sequence. This Nav1.6 channel has been rendered resistant to TTX (Nav1.6r) by a Y371S substitution. pcDNA3-Nav1.2 construct was rendered resistant to tetrodotoxin (TTXr) by a F385S substitution. Fusion proteins CD4-Nav1.6-Loop2 and CD4-Nav1.6-CTD were designed in-house and purchased from GenScript. Briefly, the extracellular and transmembrane segments of CD4 (amino acid 1-418) were fused with Nav1.6-Loop2 (amino acid 976-1193) or Nav1.6-CTD (amino acid 1768-1978). All mutations (Nav1.6 C1169,1170A, Nav1.6 C1978A, Nav1.2 K2005C, CD4-CTD CA, CD4-Loop2 CCAA) were introduced into the wild-type cDNA constructs using QuickChange II XL site-directed mutagenesis kit from Agilent Technologies according to the manufacture’s instructions. Mutant constructs were fully sequenced (ACGT, Inc.) to confirm correct mutation and absence of additional mutations.

Cell culture and transfection

The neuronal cell line ND7/23 was used to transiently express WT and mutant Nav1.6r and Nav1.2r channels for functional characterization, the HEK 293 cell line was used to transiently express WT and mutant CD4-Nav1.6 fusion proteins and to stably express Nav1.6 for acyl-biotin exchange assays. The cells were grown under standard tissue culture conditions and were transfected using Invitrogen Lipofectamine 2000 Transfection Reagent according to the manufacturer’s instructions. Briefly, lipid-DNA mixture (5μg channel construct and 0.5μg enhanced green fluorescent protein (EGFP) construct) in Opti-MEM medium was added to cells for 4h, after which transfected cells were split onto 35mm dish with fresh medium. Transfected cells were incubated at 30ºC overnight to increase channel surface expression. 24-32h after transfection, whole-cell voltage-clamp recordings were performed. Transfected cells were identified by EGFP expression under a fluorescent microscope. To manipulate S-palmitoylation status, 25 μM 2-bromo-palmitate (2BP), 10 μM palmitic acid (PA) or DMSO were added to medium 8 h after transfection for overnight incubation.

Rat DRG neurons were dissociated and cultured as previously described [65]. Briefly, young adult male Sprague Dawley rats, in adherence with animal procedures approved by the Indiana University School of Medicine and the School of Science Institutional Animal Care and Use Committees, were euthanized by carbon dioxide overexposure followed by decapitation. All DRGs were harvested and subsequently incubated in collagenase (1 mg/ml) and protease (1 mg/ml) for 70min. DRGs were then spun down and washed in DMEM with 10% FBS. After trituration, cells were plated on coverslips coated with poly-D-lysine and laminin. Transient transfection was performed using the Helios Gene Gun (Bio-Rad...
Laboratories) as previously described [57, 66]. Cells were co-transfected with Nav1.6r cDNA and Nav1.8 shRNA with an internal ribosome entry site–EGFP (IRES-EGFP). Previous data has shown that the Nav1.8 shRNA reduces endogenous Nav1.8 current in DRG neurons by 98% [27, 57]. Patch clamp recordings were performed 48h after transfection.

**Acyl-biotin exchange (ABE)**

Performed as previously described with minor modifications [9, 67]. Briefly, HEK 293 cells stably expressing Nav1.6 or transiently transfected with WT CD4-Loop2, CD4-CTD and their mutant variants were lysed and treated with N-ethylmaleimide (NEM) overnight to block free cysteines at 4ºC with end-to-end rotation. The next day, NEM was removed by chloroform-methanol precipitation and dissolved in 4% SDS buffer. The soluble protein was then divided into two equal parts and treated with either NH2OH (0.7 M hydroxylamine, 1 mM biotin, 0.2% Triton X-100 and 1 x protease inhibitor) or tris buffered solution (200 mM tris, 1 mM biotin, 0.2% Triton X-100 and 1 x protease inhibitor). The reaction was carried out in the dark for 1h. Chemicals were removed by chloroform-methanol precipitation. Protein was resolubilized in 2% SDS buffer and subsequently diluted with lysis buffer to achieve 0.1% SDS concentration for streptavidin-agarose beads capture. BCA protein assay was performed to ensure equal amount of proteins from different groups were subjected to the streptavidin-agarose beads capture. After 1h incubation at room temperature, beads were washed four times with lysis buffer containing 1% Triton X-100 and 0.1% SDS. Protein was eluted in LDS sample buffer (Invitrogen) with 2% β-mercaptoethanol and heated at 65ºC for 5 min before probing with western blotting.

**Gel electrophoresis and western immunoblotting**

Gel electrophoresis and protein transfer was performed according to the standard protocol (Life Technologies). Primary antibodies include mouse pan-Nav antibody (Sigma, 1:1000) and rabbit CD4 antibody (Abcam, 1:2000). Fluorescently labelled secondary antibodies used were goat anti-mouse IgG H&L IRDye® 800CW (Abcam, 1:20000) and goat anti-rabbit IgG IRDye 800CW (Li-Cor, 1:10000).

**Whole-cell patch clamp recordings**

All recordings were obtained at room temperature (~22ºC) using a HEKA EPC-10 amplifier and the PatchMaster program (v2x73.2, HEKA Electronic) as previously described [68]. For voltage-clamp recordings, electrodes were fabricated from 1.7mm capillary glass and fire-polished to a resistance of 0.8–1.0 MΩ using a Sutter P-1000 Micropipette puller (Sutter Instrument Company). The series of recording protocols was started 3 min after break-in for each cell, which controlled for time-dependent shifts in channel properties. Cells were not considered for analysis if the initial seal resistance was < 1 GΩ or if they had a series resistance > 3 MΩ. Voltage errors were minimized using >80% series resistance compensation and passive leak currents were cancelled by subtraction. The intracellular solution contained (in mM): 140 CsF, 10 NaCl, 1.1 EGTA, and 10 HEPES, adjusted to a pH of 7.30 with CsOH. For recordings in ND7/23 cells, the extracellular solution contained (in mM): 140 NaCl, 20 TEA-Cl, 3 KCl, 1 MgCl2, 1 CaCl2 and 10 HEPES, adjusted to a pH of 7.30 with NaOH. For recordings in DRG neurons, the extracellular solution contained (in mM): 140 NaCl, 20 TEA-Cl, 3 KCl, 1 MgCl2, 1 CaCl2, 0.1 CdCl2 and 10 HEPES, adjusted to a pH of 7.30 with NaOH. 500nM TTX was added to the extracellular solution to block endogenous sodium currents and isolate TTX-resistant current generated by transfected channels. Osmolarity of all solutions was adjusted to 300 mOsm.

For current-clamp recordings, electrodes were fabricated from 1.2mm capillary glass to achieve a resistance of 4.0–6.0 MΩ. The series of recording protocols was started 2 min after break-in for each cell. The intracellular solution contained (in mM): 140 KCl, 5 MgCl2, 5 EGTA, 2.5 CaCl2 and 10 HEPES, adjusted to a pH of 7.30 with KOH. The extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl and 10 HEPES, adjusted to a pH of 7.30 with NaOH. Osmolarity of solutions was adjusted to 300 mOsm.

**Patch clamp protocols and data analysis**

**Activation protocol.**

Transient sodium current (I\(_{NaT}\)) was measured during a 50ms depolarizing step (–80mV to +45mV; 5mV increment) from a holding potential of –120mV. The current density was calculated by
dividing the measured \( I_{NaT} \) by the capacitance of the cell. Sodium current conductance \( (G_{Na}) \) was converted from \( I_{NaT} \) using the equation
\[
G_{Na} = \frac{I_{NaT}}{V - V_{rev}},
\]
where \( V_{rev} \) is the reversal potential of \( Na^+ \) obtained in FitMaster (v2x7.5, HEKA Electronic) for each cell. Activation curves were generated by plotting normalized \( G_{Na} \) against depolarizing potentials and fitting it with the Boltzmann function in the form of
\[
\frac{G_{Na}}{G_{max}} = \frac{1}{1 + \exp\left(\frac{V_{50,act} - V}{k_{act}}\right)},
\]
where \( G_{max} \) is the maximal \( G_{Na} \), \( V_{50,act} \) is the potential at which activation is half-maximal, \( V \) is the depolarizing potential, and \( k_{act} \) is the slope factor.

**Steady-state inactivation.**

Availability of sodium channels was measured by the peak sodium current during a 20ms test pulse at 0mV following a 500ms prepulse (–140mV to +10mV; 10mV increment) that allows channels to enter equilibrium states. Steady-state inactivation curves were generated by plotting normalized sodium current against prepulse potentials and fitting it with the Boltzmann function in the form of
\[
\frac{I}{I_{max}} = \frac{1}{1 + \exp\left(\frac{V_{50,inact} - V}{k_{inact}}\right)},
\]
where \( I_{max} \) is the maximal sodium current obtained in this protocol, \( V_{50,inact} \) is the potential at which half of the sodium channels are available for activation, \( V \) is the prepulse potential, and \( k_{inact} \) is the slope factor.

**Recovery from inactivation.**

A 20ms depolarization prepulse at 0mV was applied to allow channel activation and subsequent inactivation, which was followed by a repolarizing step to –80mV for durations ranging from 0ms to 50ms with 2ms increment. The non-inactivated sodium currents were measured during a subsequent 20ms test pulse at 0mV and normalized to the maximum current obtained in this protocol. The normalized non-inactivated sodium current was plotted against the duration of repolarizing step and fitted with a single exponential function.

**Statistics**

GraphPad Prism (v 6.00, GraphPad Software) was used for statistical analysis and curve fitting with the nonlinear least-squares minimization method. The data acquired from voltage-clamp recording are presented as mean ± standard error of mean (SEM) of the indicated number of cells (n). The data acquired from current-clamp recording are presented as mean ± standard deviation (SD) and plotted with individual values. For comparisons of current density from both ND7/23 cells and DRG neurons, two-way ANOVA was performed. For comparisons of all the other parameters, One-way ANOVA followed by Tukey multiple comparisons test was performed for treatment groups in the same channel variant transfected in ND7/23 cells; Student’s t test was performed for WT and individual mutant channels transfected in ND7/23 cells and DRG neurons.
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1. Catterall, W.A., Voltage-gated sodium channels at 60: structure, function and pathophysiology. The Journal of physiology, 2012. 590(11): p. 2577-2589.
2. Shen, H., et al., Structures of human Nav1.7 channel in complex with auxiliary subunits and animal toxins. Science, 2019. 363(6433): p. 1303-1308.
3. Pan, X., et al., Molecular basis for pore blockade of human Na+ channel Nav1.2 by the μ-conotoxin KIIIA. Science, 2019. 363(6433): p. 1309-1313.
4. Bagal, S.K., et al., Voltage-gated sodium channels as drug discovery targets. Channels, 2015. 9(6): p. 360-366.
5. Pei, Z., Y. Pan, and T.R. Cummins, Posttranslational modification of sodium channels. 2017.
6. Naumenko, V.S. and E. Ponimaskin, Palmitoylation as a functional regulator of neurotransmitter receptors. Neural plasticity, 2018. 2018.
7. Globa, A.K. and S.X. Bamji, Protein palmitoylation in the development and plasticity of neuronal connections. Current opinion in neurobiology, 2017. 45: p. 210-220.
8. Fukata, Y. and M. Fukata, Protein palmitoylation in neuronal development and synaptic plasticity. Nature Reviews Neuroscience, 2010. 11(3): p. 161.
9. Pei, Z., et al., Cardiac sodium channel palmitoylation regulates channel availability and myocyte excitability with implications for arrhythmia generation. Nature communications, 2016. 7: p. 12035.
10. Bosmans, F., M. Milescu, and K.J. Swartz, Palmitoylation influences the function and pharmacology of sodium channels. Proceedings of the National Academy of Sciences, 2011. 108(50): p. 20213-20218.
11. Itoh, M., et al., Perturbed expression pattern of the immediate early gene Arc in the dentate gyrus of GluA1 C-terminal palmitoylation-deficient mice. Neuropsychopharmacology reports, 2019. 39(1): p. 61-66.
12. Itoh, M., et al., Deficiency of AMPAR–Palmitoylation Aggravates Seizure Susceptibility. Journal of Neuroscience, 2018. 38(47): p. 10220-10235.
13. Hayashi, T., G. Rumbaugh, and R.L. Huganir, Differential regulation of AMPA receptor subunit trafficking by palmitoylation of two distinct sites. Neuron, 2005. 47(5): p. 709-723.
14. Chien, A.J., et al., Identification of palmitoylation sites within the L-type calcium channel β2a subunit and effects on channel function. Journal of Biological Chemistry, 1996. 271(43): p. 26465-26468.
15. Roth, A.F., et al., Global analysis of protein palmitoylation in yeast. Cell, 2006. 125(5): p. 1003-1013.
16. Fukata, M., et al., Identification of PSD-95 palmitoylating enzymes. Neuron, 2004. 44(6): p. 987-996.
17. Ponimaskin, E., et al., Fibroblast growth factor-regulated palmitoylation of the neural cell adhesion molecule determines neuronal morphogenesis. Journal of Neuroscience, 2008. 28(36): p. 8897-8907.
18. Lievens, P.M.-J., et al., ZDHHC3 tyrosine phosphorylation regulates neural cell adhesion molecule palmitoylation. Molecular and cellular biology, 2016. 36(17): p. 2208-2225.
19. Brigidi, G.S., et al., Palmitoylation of δ-catenin by DHHC5 mediates activity-induced synapse plasticity. Nature neuroscience, 2014. 17(4): p. 522.
20. Noritake, J., et al., Mobile DHHC palmitoylating enzyme mediates activity-sensitive synaptic targeting of PSD-95. The Journal of cell biology, 2009. 186(1): p. 147-160.
21. Wood, J.N., et al., *Novel cell lines display properties of nociceptive sensory neurons*. Proceedings of the Royal Society of London. Series B: Biological Sciences, 1990. 241(1302): p. 187-194.

22. Rogers, M., et al., *Characterization of endogenous sodium channels in the ND7-23 neuroblastoma cell line: implications for use as a heterologous ion channel expression system suitable for automated patch clamp screening*. Assay and drug development technologies, 2016. 14(2): p. 109-130.

23. Gasser, A., et al., *An ankyrin-G-binding motif is necessary and sufficient for targeting Nav1.6 sodium channels to axon initial segments and nodes of Ranvier*. Journal of Neuroscience, 2012. 32(21): p. 7232-7243.

24. Ren, J., et al., *CSS-Palm 2.0: an updated software for palmitoylation sites prediction*. Protein Engineering, Design & Selection, 2008. 21(11): p. 639-644.

25. Jindal, H.K., et al., *Posttranslational modification of voltage-dependent potassium channel Kv1.5: COOH-terminal palmitoylation modulates its biological properties*. American Journal of Physiology-Heart and Circulatory Physiology, 2008. 294(5): p. H2012-H2021.

26. Hayashi, T., G.M. Thomas, and R.L. Huganir, *Dual palmitoylation of NR2 subunits regulates NMDA receptor trafficking*. Neuron, 2009. 64(2): p. 213-226.

27. Barbosa, C., et al., *Navβ4 regulates fast resurgent sodium currents and excitability in sensory neurons*. Molecular pain, 2015. 11(1): p. 60.

28. Chen, L., et al., *Conditional knockout of Na V 1.6 in adult mice ameliorates neuropathic pain*. Scientific reports, 2018. 8(1): p. 1-17.

29. Howie, J., et al., *Substrate recognition by the cell surface palmitoyl transferase DHHC5*. Proceedings of the National Academy of Sciences, 2014. 111(49): p. 17534-17539.

30. Lemonidis, K., M.C. Sanchez-Perez, and L.H. Chamberlain, *Identification of a novel sequence motif recognized by the ankyrin repeat domain of zDHHC17/13 S-acyltransferases*. Journal of Biological Chemistry, 2015. 290(36): p. 21939-21950.

31. Thomas, G.M., et al., *Palmitoylation by DHHC5/8 targets GRIP1 to dendritic endosomes to regulate AMPA-R trafficking*. Neuron, 2012. 73(3): p. 482-496.

32. Nadolski, M.J. and M.E. Linder, *Molecular recognition of the palmitoylation substrate Vac8 by its palmitoyltransferase Pfa3*. Journal of Biological Chemistry, 2009. 284(26): p. 17720-17730.

33. Huang, K., et al., *Neuronal palmitoyl acyl transferases exhibit distinct substrate specificity*. The FASEB Journal, 2009. 23(8): p. 2605-2615.

34. Hou, H., et al., *Analysis of DHHC acyltransferases implies overlapping substrate specificity and a two-step reaction mechanism*. Traffic, 2009. 10(8): p. 1061-1073.

35. Plain, F., et al., *An amphipathic α-helix directs palmitoylation of the large intracellular loop of the sodium/calcium exchanger*. Journal of Biological Chemistry, 2017. 292(25): p. 10745-10752.

36. Li, Y., et al., *DHHC5 interacts with PDZ domain 3 of post-synaptic density-95 (PSD-95) protein and plays a role in learning and memory*. Journal of Biological Chemistry, 2010. 285(17): p. 13022-13031.

37. Brigidi, G.S., et al., *Activity-regulated trafficking of the palmitoyl-acyl transferase DHHC5*. Nature communications, 2015. 6: p. 8200.

38. Fredericks, G.J., et al., *Stable expression and function of the inositol 1,4,5-triphosphate receptor requires palmitoylation by a DHHC6/selenoprotein K*
complex. Proceedings of the National Academy of Sciences, 2014. **111**(46): p. 16478-16483.

39. Rocks, O., et al., *The palmitoylation machinery is a spatially organizing system for peripheral membrane proteins*. Cell, 2010. **141**(3): p. 458-471.

40. Greaves, J., J.A. Carmichael, and L.H. Chamberlain, *The palmitoyl transferase DHHC2 targets a dynamic membrane cycling pathway: regulation by a C-terminal domain*. Molecular biology of the cell, 2011. **22**(11): p. 1887-1895.

41. Gorleku, O.A., et al., *Endoplasmic reticulum localization of DHHC palmitoyltransferases mediated by lysine-based sorting signals*. Journal of Biological Chemistry, 2011. **286**(45): p. 39573-39584.

42. Mill, P., et al., *Palmitoylation regulates epidermal homeostasis and hair follicle differentiation*. PLoS genetics, 2009. **5**(11): p. e1000748.

43. Fernández-Hernando, C., et al., *Identification of Golgi-localized acyl transferases that palmitoylate and regulate endothelial nitric oxide synthase*. The Journal of cell biology, 2006. **174**(3): p. 369-377.

44. Herzog, R.I., et al., *Distinct repriming and closed - state inactivation kinetics of Nav1. 6 and Nav1. 7 sodium channels in mouse spinal sensory neurons*. The Journal of physiology, 2003. **551**(3): p. 741-750.

45. Cummins, T.R., et al., *Voltage-clamp and current-clamp recordings from mammalian DRG neurons*. Nature protocols, 2009. **4**(8): p. 1103.

46. Rush, A.M., T.R. Cummins, and S.G. Waxman, *Multiple sodium channels and their roles in electrogenesis within dorsal root ganglion neurons*. The Journal of physiology, 2007. **579**(1): p. 1-14.

47. Maurice, N., et al., *D1/D5 dopamine receptor activation differentially modulates rapidly inactivating and persistent sodium currents in prefrontal cortex pyramidal neurons*. Journal of Neuroscience, 2001. **21**(7): p. 2268-2277.

48. Khaliq, Z.M., N.W. Gouwens, and I.M. Raman, *The contribution of resurgent sodium current to high-frequency firing in Purkinje neurons: an experimental and modeling study*. Journal of Neuroscience, 2003. **23**(12): p. 4899-4912.

49. Do, M.T.H. and B.P. Bean, *Sodium currents in subthalamic nucleus neurons from Nav1. 6-null mice*. Journal of neurophysiology, 2004. **92**(2): p. 726-733.

50. Van Wart, A. and G. Matthews, *Impaired firing and cell-specific compensation in neurons lacking nav1. 6 sodium channels*. Journal of Neuroscience, 2006. **26**(27): p. 7172-7180.

51. Enomoto, A., et al., *Sodium currents in mesencephalic trigeminal neurons from Nav1. 6 null mice*. Journal of neurophysiology, 2007. **98**(2): p. 710-719.

52. Mercer, J.N., et al., *Nav1. 6 sodium channels are critical to pacemaking and fast spiking in globus pallidus neurons*. Journal of Neuroscience, 2007. **27**(49): p. 13552-13566.

53. Osorio, N., et al., *Persistent Nav1. 6 current at axon initial segments tunes spike timing of cerebellar granule cells*. The Journal of physiology, 2010. **588**(4): p. 651-670.

54. Royeck, M., et al., *Role of axonal NaV1. 6 sodium channels in action potential initiation of CA1 pyramidal neurons*. Journal of neurophysiology, 2008. **100**(4): p. 2361-2380.

55. Smith, M.R., et al., *Functional analysis of the mouse Scn8a sodium channel*. Journal of Neuroscience, 1998. **18**(16): p. 6093-6102.

56. Raman, I.M., et al., *Altered subthreshold sodium currents and disrupted firing patterns in Purkinje neurons of Scn8a mutant mice*. Neuron, 1997. **19**(4): p. 881-891.
57. Jarecki, B.W., et al., Human voltage-gated sodium channel mutations that cause inherited neuronal and muscle channelopathies increase resurgent sodium currents. The Journal of clinical investigation, 2010. 120(1): p. 369-378.

58. Landrum, M.J., et al., ClinVar: public archive of interpretations of clinically relevant variants. Nucleic acids research, 2015. 44(D1): p. D862-D868.

59. Pankow, S., C. Bamberger, and J.R. Yates, A posttranslational modification code for CFTR maturation is altered in cystic fibrosis. Sci. Signal., 2019. 12(562): p. eaan7984.

60. Ho, G.P., et al., S-nitrosylation and S-palmitoylation reciprocally regulate synaptic targeting of PSD-95. Neuron, 2011. 71(1): p. 131-141.

61. Shipston, M.J., Ion channel regulation by protein S-acylation. The Journal of general physiology, 2014. 143(6): p. 659-678.

62. Tian, L., et al., Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels. Proceedings of the National Academy of Sciences, 2008. 105(52): p. 21006-21011.

63. Summers, D.W., J. Milbrandt, and A. DiAntonio, Palmitoylation enables MAPK-dependent proteostasis of axon survival factors. Proceedings of the National Academy of Sciences, 2018. 115(37): p. E8746-E8754.

64. Woolfrey, K.M., et al., CaMKII regulates the depalmitoylation and synaptic removal of the scaffold protein AKAP79/150 to mediate structural long-term depression. Journal of Biological Chemistry, 2018. 293(5): p. 1551-1567.

65. Cummins, T.R., et al., Sodium channels and the molecular pathophysiology of pain, in Progress in brain research. 2000, Elsevier. p. 3-19.

66. Herzog, R.I., et al., Calmodulin binds to the C terminus of sodium channels Nav1.4 and Nav1.6 and differentially modulates their functional properties. Journal of Neuroscience, 2003. 23(23): p. 8261-8270.

67. Wan, J., et al., Palmitoylated proteins: purification and identification. Nature protocols, 2007. 2(7): p. 1573.

68. Pan, Y. and T.R. Cummins, Distinct functional alterations in SCN8A epilepsy mutant channels. The Journal of physiology, 2020. 598(2): p. 381-401.
|                      | Max I density (pA/pF) | n   | Vhalf (mV) | K    | n   | Vhalf (mV) | K    | Recovery τ (ms) | n   |
|----------------------|-----------------------|-----|------------|------|-----|------------|------|----------------|-----|
|                      |                       |     |            |      |     |            |      |                |     |
| Nav1.6 WT            | DMSO                  | -80.17 ± 8.30 | 29  | -64.46 ± 0.21 | 5.32 ± 0.138 | 30  | -19.7 ± 0.18   | 7.045 ± 0.13 | 28  | 9.842 ± 0.76  | 24  |
|                      | 2BP                   | -26.30 ± 3.29 | 21  | -72.34 ± 0.28 | 6.51 ± 0.243 | 28  | -22.48 ± 0.84 | *7.802 ± 0.16 | 22  | 13.18 ± 1.28 *| 18  |
|                      | PA                    | -142.8 ± 17.24 | 33  | -66.12 ± 0.18 | 5.162 ± 0.08  | 33  | -21.32 ± 0.2  | 6.92 ± 0.20 | 33  | 10.54 ± 0.45 | 18  |
|                      | 2BP+PA                | -32.12 ± 7.43 | 12  | **         | **          | **  | **          | **    | 9.62 ± 0.45 | 20  |
| Nav1.6 CCAA          | DMSO                  | -94.17 ± 17.23 | 19  | -75.50 ± 0.39 | 7.205 ± 0.19 | 26  | -22.59 ± 0.93 | ^7.93 ± 0.37 | 18  | 10.70 ± 0.38 | 21  |
|                      | 2BP                   | -52.09 ± 5.11 | 19  | -80.48 ± 0.30 | 6.824 ± 0.22 | 22  | -25.88 ± 0.83 | *8.10 ± 0.22 | 17  | 12.69 ± 0.69 *| 10  |
|                      | PA                    | -163.3 ± 24.30 | 18  | *####      | ####       | #   | **          | **    | 8.15 ± 0.18 | 17  |
| Nav1.6 C1978A        | DMSO                  | -113.2 ± 13.68 | 35  | -63.79 ± 0.23 | 5.446 ± 0.08  | 34  | -19.81 ± 0.76 | 7.228 ± 0.12 | 35  | 10.32 ± 0.31 | 21  |
|                      | 2BP                   | -74.20 ± 12.70 | 27  | -73.59 ± 1.39 | 7.107 ± 0.14 | 22  | -21.66 ± 0.99 | 7.76 ± 0.57 | 17  | 12.82 ± 0.57 | 26  |
|                      | PA                    | -120.5 ± 16.18 | 27  | #          | #          | #   | **          | **    | 9.23 ± 0.15 | 23  |
| Nav1.6 CCCAAA        | DMSO                  | -115.8 ± 15.01 | 25  | -72.91 ± 0.44 | 7.143 ± 0.18 | 21  | -18.75 ± 0.2  | 8.58 ± 0.16 | 23  | 12.68 ± 0.54 | 19  |
|                      | 2BP                   | -83.50 ± 9.69 | 22  | *P=0.011    | 7.916 ± 0.18 | 21  | -21.61 ± 0.2  | 8.491 ± 0.10 | 16  | 14.36 ± 0.68 | 15  |
|                      | PA                    | -117.5 ± 12.84 | 26  | #          | #          | #   | **          | **    | 9.671 ± 1.18 | 19  |
| DrG                  | WT                    | -2006 ± 294.9 | 20  | -78.30 ± 1.27 | 5.936 ± 0.197 | 20  | -34.57 ± 2.16 | 7.113 ± 0.625 | 18  | 11.35 ± 1.17 | 20  |
| Nav1.6 CCCAAA        | -1026 ± 124.7         | 29  | -85.17 ± 2.24 | *          | 9.952 ± 0.659 | 25  | -31.63 ± 1.37 | 8.721 ± 0.403 | 27  | 9.355 ± 0.56 | 26  |
| Nav1.2 WT            | DMSO                  | -648.7 ± 69.50 | 30  | -57.79 ± 0.18 | 4.868 ± 0.06 | 26  | -15.01 ± 0.2  | 6.743 ± 0.24 | 26  | 7.968 ± 0.38 | 22  |
|                      | 2BP                   | -397.3 ± 47.31 | 28  | *           | 5.474 ± 0.15 | 24  | -14.59 ± 0.1  | 7.479 ± 0.16 | 27  | 10.41 ± 0.80 *| 21  |
|                      | PA                    | -641.2 ± 92.95 | 23  | *           | 4.705 ± 0.10 | 21  | -12.43 ± 0.2  | 6.704 ± 0.23 | 21  | 7.437 ± 0.39 | 19  |
| K2005C               | DMSO                  | -562.1 ± 62.34 | 28  | -57.20 ± 0.18 | 4.88 ± 0.088 | 28  | -14.82 ± 0.2  | 6.608 ± 0.21 | 27  | 8.702 ± 0.53 | 22  |
|                      | 2BP                   | -416.5 ± 52.65 | 29  | P=0.162     | 5.33 ± 0.078 | 29  | -17.29 ± 0.2  | 7.212 ± 0.17 | 25  | 11 ± 0.579   | 26  |
|                      | PA                    | -837.1 ± 134.8 | 31  | *P=0.047    | 4.849 ± 0.08 | 27  | -16.13 ± 0.2  | 6.028 ± 0.30 | 27  | 7.384 ± 0.34 | 23  |

****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 compared to DMSO of the same DNA

# PA compared to 2BP of the same DNA

^ Mutant compared to WT
Figure 1. Topology of a voltage-gated sodium channel showing positions of the three predicted S-palmitoylation sites in Nav1.6.
Figure 2. S-palmitoylation post-translationally modifies Nav1.6 and functionally regulates Nav1.6 current amplitude and voltage-dependence of inactivation.

(A) Acyl-biotin exchange assay on mouse cerebellum probed with Nav1.6 antibody (left) and on HEK cell line stably expressing Nav1.6 probed with pan-Nav antibody (right). (B-E) Voltage-clamp experiments on ND7/23 cells transiently transfected with TTXr-Nav1.6. Transfected cells were treated with DMSO as solvent control, 2-Br-palmitate (2BP) to block S-palmitoylation and palmitic acid (PA) to enhance S-palmitoylation 24h before recording. (B) Representative current traces from each treatment group elicited with the activation protocol. Maximum current traces are highlighted with black, red and blue respectively. (C) Current density-voltage plot. * P < 0.05 compared to DMSO in two-way ANOVA. (D) Steady-state inactivation and activation curves fitted with Boltzmann functions. (E) Recovery from inactivation with recovery time duration from 0 to 50ms. Data presented as mean ± SEM. Values and statistic significance are reported in Table.
Figure 3. S-palmitoylation of C1169, C1170 regulates Nav1.6 voltage-dependence of inactivation, but not Nav1.6 current amplitude.

(A) A segment of amino acid alignment of the Nav family. Arrow: C1163, C1169, C1170 in Nav1.6. (B) Representative current traces elicited from Nav1.6-CCAA with different treatments. (C) Current density-voltage plot. * P < 0.05 compared to DMSO in two-way ANOVA. (D)(E) Steady-state inactivation and activation curves of Nav1.6-WT and Nav1.6-CCAA fitted with Boltzmann functions. (F) Recovery from inactivation with recovery time duration from 0 to 50ms. Data presented as mean ± SEM. Values and statistic significance are reported in Table. (G) Schematic illustration of the CD4-Nav1.6-loop2 fusion protein showing the last five amino acids of the CD4 transmembrane segment and the first five amino acids of the Nav1.6-loop2. (H) Acyl-biotin exchange assays on fusion protein-transfected HEK cells probed with CD4 antibody: (left) WT fusion protein transfectioned cells treated with DMSO, 2BP and PA and (right) WT, C1163A, CCAA and CCCCCAA fusion proteins.
Figure 4. C1978A eliminates Nav1.6 current enhancement by PA without altering voltage-dependence response to S-palmitoylation manipulation.

(A) A segment of amino acid alignment of the Nav family. Arrow: C1978 in Nav1.6. (B) Representative current traces elicited from Nav1.6-C1978A with different treatments. (C)(I) Current density-voltage plots of Nav1.6-C1978A and Nav1.6-CCCAA. # P < 0.05 PA compared to 2BP in two-way ANOVA. (D)(E)(J) Steady-state inactivation and activation curves of Nav1.6-C1978A and Nav1.6-CCCAA fitted with Boltzmann functions. (F)(K) Recovery from inactivation of Nav1.6-C1978A and Nav1.6-CCCAA with recovery time duration from 0 to 50ms. Data presented as mean ± SEM. Values and statistic significance are reported in Table. (G) Schematic illustration of the CD4-Nav1.6-CTD fusion protein showing the last five amino acids of the CD4 transmembrane segment and the first five amino acids of the
Nav1.6-CTD. (H) Acyl-biotin exchange assays on WT and C1978A fusion protein-transfected HEK cells probed with CD4 antibody.
Figure 5. An exogenous cysteine renders Nav1.2 current amplitude enhanced by S-palmitoylation. Voltage-clamp recordings from ND7/23 cells transiently transfected with Nav1.2r-WT and Nav1.2r-K2005C. Transfected cells were treated with DMSO as solvent control, 2-Br-palmitate (2BP) to block S-palmitoylation and palmitic acid (PA) to enhance S-palmitoylation 24h before recording. (A) Representative current traces from different treatment groups elicited by activation protocol. (B)(E) Current density-voltage plots. * P < 0.05 compared to DMSO, # P < 0.05 PA compared to 2BP in two-way ANOVA. (C)(F) Steady-state inactivation and activation curves fitted with a Boltzmann function. (D)(G) Recovery from inactivation with recovery time duration from 0 to 50ms. Data presented as mean ± SEM. Values and statistic significance are reported in Table.
Figure 6. Loss of S-palmitoylation at C1169, C1170 and C1978 reduced Nav1.6 current, channel availability and dampened Nav1.6-mediated excitability in DRG neuron.

(A) Representative TTXr current traces elicited from Nav1.6-WT and Nav1.6-CCCAA transfected DRG neurons with endogenous Nav1.8 knocked down. (B) Current density-voltage plot. * P < 0.05 in two-way ANOVA. (C) Steady-state inactivation and activation curves fitted with Boltzmann functions. (D) Recovery from inactivation with recovery time duration from 0 to 50ms. Data presented as mean ± SEM in (B-D). Values and statistic significance are reported in Table. (E) Representative traces of stimulated action potentials from Nav1.6-WT and Nav1.6-CCCAA transfected DRG neurons with endogenous Nav1.8 knocked down. (F)(G) Current and voltage thresholds for evoked action potential during 500ms stimulation. * P < 0.05 in t test. (H) Maximum number of evoked action potentials during 500ms stimulations from 0pA to 1000pA in 50pA increments. (I) Percentage of cells fired versus did not fire action potential upon 1ms stimulations from 0pA to 1000pA in 50pA increments. * P < 0.05 in Chi-square test. (J) Resting membrane potential, input resistance and cell capacitance of Nav1.6-WT and Nav1.6-CCCAA transfected DRG neurons. Data presented as mean ± SD in (F-H, J).
S-palmitoylation of the sodium channel Nav1.6 regulates its activity and neuronal excitability
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