Membrane Lipid Domains Modulate HCN Channels in Nociceptor DRG Neurons

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Abstract

Cell membranes consist of heterogeneous lipid domains that influence key cellular processes, including signal transduction, endocytosis, and electrical excitability. Using FRET-based fluorescent assays and fluorescence lifetime imaging microscopy (FLIM), we found that the dimension of cholesterol-enriched ordered membrane domains (OMD) varies considerably, depending on specific cell types. The size of OMDs is also dependent on cholesterol levels and the structure of lipid tails. Particularly, nociceptor dorsal root ganglion (DRG) neurons exhibit large OMDs. Disruption of OMDs potentiated action potential firing in nociceptor DRG neurons and facilitated opening of native hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. This increased neuronal firing could be partially due to an increased open probability of HCN channels. In animal models of neuropathic pain, we observed shrunken OMDs and relocalization of HCN channels from OMDs to disordered lipid domains. The gating effect on HCN channels was likely a result of direct modulation of the voltage sensor by OMDs. These findings suggest that disturbances in lipid domains play a role in regulating HCN channels within nociceptor DRG neurons, influencing pain modulation.

Introduction

Membrane lipids form distinct compartments known as lipid domains, which serve as specialized regions within cell membranes for localized cell signaling. In compartmentalized membranes, a distinctive type known as detergent-resistant lipid-ordered membrane domain (OMD) stands apart from other regions, such as the disordered membrane domains. This distinction arises from the composition of OMDs, which is characterized by a heightened presence of cholesterol and sphingolipids[1-3]. The OMDs possess an intriguing variation in size, ranging from a few nanometers to hundreds of nanometers. This dynamic nature allows the lipid domains to undergo rapid changes, coalescing or splitting apart, in response to different physiological conditions. Techniques such as super-resolution microscopy, single-molecule fluorescence, and cryo-electron tomography have been used to visualize and characterize these membrane domains[4-7]. Nevertheless, many details about the structure, function, and significance of OMDs remain elusive.

The significance of lipid domains has been recognized in diverse biological processes[1,8]. For instance, in Alzheimer's disease, the dysregulation of lipid domains has been implicated in the accumulation of amyloid-β plaques[9-11]. In viral infections, these domains can serve as entry sites for the viruses into cells[12,13]. Inflammatory responses involve the reorganization of lipid domains, influencing cell signaling and immune responses. Moreover, emerging evidence suggests a link between lipid domains and chronic pain, particularly neuropathic pain, which is often caused by nerve injuries[14,15]. In an animal model of chemotherapy-induced peripheral neuropathy and arthritis, targeting of lipid domains was effective to alleviate the pathological symptoms[16]. However, the connection between OMDs and neuropathic pain remains understudied. Manipulating these domains may hold potential for developing targeted treatments for diseases related to lipid domain dysfunction, including neurodegenerative disorders, viral infections, inflammatory conditions, and neuropathic pain.

Pacemaker hyperpolarization-activated cyclic nucleotide-gated (HCN) channels play critical roles in several important physiological processes[17]. These channels are involved in establishing the resting membrane potential (RMP) and regulating the frequency of action potentials, thereby contributing to the automaticity of excitable cells[17]. Moreover, HCN channels are important for pain sensation, as evidenced by their involvement in repetitive firing of primary nociceptor neurons in the dorsal root ganglion[18-20]. HCN2-channel current is responsible for eliciting repetitive firing in nociceptor neurons[21,22]. In support of this, knocking out the HCN2 gene reduces neuropathic pain in animal models[18,23]. Conversely, increased HCN channel activity correlates with spontaneous action potential

firing and chronic pain, while pharmacological blockade of HCN channel activity decreases inflammatory and neuropathic pain[18,20,24]. The lipid domain-specific localization of HCN channels appears to significantly influence their function, thereby impacting the neuronal and cardiac excitability[25-27]. The function of cardiac HCN4 channels depends on compartmentalized cAMP signaling in lipid microdomains[28,29]. We showed that the voltage-sensing process of HCN channels, including the voltage-sensor movement, is directly regulated by OMDs[30]. In addition, the voltage-sensing modules of HCN channels interact intimately with membrane lipids, including signaling phosphoinositides[31-34]. In summary, these studies corroborate a significant connection between pain sensation and the regulation of pacemaker HCN channels by membrane lipids.

In this current study, we performed fluorescence lifetime imaging microscopy (FLIM) and Förster resonance energy transfer (FRET), which are well-suited for evaluating the nanometer sized OMDs in various cell types. Nociceptor DRG neurons exhibit larger OMDs than non-nociceptive DRG neurons. When OMDs are disrupted in nociceptor DRG neurons, the action potential firing becomes more frequent. HCN2 channels, abundantly expressed in nociceptor DRG neurons, tend to localize within the OMD and are sensitive to the disruption of lipid domains. Furthermore, the voltage sensor movement of HCN2 channels is modulated by the OMD localization directly. This modulation appears to impact the gating of the native HCN channels in nociceptor DRG neurons. In addition, significant reductions in the OMD size are associated with both a chemotherapy-induced model and a nerve injury-induced model of neuropathic pain. These findings indicate that perturbations in lipid domains and a resulting increase in the HCN channel opening near the RMP may contribute to the mechanisms underlying neuropathic pain.

Results

Imaging the OMDs of living cells using FLIM-FRET

We employed cholera toxin subunit B (CTxB)-conjugated FRET donors and acceptors as probes to investigate lipid nanodomains. CTxB, derived from *Vibrio cholerae,* acts as a pentameric multivalent ligand with exceptionally high affinity (picomolar) for GM1 gangliosides[35,36]. These gangliosides, specifically GM1, are distinct sphingolipid molecules prominently present in OMDs. Conventional fluorescence microscopy encounters limitations due to the diffraction limit of light (250 nm), often making it impossible to visualize small-sized lipid domains. FRET-based fluorescence microscopy can overcome this limitation by acting as a spectroscopic distance ruler with nanometer sensitivity. This sensitivity is ideal for detecting nanoscale lipid domains of the dimensions thought to be critical in biological membranes[37]. We used Alexa Fluor 488 (AF-488) CTxB conjugates as FRET donors and Alexa Fluor 647 (AF-647) CTxB conjugates serving as FRET acceptors (Fig. 1a). This specific FRET pair was previously established, with an Förster critical distance (R_0) value of approximately 52 Å[38]. When the size of lipid domains exceeds a radius of approximately 10 nm, the likelihood of accommodating two CTxB pentamers within the same lipid domain increases significantly[36]. In contrast, when the lipid domain has a radius of less than 5 nm, it can accommodate only one CTxB pentamer[36], resulting in minimal FRET between AF-488 and AF-647. This approach can effectively quantify the relative size of lipid domains.

To conduct live-cell time-resolved FRET imaging, we used a confocal scanning FLIM system to measure FRET efficiency (see Methods). We labeled the plasma membrane of tsA-201 cells — a modified version of human embryonic kidney (HEK) cells — by supplementing the cell culture medium with 20 nM CTxB AF-488 conjugates (the FRET donor). By using a pulsed laser for excitation, the frequency-domain lifetime information corresponding to each image pixel in the intensity-based image was transformed into a pixel on a Cartesian coordinate system, known as “phasor plot”, which exhibits a semicircular shape (Fig. 1b and Supplementary Fig. 1)[39]. This semicircle serves as a reference for pure single-exponential lifetimes, while multiexponential lifetimes are observed within the boundaries of the semicircle. Notably, shorter lifetimes demonstrate a clockwise movement along the semicircle. The phasor approach offers several advantages, including unbiased measurement of fluorescence lifetimes without the necessity of exponential fitting, and the capability to map multiple cursors to identify distinct lifetime species within confocal images[39-42] (Supplementary Fig. 1). In the subsequent phasor analysis of FRET, we specifically focused on the lifetime species originating from membrane-specific

fluorescence while excluding those from the internalized CTxB AF-488 due to endocytosis (see Supplementary Fig. 1 and Methods).

Figure 1

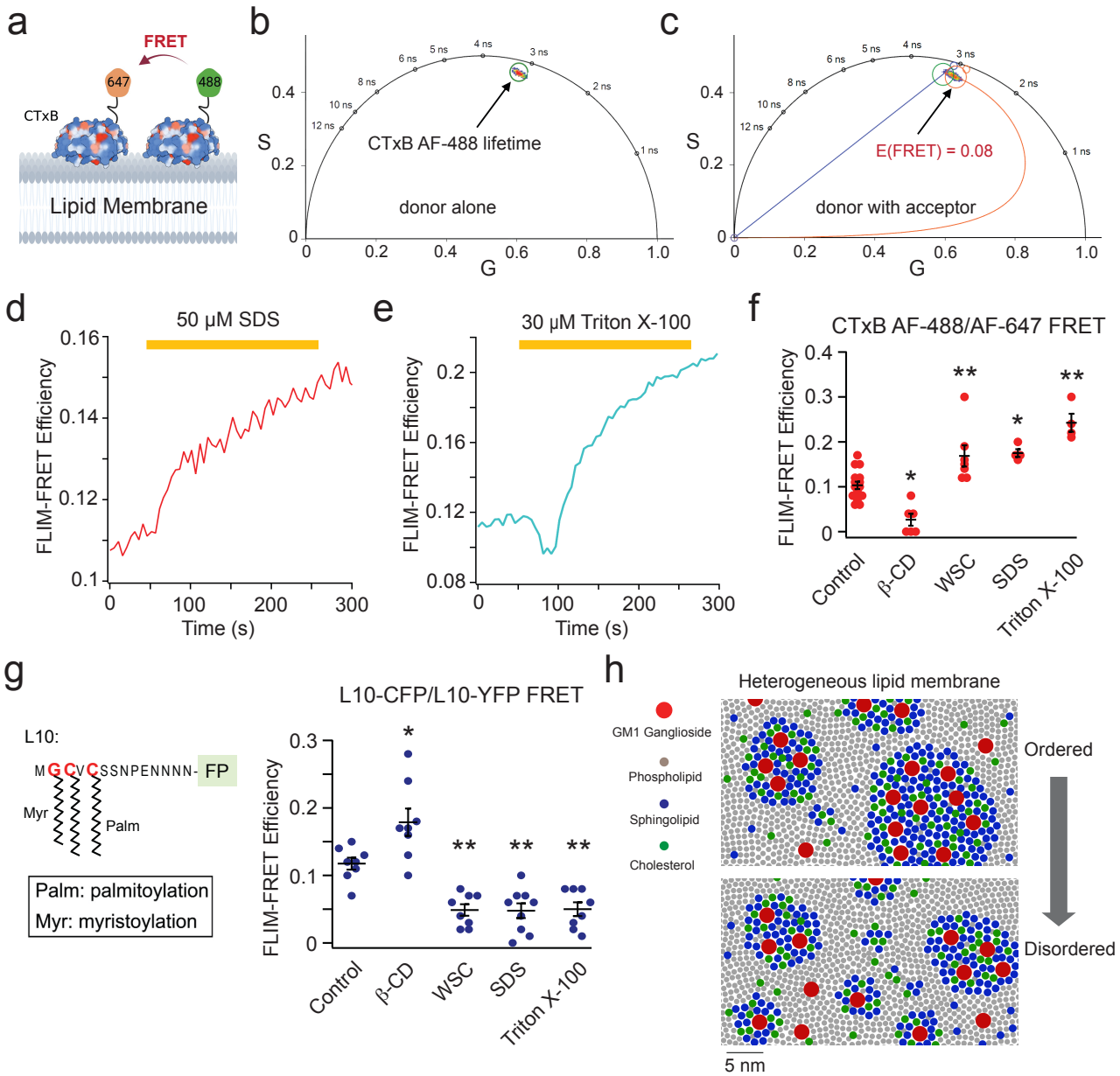

Figure 1. Assessing the OMD size using FLIM-FRET and FRET-based OMD probes.
(**a**) Experimental design using the FRET donor CTxB AF-488 together with the FRET acceptor CTxB AF-647. (**b-c**) Representative phasor plots of membrane-localized fluorescence of tsA201 cells from donor alone (CTxB AF-488) or together with the FRET acceptor (CTxB AF-647) conditions. Lifetime decreased with the addition of the acceptor. The FRET efficiency was calculated using the FRET trajectory (in red), showing that most of the lifetime species are positioned under a cursor with an average FRET efficiency of 0.11. (**d**) Time course of the increase of the FRET efficiency after applying 50 μM SDS. (**e**) Time course of the increase of the FRET efficiency after applying 30 μM

Triton X-100. (**f**) Summary data of the effects produced by the acute application of 5 mM β-cyclodextrin, 1 mg/ml WSC, 50 μM SDS and 30 μM Triton X-100 as shown in the panel D and E. Data shown are mean ± s.e.m., n = 4 – 16 cells, *p < 0.05; **p < 0.01. (**g**) Diagram of the L10 probe with N-terminal fluorescent protein; summary of the effects produced by the acute application of 5 mM β-cyclodextrin, 1 mg/mL WSC, 50 μM SDS and 30 μM Triton X-100. Data shown are mean + s.e.m., n = 8 – 9 cells, *p < 0.05; **p < 0.01. (**h**) Illustration depicting ordered and disordered lipid membrane domains featuring GM1 gangliosides, phospholipids, sphingolipids, and cholesterol. Smaller OMDs can only host a single CTxB, whereas larger OMDs have the capacity to bind multiple CTxB molecules. The scale bar corresponds to the estimated diameter of 7 Å for the area of a single phospholipid.

When both FRET donors (CTxB AF-488) and acceptors (CTxB AF-647) were present at a concentration of 20 nM, the lifetime characteristics observed in labeled tsA cells exhibited a clockwise shift towards shorter lifetimes, indicating the occurrence of FRET (Fig. 1c). To calculate the FRET efficiency, we used the FRET trajectory method enabled by the phasor plot. The FRET trajectory begins at the zero FRET efficiency position, aligning with the donor-alone cursor, and proceeds through the measured phasor species. Ultimately, it reaches the theoretical 100% FRET efficiency located at the origin coordinate (0, 0). At this point, the background noise primarily consists of white noise with an infinite lifetime contribution. Moreover, the FRET efficiency for each pixel was determined using the FRET trajectory function, generating a heatmap based on FRET efficiency, to provide an overview of the FRET distribution of a single cell. The observed FRET values in tsA cells were low, implying a potential scenario in which numerous OMDs had small nanometer sizes and bound only a single CTxB [36]. This observed size range aligns with the estimated dimensions of lipid domains derived from the correlation length of the critical fluctuations in plasma membrane vesicles[43,44].

We further investigated the impact of the cholesterol-extracting reagent β-cyclodextrin (β-CD) and amphipathic ionic detergents on FLIM-FRET efficiency in tsA-201 cells. Our findings indicate that the acute (~1 min) application of β-CD (5 mM) significantly reduced FRET, while supplementing cholesterols using 1 mg/ml preloaded methyl-β-CD-cholesterol complexes “water soluble cholesterol” (WSC) elevated FRET. In addition, the treatment of micromolar concentrations of ionic detergents, such as sodium dodecyl sulfate (SDS) and Triton X-100, increased FRET levels (Fig. 1 d-f). Amphipathic detergents are regarded as "cholesterol-phobic," indicating their tendency to compete with cholesterol in disordered domains. This competition promotes cholesterols to migrate towards larger OMDs. These results suggest that the extraction of cholesterols leads to separation of OMDs, the addition of cholesterol expands OMDs, whereas low concentrations of detergents (50 μM SDS or 30 μM Triton X-100) produce the merging of OMDs. Previous research has demonstrated that application of amphipathic detergents triggers clathrin-independent massive endocytosis (MEND) during the application of Triton X-100 or after the application of SDS[3,45]. MEND was able to internalize a

substantial portion of the plasma membrane[3,45,46]. Our results lend new support to the notion that detergent-induced MEND involves the coalescence of OMDs.

In addition, we employed a genetically encoded fluorescent marker of the OMD, L10, derived from the initial 10 amino acids of the Lck kinase[30,47,48], to reinforce our conclusions (Fig. 1g). The membrane-localized L10 probe, palmitoylated at two cysteines, exhibits colocalization with the OMDs in a GM1-independent manner. Using FLIM-FRET between L10-CFP and L10-YFP pairs, similar to the CTxB-based method, enabled us to monitor the OMD size. Consistent with prior research using this probe[48], treatment with β-CD increased FRET, while WSC treatment decreased FRET between L10-CFP and L10-YFP (Fig. 1g). Note that the directional change in FRET here opposes that observed with CTxB-based FRET, as the L10 probes come closer in distance when the OMD is smaller. In contrast, a smaller OMD size accommodates fewer CTxB molecules, often just one, leading to lower FRET (Fig. 1h). Therefore, while the CTxB and L10-based FRET pairs produce opposite FRET changes, the results imply consistent conclusions regarding OMD size. The FRET efficiency significantly decreased with low concentrations of detergents in the case of L10 probes, affirming OMD coalescence under these conditions (Fig. 1g). Furthermore, using another similarly engineered marker, S15, which tends to colocalize with disordered membrane regions, revealed a contrasting change in the S15-CFP/S15-YFP FRET after β-CD treatment (Supplementary Fig. 3). These results serve to further validate our approach using CTxB-based FRET pairs.

Domain size of OMDs varies depending on the cell type

Using FLIM-FRET and CTxB Alexa Fluor 488 or 647 conjugates, we conducted measurements to determine FRET efficiency indicative of OMD size in various primary cells and cultured cell lines (Fig. 2). These included isolated rat dorsal root ganglion (DRG) somatosensory neurons, isolated mouse primary hepatocytes, isolated mouse dermal fibroblasts, isolated mouse cardiac pacemaker cells, cultured embryonic rat cardiomyocytes (H9c2 cells), and tsA-201 cells. Importantly, significant differences were observed between the different cell types, with pacemaker cells demonstrating the highest FRET levels (Fig. 2 a-d). Hepatocytes, known for their cholesterol synthesis, also showed high FRET, indicative of large OMDs. Conversely, fibroblasts, embryonic kidney cells, and cultured H9c2 cardiomyocytes exhibited low FRET. These results document substantial heterogeneity in lipid domain sizes in the plasma membrane of different cell types.

Figure 2

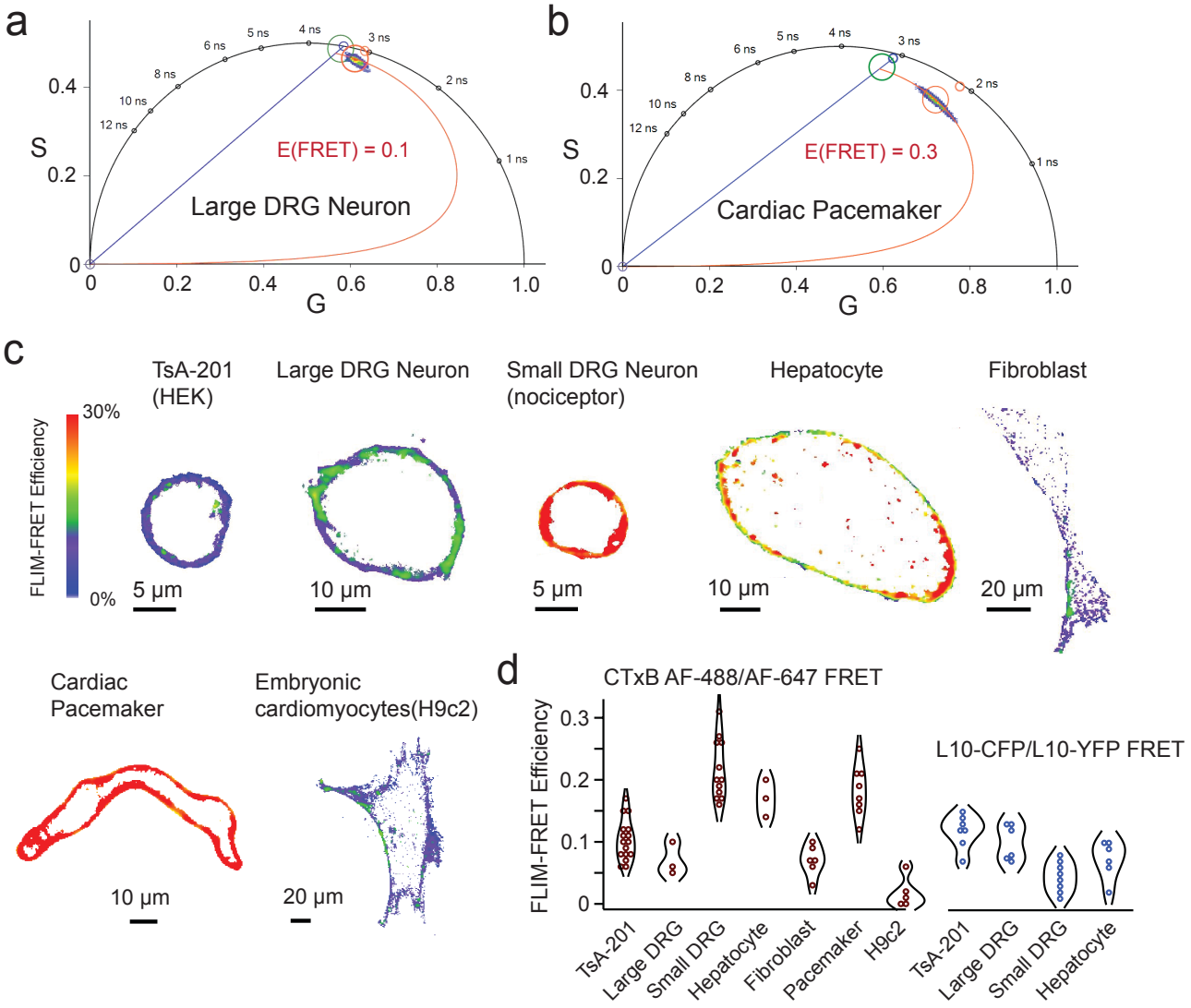

Figure 2. Live-cell FLIM-FRET reports the OMD abundance in different cell types.
(**a**) Representative phasor plot of a large DRG neuron showing the CTxB AF-488/CTxB AF-647 FLIM-FRET. Using the FRET trajectory, the average FRET efficiency is estimated at 0.1. (**b**) Representative phasor plot of a cardiac pacemaker cell. Using the same FRET pair and FRET trajectory method, the average FRET efficiency of this cell is estimated at 0.3. (**c**) Representative single-cell heatmap images of the localized FLIM-FRET efficiencies of different cell types as illustrated. (**d**) Summary data of the FRET efficiencies of different cell types tested in the panel C using the CTxB-based FRET pairs; n = 3 – 16 cells. Summary data of comparable experiments using transiently transfected L10-based FRET pairs are also shown; n = 6 – 8 cells.

DRG neurons, which are pseudo-unipolar sensory neurons, play a crucial role in transmitting sensory information from the periphery to the spinal cord, particularly for pain sensation[49,50]. DRG neurons that have a relatively small diameter, and mostly unmyelinated axons (C-fibers) are

responsible for relaying nociceptive, thermal, and mechanoreceptive signals. Conversely, large-diameter DRG neurons, which are non-nociceptive, mostly transmit mechanoreceptive and proprioceptive signals through large-diameter myelinated axons[50]. We found that the CTxB-based FLIM-FRET efficiency in the smaller neurons with cell capacitance less than ~28 pF was significantly greater than that in neurons of larger size (Fig. 2d). To further substantiate this finding, we extended the application of L10 probe-based FRET pairs to primary cells, including small and large DRG neurons as well as hepatocytes. In line with the observed larger OMD size in small DRG neurons and hepatocytes, the L10 FRET pairs exhibited reduced FRET efficiencies in these cell types in comparison to large DRG neurons and tsA cells (Fig. 2d). These results suggest that heterogeneity in the OMD size of sensory neurons could potentially affect the excitability of these neurons.

Lipid tail structure influences the OMD size in an *in vitro* fatty liver model

The formation of OMDs relies on the tight packing of sphingolipids with saturated lipid tails and cholesterols. To investigate the impact of supplementing fatty acids such as palmitate and oleate on the FRET between CTxB-conjugated AF-488 and AF-647, we implemented protocols commonly used to generate *in vitro* models of fatty liver disease. For hepatocytes, the overnight treatment of additional 300 μM palmitate in the culture medium in the presence of albumin resulted in a significant increase in FRET efficiency (Supplementary Fig. 4 a and b). However, combining 200 μM palmitate with 100 μM oleate nearly abolished the FRET signal, indicating a division of the OMDs (Supplementary Fig. 4 a and b).

In this cell model of fatty liver, the addition of palmitate induces cellular stress on the cells, potentially leading to lipotoxicity[51,52]. Conversely, the inclusion of oleate can act as a preventive measure against such cellular stress[51]. Notably, oleate is a monounsaturated fatty acid (18:1) with a double bond that induces a tilt and increased flexibility in the carbon chain, in contrast to the saturated aliphatic chain (16:0) of palmitate. Introducing the tilted aliphatic chain to the lipid membrane greatly impedes the formation of OMDs. These results suggest that the lipotoxicity introduced by palmitate is associated with a substantial increase in the abundance of OMDs, and oleate supplementation can rescue this effect. Moreover, these findings further validate the reliability of our approach using the CTxB-based probes in assessing the size and abundance of OMDs.

Disrupting OMDs accelerates action potential firing of nociceptor DRG neurons

Following the validation experiments using FLIM-FRET to measure OMDs in various cell types, we shifted our focus to DRG sensory neurons. To investigate the potential effects of disrupting OMDs on the firing behavior of small nociceptor DRG neurons, we conducted whole-cell current clamp experiments. Specifically, we tested whether altering the size of these domains has an impact on the generation of action potentials. To achieve this, we used an acute application (30-60 sec) of β-CD (5 mM) to disrupt OMDs, as confirmed by the observed reduction in FRET efficiency using the CTxB FRET pairs following its application (Fig. 3a). OMD disruption was also confirmed by an increase in L10 probe-based FRET following β-CD treatment (Fig. 3b).

We found that the β-CD treatment led to an increase in the spontaneous action potential firing activity of nociceptor DRG neurons (Fig. 3c). We supplemented our cell culture with nerve growth factor (NGF), which promoted the spontaneous firing of DRG neurons[53], mimicking a moderate pain condition. Furthermore, with positive current injections, the action potential firing frequency increased as expected. Cells treated with β-CD also showed more frequent current injection-elicited firing than control cells (Supplementary Fig. 5 a and b). The observed augmentation in the spontaneous and the elicited firings of these DRG neurons by the disruption of OMDs suggests that these neurons exhibit a state of hyperactivity. This finding is consistent with a pain phenotype, suggesting that the alteration of OMDs contributes to the modulation of neuronal excitability during the development of chronic pain. We also tested the addition of cholesterol using acute 1 mg/ml WSC treatment. In the presence of NGF, the percentage of spontaneous firing reduced from 50% for the control to 30% after the WSC treatment. The ability of nociceptor DRG neurons to fire action potentials with positive current injections was also compromised after the WSC treatment (Supplementary Fig. 5c). These results suggest that expanding the OMD by cholesterol enrichment could be used to rescue membrane hyperexcitability and potentially alleviate pain.

Figure 3

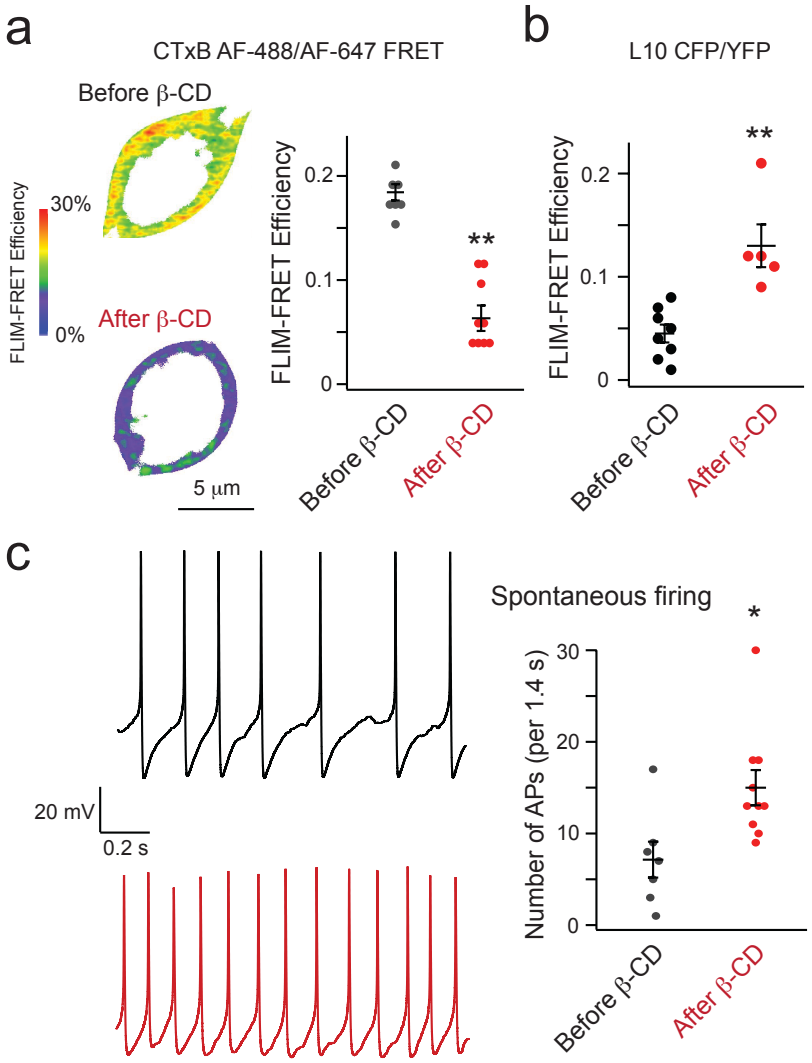

Figure 3. Disrupting the OMD accelerates the action potential firing of DRG neurons.
(**a**) Representative heatmap FRET images of small nociceptor DRG neurons before and after the acute β-cyclodextrin treatment. Summary data of the effect of β-cyclodextrin on the FRET efficiency between CTxB 488/647 probes. Data shown are mean ± s.e.m., n = 7 – 9; **p < 0.01. (**b**) Summary data of the effect of β-cyclodextrin on the FRET efficiency between the L10-CFP and L10-YFP pair. Data shown are mean ± s.e.m., n = 5 – 8, **p < 0.01. (**c**) Representative spontaneous action potential firings of small DRG neurons before and after the acute β-cyclodextrin treatment. Summary data of the effect of β-cyclodextrin on number of the spontaneous action potential firings during the 1.4 second recording period. Data shown are mean ± s.e.m., n = 7 – 10, *p < 0.05.

Considering the role of oleate supplementation in disrupting membrane order, we applied an overnight treatment of 100 μM bovine serum albumin (BSA)-conjugated oleate to DRG neurons. We found that compared to the BSA alone control treatment, the oleate treatment potentiated the action potential firing of small DRG neurons (Supplementary Fig. 8a). These results suggest that oleic acids may play a role in regulating sensory neurons through perturbing the OMD of the membrane.

Paclitaxel, a drug that produces chemotherapy-induced neuropathy, reduces the OMD size

Chemotherapy-induced peripheral neuropathy (CIPN) affects approximately 40% of chemotherapy patients and lacks effective treatments at present[54]. Paclitaxel (Taxol), a widely used chemotherapeutic agent, is known to induce both acute and chronic CIPN. Considering the role of OMDs in regulating voltage-gated ion channels and the previously reported role of paclitaxel in regulating membrane lipid domains[14,30,55,56], we postulated that the paclitaxel-triggered neuropathy might be associated with alterations in the lipid domain organization of nociceptor DRG neurons. Using FLIM-FRET and the same CTxB-mediated labeling approach, our results revealed that overnight treatment using either a low concentration (10 nM) or a saturating concentration (1 μM) of paclitaxel resulted in a significant reduction in the FLIM-FRET efficiency of small DRG neurons (Fig. 4 a-d). In contrast, the same treatment of paclitaxel did not produce a considerable reduction in the FLIM-FRET efficiency of large DRG neurons, which could be because the smaller OMDs in large DRG neurons are more resistant to this treatment (Fig. 4d). In addition, consistent with the results seen with the β-CD treatment, the action potential firing of nociceptor DRG neurons accelerated after the treatment of 10 nM paclitaxel (Fig. 4 e and f). This finding implies that the disruption of OMDs is associated with and may play a direct role in paclitaxel-induced neuropathy. Since paclitaxel targets tubulin and microtubules, this finding may suggest a linkage between OMDs and cellular cytoskeleton.

Figure 4

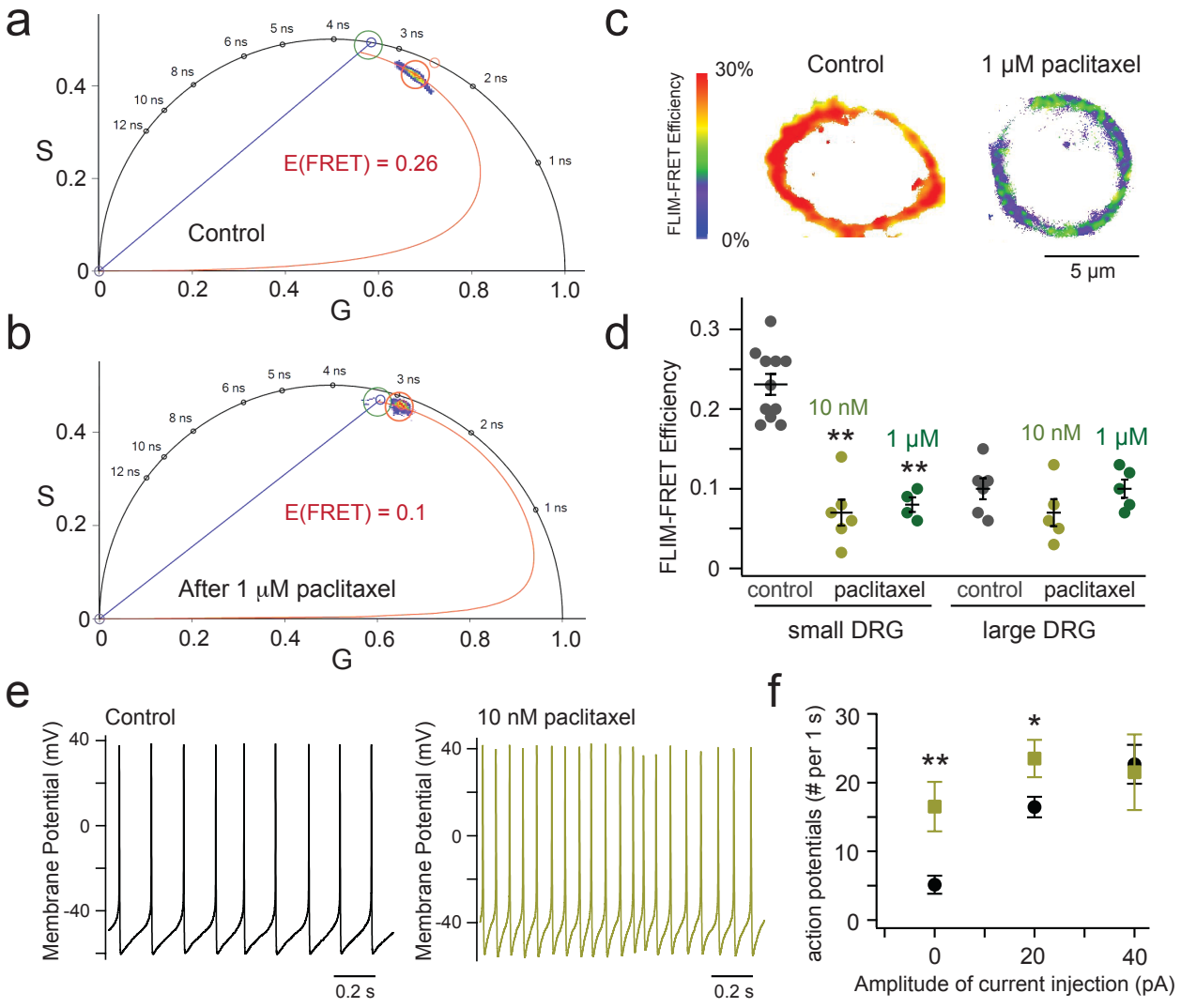

Figure 4. Effects of the CIPN-related drug paclitaxel on the OMD size and excitability of DRG neurons. (**a**-**b**) Representative phasor plots of a small nociceptor DRG neuron before and after the overnight 1 μM paclitaxel treatment, using the CTxB-AF 488/CTxB-AF 647 FLIM-FRET. (**c**) Representative heatmap FRET images of small nociceptor DRG neurons before and after the paclitaxel treatment. (**d**) Summary data of the effect on FRET efficiencies of small and large DRG neurons caused by the 10 nM or 1 μM paclitaxel treatments. Data shown are mean ± s.e.m., n = 4 – 11, *p < 0.05; **p < 0.01. (**e**) Representative spontaneous action potential firings of small DRG neurons before and after the overnight 10 nM paclitaxel treatment. (**f**) Summary data results of the number of action potentials of small DRG neurons plotted against of the amplitude of current injection. Data shown as mean ± s.e.m. n = 4 – 11, *p < 0.05; **p < 0.01.

Facilitated native HCN currents of nociceptor DRG neurons after OMD disruption

Pacemaker hyperpolarization-activated HCN channels are key players in regulating the electrical excitability of DRG neurons[18,22]. With physiological electrochemical driving force, they conduct inward currents primarily carried by sodium ions, which depolarize the membrane potential, bringing it closer to the threshold of firing an action potential. The native HCN channel currents of small nociceptor DRG neurons were elicited by a series of hyperpolarizing voltages using whole-cell voltage clamp, exhibiting a conductance-voltage (G-V) relationship with a voltage for the half maximal activation ($V_{1/2}$) of -94 mV, close to that of HCN2 channels (Fig. 5 a, c, d)[18,22]. Addition of a saturating concentration of cyclic adenosine monophosphate (cAMP, 0.5 mM) in the pipette solution shifted the G-V relationship to more depolarized voltages, with a $V_{1/2}$ of -84 mV (Fig. 5 b, c, d). Contrastingly, large DRG neurons abundantly express the HCN1 isoform, which is not particularly sensitive to cAMP[22]. The change in the $V_{1/2}$, by 0.5 mM cAMP for the G-V relationship of endogenous HCN channels within large DRG neurons is around 5 mV (Supplementary Fig. 10 b), only half of that observed in small DRG neurons.

We found that disrupting OMDs using the same acute application of β-CD altered the hyperpolarization-dependent activation of endogenous HCN channels of small nociceptor DRG neurons. In the absence of cAMP supplementation, β-CD treatment did not shift the $V_{1/2}$, but instead increased the slope factor of the G-V relationship according to the Boltzmann sigmoidal fit, suggesting less gating charge movement during the channel activation (Fig. 5 c-e). The estimated number of charge movement for the channel activation was 2.83 ± 0.07 for the control and 2.48 ± 0.1 after the β-CD treatment ($p < 0.05$) (see Methods). Furthermore, with 0.5 mM cAMP in the pipette solution, β-CD treatment also failed to further shift the $V_{1/2}$ for the channel activation to more depolarized voltages. Nevertheless, the slope factor associated with the Boltzmann fit of the G-V relationship increased significantly, suggesting that in the presence of cAMP, the estimated gating charge movement across the membrane was decreased (Fig. 5 c-e). With cAMP, the calculated number of charge movement for the channel activation was 2.98 ± 0.8 for the control and 2.41 ± 0.2 after the β-CD treatment ($p < 0.05$). This change of the effective gating charge movement could be related to the membrane-thinning effect after disrupting the OMD, which might narrow the focused electric field of the membrane. On the other hand, the shallower slope of the G-V relationship produces a much higher channel open probability at the RMP (around -60 mV). Specifically, the relative open probability of HCN channels quantified at -60 mV and -70 mV increased after the β-CD treatment in both the presence and absence of cAMP (Supplementary Fig. 7 a and b). This increase in open probability of HCN channels near the RMP could effectively depolarize the membrane to promote action potential firing. In addition, using the obtained

parameters from the Boltzmann fits, the free energy (ΔG) associated with the HCN channel activation can be calculated. It is evident that a lower amount of free energy was required to open the channel after the β-CD treatment, regardless of the presence of cAMP (Fig. 5f). This observation suggests that disruption of the OMD led to an energetic facilitation of the HCN channel opening process, which is physiologically important at the RMP.

Next, we evaluated the effect of 10 nM paclitaxel on the gating of HCN channels in small DRG neurons (Fig. 5 d-f). Similar to the influence of β-CD on HCN currents, the HCN G-V relationship after paclitaxel treatment manifested a mostly unchanged $V_{1/2}$, but a significantly shallower slope. With paclitaxel, the measured number of charge movement for the channel activation was decreased to 2.42 ± 0.14 in the absence of cAMP and 2.43 ± 0.14 in the presence of 0.5 mM cAMP. The ΔG associated with the HCN channel activation was also decreased considerably after the paclitaxel treatment (Fig. 5f). On the contrary, the administration of 10 nM paclitaxel did not produce a significant effect on the gating parameters of HCN channels in large DRG neurons (Supplementary Fig. 10). These findings suggest that the paclitaxel-induced change in HCN channel gating, likely specific to small DRG neurons, might contribute to the hyperexcitability observed in DRG neurons following this type of chemotherapy. Moreover, the overnight oleate treatment similarly decreased the apparent gating charge movement for native HCN channels in small DRG neurons, suggesting a similar mechanism of disrupting OMDs underlying the oleate-induced neuronal hyperexcitability (Supplementary Fig. 8 b-d).

OMD disruption impacts the domain localization and voltage sensor conformations of HCN2 channels

We also tested whether the primary HCN-channel type HCN2 channels expressed in nociceptor DRG neurons are localized in OMDs. Using the phasor FLIM-FRET method and the OMD-specific probe L10-CFP, we found considerable FRET between L10-CFP and a human HCN2-YFP construct expressed in tsA 201 cells (Supplementary Fig. 6). L10-CFP was shown to have low FRET with human HCN1-YFP but exhibited higher FRET with the human HCN4-YFP[30]. When transiently transfected into small DRG neurons, the coexpression of L10-CFP probes and HCN2-YFP channels resulted in significant FRET (Fig. 5 g and i). These results indicate that, like human HCN4 channels, human HCN2 channels exhibit a tendency to localize to OMDs. Additionally, both acute treatment with 5 mM β-CD and overnight exposure to 10 nM paclitaxel led to a substantial reduction in FRET between L10-CFP and HCN2-YFP (Fig. 5 h and i). Consistent with the OMD localization of hHCN2, the FRET between

S15-CFP and HCN2-YFP was significantly lower compared to that observed with L10-CFP (Supplementary Fig. 6).

Figure 5

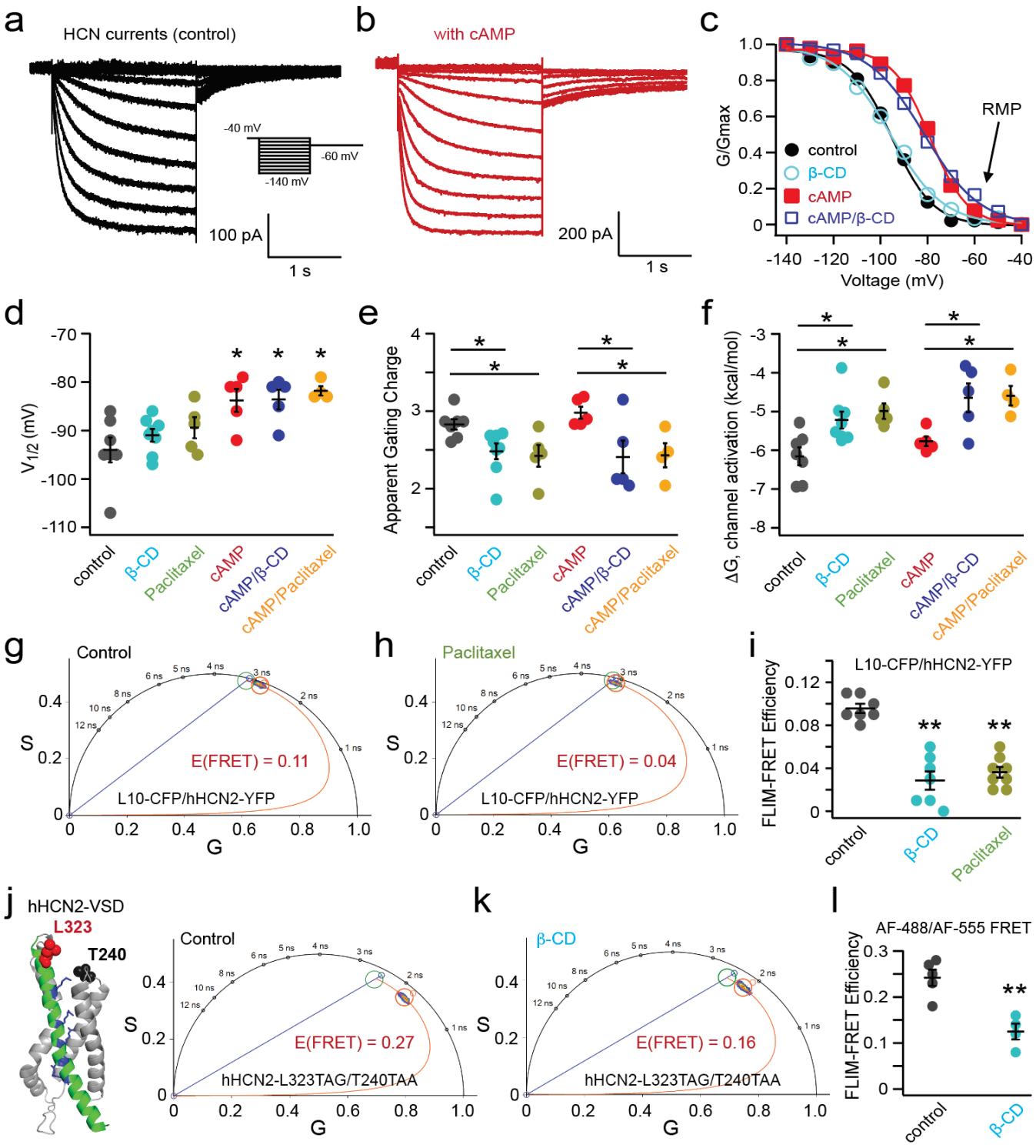

Figure 5. Impact of OMD disruption on the HCN channel behavior and localization: unraveling a potential mechanism for modulating nociceptor DRG neurons.
(**a**-**b**) Endogenous HCN currents elicited by a series of hyperpolarizing voltage pulses from -40 mV to -140 mV, in the presence (red) or in the absence (black) of 0.5 mM cAMP in the pipette solution. (**c**) Representative conductance-voltage relationships of the endogenous HCN currents in the following conditions: control, with β-cyclodextrin, with cAMP, and with both β-cyclodextrin and cAMP. (**d**-**f**) Summary data of effects of β-cyclodextrin, 10 nM paclitaxel, and cAMP on the gating parameters of endogenous HCN currents: $V_{1/2}$, estimated gating charge, and the free energy for the channel activation. Data shown are mean ± s.e.m., n = 4 – 8, *p < 0.05; **p < 0.01. (**g**-**h**) Representative phasor plots showing FRET between L10-CFP and hHCN2-YFP expressed in small DRG neurons before and after 10 nM paclitaxel treatment. (**i**) Summary data of the effects of the β-cyclodextrin and paclitaxel on the FRET between L10-CFP and hHCN2-YFP. Data shown are mean ± s.e.m., n = 7 – 8, *p < 0.05; **p < 0.01. (**j**) Cartoon showing the design of the FRET sites at the VSD of HCN2 channels and the representative phasor plot showing FRET between donor AF-488 labeled to the HCN2-L323 site and the FRET acceptor AF-555 labeled to the HCN2-T240TAA site (**k**) Representative phasor plot of the same FRET pair as in panel J following acute 5 mM β-cyclodextrin treatment. (**l**) Summary data of the effect of the β-cyclodextrin on the FRET efficiency of hHCN2-L323TAG/T240TAA channels. Data shown are mean ± s.e.m., n = 7 – 8, *p < 0.05; **p < 0.01.

We proceeded to test whether the voltage sensor S4 helix of human HCN2 undergoes rearrangement following disruption of the OMD, similar to previous findings in human HCN4 channels[30]. The structural foundation of gating charge is established by positively charged arginines within the S4 transmembrane helix, which traverses the electric field across the membrane. Employing a dual stop codon (amber TAG and ochre TAA)-mediated suppression strategy, we incorporated two noncanonical amino acids, trans-cyclooct-2-ene-L-lysine (TCO*K) and N-propargyl-L-lysine (ProK), using click chemistry for site-specific fluorescence labeling, same as in prior studies[30,57]. This system uses two orthogonal archaea pyrrolysine-based stop-codon suppression strategies in tsA201 cells[57]. For fluorescence labeling of these noncanonical amino acids, we employed the strain-promoted inverse electron-demand Diels–Alder (IEDDA) cycloaddition reaction to conjugate TCO*K with tetrazine-conjugated AF-488[57]. For the site of ProK, copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) was used to attach a picolyl azide-conjugated Alexa Fluor 555 (AF-555)[57]. The FRET pair for hHCN2 was established with L323TAG at the N-terminal side of the S4 helix and T240TAA in the S1-S2 helix loop of the voltage-sensing domain of the channel (Fig. 5j). Upon the acute application of 5 mM β-CD, we observed a significant decrease in FRET between AF-488 and AF-555 using the phasor FLIM-FRET method (Fig. 5 k and l). This reduction closely mirrored the changes in FRET between corresponding sites in hHCN4 channels[30]. In contrast, minimal FRET change was detected between comparable sites in hHCN1 channels following β-CD treatment, as shown previously[30]. When using a high potassium (120 mM KCl) solution to depolarize the membrane, increased FRET between AF-488 and AF-555 was detected (Supplementary Fig. 9 f and g). However, the effect of β-CD treatment on the FRET efficiency was much attenuated with the membrane depolarization, suggesting a dependence of the OMD-mediated modulation on the functional state of the voltage sensor (Supplementary Fig. 9 f and g). This state dependence of FRET change by OMD disruption was previously observed in hHCN4 channels[30].

Collectively, these findings suggest that the voltage sensor S4 helix of hHCN2 undergoes rearrangements when the channel translocates from the ordered membrane domain (OMD) to disordered membrane regions. This may underlie the structural mechanism of the observed decrease in apparent gating charge of HCN channels after disrupting the OMD in DRG neurons.

Modulation of HCN channels by OMDs in a nerve injury-induced model of neuropathic pain

Mechanical allodynia and neuropathic pain are known to manifest following peripheral nerve damage. To define the potential correlation between nerve injury-induced neuropathic pain and changes in the size of OMDs, we also used the FLIM-FRET method to assess OMD size in DRG neurons in a spared nerve injury (SNI) model of neuropathic pain[58] (Fig. 6). In the SNI model, we measured the percentage of spontaneous action potential firing of small nociceptor DRG neurons, which elevated from 40% (n = 10) on the contralateral side of the injury to 80% on the ipsilateral side (n = 11). Furthermore, our study revealed that the FRET efficiency, which reports OMD size, displayed a reduction in both large and small DRG neurons (Fig. 6b). Specifically, the FRET efficiency measured from small DRG neurons decreased from 10.4 ± 1.8% for the control neurons on the contralateral side of the injury to 4.3 ± 0.5% for the neurons on the ipsilateral side of the injury. And for large DRG neurons that are characterized by small OMDs, the FRET changed from 2.5 ± 1.0% for the contralateral ones to 1 ± 0.6% for the ipsilateral ones. These results suggest that downsizing the OMD is not only observed in the *in vitro* paclitaxel-induced neuropathy model but also in the *in vivo* nerve injury-induced pain model, implying that it might be a general phenomenon associated with neuropathic pain (Fig. 6). Additionally, we coexpressed L10-CFP probes and hHCN2-YFP channels in small DRG neurons within the SNI model to assess the colocalization of HCN2 channels with the OMD. We observed pronounced FRET for the control neurons on the contralateral side, whereas this FRET was significantly lower for neurons on the ipsilateral side of the injury (Fig. 6 c and d). These results indicate the disruption of OMDs in the SNI pain model, consequently leading to a predominant localization of HCN2 channels in disordered lipid domains.

We assessed the gating parameters of native HCN channels in neurons of the SNI pain model. This included determining the $V_{1/2}$ for channel activation, the apparent gating charge associated with channel activation, and the ΔG required for channel activation, with and without cAMP, in neurons from both the contralateral and ipsilateral sides of the injury. Our findings aligned with observations from β-CD treated neurons and those from the paclitaxel-induced CIPN pain model. While the $V_{1/2}$ for channel activation remained unchanged when comparing the contralateral and ipsilateral sides of the injury (Fig.

6e), there was a notable reduction in the apparent gating charge on the ipsilateral side (Fig. 6f). Consequently, the calculated ΔG was significantly diminished in neurons from the ipsilateral side (Fig. 6g). Moreover, with 0.5 mM cAMP present in the pipette solution, there was an increase in $V_{1/2}$ for channel activation (Fig. 6e and Supplementary Fig. 11). The effects on the gating charge and ΔG for channel activation persisted with the supplementation of cAMP (Fig. 6 f and g), consistent with a direct modulation of the voltage sensor of HCN channels by OMDs and an independence of cAMP binding.

Figure 6

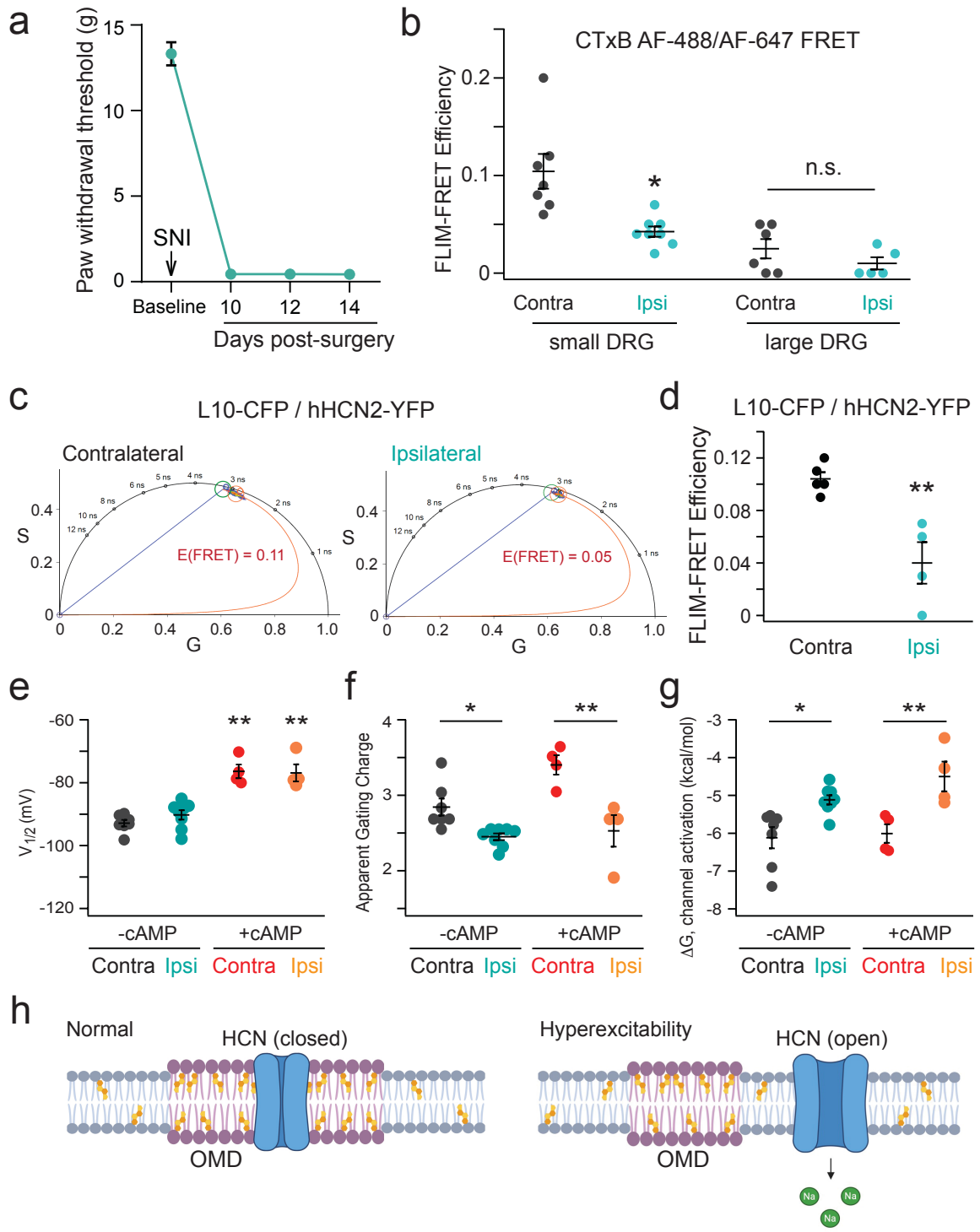

Figure 6. Assessing the OMD size and HCN2 channel localization in DRG neurons using a spared nerve injury model of neuropathic pain.

(**a**) Rats develop mechanical hypersensitivity after spared nerve injury. Neuropathic pain was induced through the spared nerve injury (SNI) model of chronic neuropathic pain in Sprague-Dawley rats. Graph showing the paw withdrawal thresholds of rats (n = 6) before and after (day 0 onwards) SNI surgery. Paw withdrawal threshold decreased after SNI showing the development of a chronic neuropathic pain state and hypersensitivity to

mechanical stimuli. (**b**) Summary data of the FRET efficiencies between CTxB AF-488 and CTxB AF-647 in contralateral versus ipsilateral small and large DRG neurons from the SNI pain model. Data shown are mean ± s.e.m., n = 5 – 8, *p < 0.05; **p < 0.01. (**c**) Representative phasor plots showing FRET between L10-CFP and hHCN2-YFP in contralateral versus ipsilateral DRG neurons. FRET efficiency is lower in ipsilateral neurons. (**d**) Summary data of the L10-CFP/hHCN2-YFP FLIM-FRET efficiencies in contralateral and ipsilateral neurons. Data shown are mean ± s.e.m., n = 4 – 5, *p < 0.05; **p < 0.01. (**e**-**g**) Summary data of effects of nerve injury on the gating parameters of endogenous HCN currents in nociceptor DRG neurons: $V_{1/2}$, estimated gating charge, and the free energy for the channel activation, either in the presence or absence of cAMP. Data shown are mean ± s.e.m., n = 4 – 8, *p < 0.05; **p < 0.01. (**h**) Cartoons depicting how the localization of HCN channels within OMDs alters channel opening and affects the membrane excitability of DRG neurons in neuropathic pain. Cholesterols are highlighted in orange and OMDs are distinguished as thicker membrane regions compared to disordered membrane areas.

Discussion

Neuropathic pain[59,60], a prevalent and debilitating condition, arises from nerve injury, infections, diabetes, and frequently, "channelopathies." Despite its widespread occurrence, effective treatments for neuropathic pain are currently lacking. To manage severe neuropathic pain, opioids are commonly prescribed; however, their use carries the risk of addiction or overdose[59,61]. Recently, pacemaker HCN channels have emerged as promising therapeutic targets for addressing neuropathic pain[23,62,63]. This current study not only enhances our understanding of HCN channels, but also provides insights into potential strategies to combat neuropathic pain. Additionally, we investigated a previously overlooked aspect for the pain research: how membrane compartmentalization, particularly OMDs, influences ion channels[3]. Our research pinpointed a modulation of the pacemaker HCN channel by OMDs, which is dependent on the altered rearrangement of voltage sensors in distinct lipid domains[30] (Fig. 6h). This finding highlights OMDs and HCN channel voltage sensors as promising therapeutic targets for achieving pain relief.

During evolution, some cells, particularly neurons, developed the ability to carry out rapid, repetitive action potential firing. Presumably, this ability required a host of biochemical and biophysical adaptations. Our research suggests that electrically active cell membranes may require larger OMDs as a protective and adaptive mechanism to maintain a stable membrane structure that supports or accommodates repetitive action potential firing. In this study, we observed that small nociceptive DRG neurons possess larger OMDs than large DRG neurons. This finding is consistent with the fact that a low percentage (~15%) of large DRG neurons display spontaneous firing under our experimental condition in the presence of NGF in the culture medium. On the other hand, a significant percentage (~50%) of small DRG neurons exhibit spontaneous firing. This correlation supports the idea that OMDs play a role in adapting to the electrical demands of the cell. Our hypothesis gains further support from the data collected from five other types of cells tested. For instance, cardiac pacemaker cells, which fire rhythmic action potentials, display the largest OMD size among the non-neuronal cell types examined in this study. Future research is needed to more thoroughly examine the concept that the lipid composition and compartmentalization of the cell membrane adapt in response to distinct electrical requirements of the cell.

Our FLIM-FRET methodologies, specifically the use of the CTxB probes for quantifying the properties of OMDs of living cells, can be interpreted within the context of thermodynamic principles governing the phase separation in heterogeneous lipid membranes[43,64,65]. This biophysical concept

involves a two-dimensional square-lattice Ising model to understand the critical liquid-ordered to liquid-disordered transition within the membrane[66]. Critical fluctuations in lipid membranes, measured using the fluorescence autocorrelation function, dramatically change near the miscibility critical point[43]. The correlation length of the critical fluctuations can report the size and lifetime of phase-separated domains[43,67]. In this framework, a heightened FRET signal, measured between the CTxB-based FRET pairs, indicates an increased correlation length and enlarged OMDs. A greater correlation length amplifies the likelihood of multiple GM1 gangliosides in the OMDs to facilitate CTxB binding. Additionally, it elevates the probability of CTxB molecules being in proximity to one another, thereby facilitating FRET.

Our study establishes novel connections between the pacemaker HCN channel, membrane compartmentalization, and neuropathic pain. We speculate that neurotrophic factors and cytokines released from injured peripheral nerves or during paclitaxel treatment could lead to altered cell signaling and decreased OMD size. This, in turn, affects the trafficking and localization as well as the function of ion channels including voltage-gated sodium channels, as previously observed[56,68]. Here, our findings demonstrate that disrupting OMDs makes HCN channels more conductive and sensitive to voltage. In a separate study, we showed that the integrity of the OMD plays a crucial role in the rearrangement of voltage sensors[30]. We believe that the movement of the unusually long S4-helix of HCN channels is highly sensitive to the “hydrophobic (mis)match” with the lipid bilayer's hydrophobic region. Considering that this hydrophobic mismatch often plays a critical role in influencing the tilt angle of transmembrane helices[69,70], we hypothesize that re-localizing HCN channels from OMDs to disordered membrane regions increases the tilt angle of the S4 helix. This phenomenon can be attributed to the difference in lipid bilayer thickness comparing OMDs with disordered membrane regions[5]. Consequently, this increase in the tilting motion of the S4 helix facilitates pore domain activation through its interaction with the channel pore domain[71]. Interestingly, disrupting OMDs leads to reduced charge movement across the electric field of HCN channels, consistent with an alteration in voltage-sensor movement of HCN channels, or alternatively, suggesting a more focused electric field of the membrane. Similar changes of the gating charge movement could happen for other types of voltage-gated ion channels when OMDs are disrupted. The modulation of ion channels by OMDs and cholesterol stands as an important feature in neuronal regulation. Notably, this modulatory influence on $GABA_A$ receptors[72] and TREK potassium channels[6,73] contributes to molecular mechanisms underlying general anesthesia. Our observations support the notion that changes in OMDs have significant implications for ion channel function and contribute to the development of chronic neuropathic pain.

Materials and Methods

Molecular Biology, Cell Culture and Transfection

Wild-type human HCN2 construct and the HCN2-L323TAG/T240TAA (Opal TGA stop codon used as the functioning stop codon) were synthesized by VectorBuilder (Chicago, IL). The construct was amplified using a mammalian gene expression vector containing a carboxyl-terminal YFP and a CMV promoter. The L10 and S15 fluorescent probes are gifts from Prof. Bertil Hille (University of Washington, Seattle, WA). DNA concentration was measured using a Nanodrop OneC spectrophotometer (Life Technologies, Grand Island, NY).

The dual stop-codon suppression was done as previously described[30,57]. The tsA201 cells were co-transfected with two plasmids: pAS_4xU6-PylT M15(UUA) FLAG-Mma PylRS (Mma PylT/RS) and pAS_4xhybPylT A41AA C55A FLAG-G1 PylRS Y125A (G1PylT/RS) tRNA/aminoacyl-tRNA synthetase pairs from Addgene (Watertown, MA, #154774 and #154773 respectively), along with a third plasmid containing the hHCN2 channel construct with dual stop-codon mutations for selective incorporation of trans-cyclooct-2-ene-L-lysine (TCO*K) and N-propargyl-L-lysine (ProK) (SiChem, Bremen, Germany). At the TCO*K site, IEDDA reaction was performed by applying 1-2 μM tetrazine dye (Click Chemistry Tools, Scottsdale, AZ) to the culture medium and incubating it with the cells at 37°C for 30 minutes. At the ProK site, the copper-catalyzed azide-alkyne cycloaddition reaction was performed using 50 μM $CuSO_4$, 250 μM THPTA (Tris(benzyltriazolylmethyl)amine), and 2.5 mM ascorbic acid. Cells were incubated with 5 μM picolyl azide dye (Click Chemistry Tools, Scottsdale, AZ) for 10 minutes at room temperature.

The tsA-201 cells (a variant of human embryonic kidney HEK cells) were obtained from Sigma-Aldrich (St. Louis, MO). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) in a humidified incubator at 37°C with 5% CO_2 in tissue culture dishes (CellTreat, Pepperell, MA). Transfection was performed on 70%-90% confluent tsA-201 cells using the lipofectamine 3000 Kit (Invitrogen, Carlsbad, CA, #L30000080), following previously established protocols[47,74]. We employed lipofectamine 3000 and electroporation transfection techniques, to introduce L10 and S15 probes into DRG neurons. We found that the lipofectamine method was more favorable for transfecting small DRG neurons, whereas electroporation exhibited higher efficiency with larger DRG neurons. For primary mouse hepatocytes, we used lipofectamine 3000 for the transient transfection of L10 probes.

Additionally, we found some large-sized primary cultured cells are resistant to lipofectamine-mediated transfection, e.g., fibroblasts were not able to be transfected using the lipofectamine method.

The cholera toxin subunit B-Alexa Fluor™ 488 and 647 conjugates were obtained from Thermo Fisher Scientific and applied to cultured cells at a concentration of 20 nM for a duration of ~10 minutes. The binding affinity of CTxB to GM1-enriched membrane falls within the picomolar range[35]. β-cyclodextrin and water-soluble cholesterol were purchased from Sigma-Aldrich (St. Louis, MO).

Isolated oocytes were purchased from EcoCyte (Austin, TX) and cultured in a saline-like solution ND96 (96 mM NaCl) supplemented with antibiotics (100 U/ml penicillin/100 μg/ml streptomycin).

Fluorescence Lifetime Measurement and FLIM-FRET

A Q2 laser scanning confocal system, equipped with a FastFLIM data acquisition module from ISS, Inc. (Champaign, IL), and two hybrid PMT detectors (Model R10467-40, Hamamatsu USA, Bridgewater, NJ), was used for digital frequency-domain fluorescence lifetime imaging as previously done[30,75]. This confocal FLIM system was connected to an Olympus IX73 inverted microscope (Olympus America, Waltham, MA). The fluorescence lifetime measurements provided both frequency-domain (instantaneous distortion-free phasor plotting) and time-domain decay information. Confocal imaging was made possible using dichroic cubes, one with a 50/50 beam splitter and the other equipped with various long-pass filters. A supercontinuum laser (YSL Photonics, Wuhan, China) was used for fluorescence excitation, with a wavelength range of 410 nm to 900 nm, a repetition rate of 20 MHz, and a pulse duration of 6 ps. Specifically, the excitation wavelengths were 445 nm for CFP, 488 nm for AF-488 and YFP, 561 nm for AF-555. The intensity of laser excitation was kept at the same level when imaging different cell samples. To detect the emission from the CFP/YFP FRET pair, an emission filter cube comprising a 475/28 nm filter for CFP, a 495 nm long-pass dichroic mirror, and a 542/27 nm filter for YFP was used. For the AF-488/AF-555 FRET pair, an emission filter cube consisting of a 525/40 nm filter for AF-488, a 552 nm long-pass dichroic mirror, and a 593/40 nm filter for AF-555. For phasor FLIM-FRET, only the donor lifetime was measured either in the absence or in the presence of the FRET acceptor. In the FRET experiments related to L10 and S15, the intensity-based ratio of FRET donors to acceptors is randomized, and no significant differences were observed for this ratio under various conditions.

Under each experimental condition, confocal images with a frame size of 256 x 256 pixels were acquired. A motorized variable pinhole with a size of 100 μm and a pixel dwell time ranging from 0.1 to 0.4 ms were used during image acquisition. For image processing, display, and acquisition, the VistaVision software (ISS, Inc., Champaign, IL) was used. This software allows for the customization of parameters such as pixel dwell time, image size, and resolution. The FLIM analysis used a fitting algorithm, phasor plotting and analysis. To enhance the display, isolation, and analysis of membrane-localized lifetime species, a median/gaussian smoothing filter and an intensity-threshold filter were applied. The phasor FLIM approach offers a notable advantage in effectively distinguishing various lifetime species within the phasor plot. It allows for a separation of cytosolic fluorescence and background fluorescence, from the lifetime species originating from the membrane-localized fluorescence signals. Specifically, lifetime species from endocytosed CTxB that are localized inside of the cell can be separated from those from the membrane-localized CTxB (Supplementary Fig. 1b). The lifetime of the membrane-localized FRET donor CTxB AF-488 is unaffected by treatments of β-CD or detergents (Supplementary Fig. 1 c-e). Overall, the fluorescence intensity of endocytosed CTxB is minor and membrane-localized fluorescence is predominant.

The VistaVision software was used to determine the phase delays (φ) and modulation ratios (m) of the fluorescence signal in response to an oscillatory stimulus with frequency ω. These values were derived by conducting sine and cosine Fourier transforms of the phase histogram, considering the instrument response function (IRF) calibrated with different fluorophores with known lifetimes in water. The choice of calibration fluorophore (Atto 425, rhodamine 110, or rhodamine B) depended on the emission spectrum of the fluorophore of interest, with respective lifetimes of 3.6 ns, 4 ns, and 1.68 ns.

The FLIM-FRET analysis was performed using the FRET trajectory function available in the VistaVision software[30,39]. To ensure accurate results, three crucial parameters were adjusted to optimize the fitting trajectory, encompassing both the unquenched donor and the donor species affected by FRET. These parameters included: the background contribution in the donor sample, the unquenched donor contribution in the FRET sample, and the background contribution in the FRET sample. To maintain accuracy, the background levels in both the donor sample and the FRET samples were set below 4%. This value corresponds to the very low emission intensity typically observed in unlabeled or untransfected cells.

Patch-clamp Electrophysiology

The whole-cell patch-clamp recordings were performed using an EPC10 patch-clamp amplifier and PATCHMASTER (HEKA) software, with a sampling rate of 10 kHz. Borosilicate patch electrodes were created using a P1000 micropipette puller from Sutter Instrument (Sutter, Novato, CA), resulting in an initial pipette resistance of approximately 3 - 5 MΩ. A Sutter MP-225A motorized micromanipulator was used for patch clamp (Sutter, Novato, CA). The recordings were conducted at a temperature range of 22-24 °C.

To record HCN currents from DRG neurons, the internal pipette solution was prepared with the following composition (in mM): 10 NaCl, 137 KCl, 1 $MgCl_2$, 10 HEPES, 1 EGTA, and pH 7.3 adjusted with KOH. In addition, 0.5 mM cAMP was added to the internal solution to facilitate the endogenous HCN currents. The external solution used was prepared with the following composition (in mM): 154 NaCl, 5.6 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 8 HEPES, 10 D-Glucose, and pH 7.4 adjusted with NaOH. Series resistance was typically lower than 15 MΩ and series resistance compensation was set at 40%. For the action potential measurements, current clamp mode of PATCHMASTER was employed instead of the whole-cell voltage-clamp configuration. In the current clamp mode, the resting membrane potential (-57 ± 1 mV, n = 6 cells) was established through the application of zero current injection. The input resistance of DRG neurons, obtained by applying small negative current injections, was found much smaller than the seal resistance that is typically higher than one giga-ohm. Subsequent current injections for evoking action potentials were administered as absolute values. Neurons frequently exhibit adaptability to injected currents, contributing to the observed variability in the number of action potential firings during the test period.

The conductance-voltage (G-V) relationships of endogenous HCN currents were measured from the instantaneous tail currents at -60 mV following voltage pulses from -40 mV to between 0 and −140 mV. The leak tail currents following pulses to -60 mV were subtracted, and the currents were normalized to the maximum tail current amplitude, reporting the relative channel conductance (G/G_{max}). The relative conductance was plotted as a function of the voltage of the main pulse and fitted with the Boltzmann equation:

$$G/G_{max}=1 / (1 + \exp[(V - V_{1/2})/V_S]) \qquad (1)$$

where V is the membrane potential, $V_{1/2}$ is the potential for half-maximal activation, and Vs is the slope factor. Vs = RT/zeF, where R is the gas constant, T is temperature in Kelvin (297 K), F is the Faraday constant, *e* is the value of elementary charge, z is the estimated number of elementary gating

charge per channel for channel activation. The Gibbs free energy needed for channel activation was calculated according to: $\Delta G = RTV_{1/2}/Vs$[76]. While the assessed gating charge of native HCN channels in primary DRG neurons is evidently underestimated, we emphasize that the focus of this paper is not the quantification of absolute gating charge movement of HCN channels but rather the examination of directional changes in the relative gating charge for the channel activation under various conditions. Even when in heterologous expression systems, the slow voltage-sensor movement of HCN channels makes the accurate estimation of the absolute gating charge associated with channel opening particularly challenging.

Primary DRG neurons

Adult Sprague-Dawley rats (Pathogen-free female 100 g, male 250 g Envigo, Placentia, CA) were kept in light (12-h light: 12-h dark cycle; lights on at 07:00 h) and temperature (23 ± 3°C) controlled rooms. Standard rodent chow and water were available *ad libitum.* All animal use was conducted in accordance with the National Institutes of Health guidelines, and the study was conducted in strict accordance with recommendations in the guidelines approved by the IACUC of Saint Louis University (Protocol #: 3014). All behavioral experiments were performed by experimenters who were blinded to treatment groups.

Dorsal root ganglia (DRG) were dissected from 100 g female Sprague-Dawley rats[77-79]. In brief, removing dorsal skin and muscle and cutting the vertebral bone processes parallel to the dissection stage exposed DRGs. DRGs were then collected, trimmed at their roots, and digested in 3 mL bicarbonate free, serum free, sterile DMEM (Catalog# 11965, Thermo Fisher Scientific, Waltham, MA) solution containing neutral protease (3.125 mg/mL, Catalog# LS02104, Worthington, Lakewood, NJ) and collagenase Type I (5 mg/mL, Cat# LS004194, Worthington, Lakewood, NJ) and incubated for 45 min at 37°C under gentile agitation. Dissociated DRG neurons (~1.5×10^6) were then gently centrifuged to collect cells and washed with DRG media DMEM containing 1% penicillin/streptomycin sulfate from 10 000 µg/mL stock, 30 ng/mL nerve growth factor, and 10% fetal bovine serum. Cells were plated onto poly-D-lysine and laminin-coated glass 12-mm coverslips.

Spared nerve injury (SNI) model of neuropathic pain

Under isoflurane anesthesia (5% induction, 2.0 % maintenance in 2 L/min O_2), skin on the lateral surface of the left hind thigh was incised. The biceps femoris muscle was bluntly dissected to

expose the three terminal branches of the sciatic nerve[58]. Briefly, the common peroneal and tibial branches were tightly ligated with 4-0 silk suture and axotomized 2.0 mm distal to the ligation. Closure of the incision was made in two layers. The muscle was sutured once with 5-0 absorbable suture; skin was auto-clipped. Animals were allowed to recover for 7 days before any testing.

Statistics and Reproducibility

The data parameters were presented as the mean ± s.e.m. of n independent cells or patches. Statistical significance was determined using the unpaired two-sided t test for comparing two groups, or a one-way ANOVA followed by Tukey's post hoc test for comparisons with more than two groups, denoted as $*p < 0.05$, $**p < 0.01$ respectively.

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Author Contributions

G.D. conceptualized the research. G.D., A.M., and K.S.M designed experiments. L. J. H. performed electrophysiology experiments, N.L.M. and G.D. performed fluorescence imaging. N.L.A.D. and L.S. performed behavior experiments on rats and prepared neuronal samples. G.D., L. J. H., N.L.M., and N.L.A.D. analyzed data. G.D. E.N.L. and N.L.M. created figures and edited the manuscript. G.D. wrote the paper with insightful inputs from other authors.

Competing Interests

The authors declare no competing interests.

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