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Original Research

Trafficking and Gating Cooperation Between Deficient $Na_v1.5$-mutant Channels to Rescue I_{Na}

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Abstract

Background: Pathogenic variants in *SCN5A*, the gene encoding the cardiac Na^+ channel α-subunit $Na_v1.5$, result in life-threatening arrhythmias, e.g., Brugada syndrome, cardiac conduction defects and long QT syndrome. This variety of phenotypes is underlied by the fact that each $Na_v1.5$ mutation has unique consequences on the channel trafficking and gating capabilities. Recently, we established that sodium channel α-subunits $Na_v1.5$, $Na_v1.1$ and $Na_v1.2$ could dimerize, thus, explaining the potency of some $Na_v1.5$ pathogenic variants to exert dominant-negative effect on WT channels, either by trafficking deficiency or coupled gating. **Objective**: The present study sought to examine whether $Na_v1.5$ channels can cooperate, or transcomplement each other, to rescue the Na^+ current (I_{Na}). Such a mechanism could contribute to explain the genotype-phenotype discordance often observed in family members carrying Na^+-channel pathogenic variants. **Methods**: Patch-clamp and immunocytochemistry analysis were used to investigate biophysical properties and cellular localization in HEK293 cells and rat neonatal cardiomyocytes transfected respectively with WT and 3 mutant channels chosen for their particular trafficking and/or gating properties. **Results**: As previously reported, the mutant channels G1743R and R878C expressed alone in HEK293 cells both abolished I_{Na}, G1743R through a trafficking deficiency and R878C through a gating deficiency. Here, we showed that coexpression of both G1743R and R878C nonfunctioning channels resulted in a partial rescue of I_{Na}, demonstrating a cooperative trafficking of $Na_v1.5$ α-subunits. Surprisingly, we also showed a cooperation mechanism whereby the R878C gating-deficient channel was able to rescue the slowed inactivation kinetics of the C-terminal truncated R1860X (ΔCter) variant, suggesting coupled gating. **Conclusions**: Altogether, our results add to the evidence that Na_v channels are able to interact and regulate each other's trafficking and gating, a feature that likely contributes to explain the genotype-phenotype discordance often observed between members of a kindred carrying a Na^+-channel pathogenic variant.

Keywords: cardiac arrhythmia; transcomplementation; $Na_v1.5$; *SCN5A*; Sodium channelopathies

1. Introduction

Loss- or gain-of function mutations in *SCN5A*, the gene encoding the $Na_v1.5$ α-subunit of the cardiac sodium channel, have been involved in several inherited cardiac arrhythmias including Brugada syndrome (BrS), cardiac conduction defect, type-3 long QT syndrome, and sick sinus syndrome [1]. The hundreds of mutations identified in *SCN5A* highlight the crucial role of $Na_v1.5$ in cardiac conduction and rhythm. Unlike potassium channel genes, which encode one fourth of tetramers constituting functional channels, $Na_v1.5$ channels were thought to be structured as monomers, since the gene encodes the entire 4-domain channel α-subunit. It was thus unexpected to report $Na_v1.5$ mutants with a dominant-negative effect on wild-type (WT) channels, i.e., a decrease of I_{Na} exceeding the 50% of current density expected in case of haploinsufficiency observed when coexpressing some mutants with WT channels in a 1:1 ratio to mimic patient heterozygosity, as we and others did [2–9]. Furthermore, we demonstrated that $Na_v1.5$ α-subunits could interact with each other and that a trafficking-efficient mutant channel was able to drive a trafficking-deficient one to the surface membrane [3]. This transcomplementation mechanism, i.e., the functional cooperation of two different entities through their physical interaction, led to a small rescue of inward Na^+ current (I_{Na}). We have also evidenced that $Na_v1.5$ α-subunits form dimers through an interaction site located in the domain I-II linker, and that $Na_v1.5$ channels not only interact but also gate as dimers [10]. More recently, we and others reported that dominant negative suppression exerted by $Na_v1.5$ mutants could also be caused by impairing the WT gating probability, a mechanism resulting from the coupled gating of Na_v channel α-subunits [6,8,11]. Altogether, these observations led us to examine the capacity of Na^+

channels to transcomplement trafficking or gating-deficient mutant channels to rescue I_{Na}.

To assess this question, we studied three $Na_v1.5$ mutations: R878C and G1743R, which abolished I_{Na} by two distinct mechanisms, i.e., gating deficiency [12,13] and trafficking deficiency [14] respectively, and the C-terminal truncated mutant R1860X (ΔCter), which displayed slow inactivation kinetics and a drastic leftward shift of the steady-state inactivation. Heterologous expression of mutant and WT channels in a 1:1 ratio led to a reduction in I_{Na} density of 80% for the G1743R trafficking-deficient channels and 50% for the R878C trafficking-efficient channels [3]. Interestingly, because of the trafficking capability of R878C and the gating capacity of G1743R, coexpression of both mutant channels led to a small I_{Na} by a mechanism of transcomplementation. Importantly, we also observed a cooperation of $Na_v1.5$ mutant channels R878C and ΔCter at the cell surface. Indeed, coexpression of the full-length but non-gating R878C channel with the ΔCter channel rescued the inactivation kinetics of the latter. This strongly suggests that the C-terminus of R878C contributes to inactivate the truncated ΔCter channel, raising the possibility of a cooperation between $Na_v1.5$-subunits C-termini, as a sort of domain swapping. Once again, our results provide evidence that $Na_v1.5$ channels are able to oligomerize, traffic and gate as multi-channel complexes. Importantly, we have shown here that such a cooperation could lead to the restoration of a small I_{Na}, a mechanism that could contribute to explain the genotype-phenotype discordance often observed between family members carrying Na^+-channel pathogenic variants.

2. Materials and Methods

2.1 SCN5A cDNA Cloning and Mutagenesis

The plasmid pcDNA3.1-GFP-hH1a (N-terminal GFP) was a gift from Dr. H. Abriel (Bern, Switzerland). $Na_v1.5$ mutants R878C, G1743R and R1860X (ΔCter) were prepared using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions and verified by sequencing. The plasmid pcDNA3-CD4-KKXX and the anti-CD4-KKXX antibody were gifts from Dr. J. Mérot (Nantes, France). This plasmid has been designed to express CD4 carrying the KKXX motif of ER retention.

2.2 HEK293 Cell Culture and Transfection

HEK293 cells were transfected with jetPEI (Polyplus Transfection, NY, USA) according to the manufacturer's instructions. For patch-clamp recordings, HEK293 cells were transfected with pcDNA3.1-GFP-hH1a WT or mutants in 35-mm culture dishes, with a total of 0.6 μg of plasmid per 35 mm dish, to avoid saturating currents. To mimic the heterozygous state of patients, cells were co-transfected with 0.3 μg of pcDNA3.1-hH1a (no tag) WT and 0.3 μg of pcDNA3.1-GFP-hH1a-mutant, or 0.3 μg of pcDNA3.1-GFP-hH1a-mutant and 0.3 μg of the second pcDNA3.1-GFP-hH1a-mutant. For biochemical analysis, cells were plated in 25-cm^2 flasks and transfected with 2 μg of pcDNA3.1-GFP-hH1a WT or mutant.

2.3 Electrophysiological Recordings

Patch-clamp recordings were carried out in the whole-cell configuration at room temperature (~ 22 °C) 36 h after transfection. Cells were bathed in an extracellular Tyrode solution containing (in mM): 135 NaCl, 4 KCL, 2 $MgCl_2$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 20 glucose, 10 HEPES, adjusted to pH 7.4 with CsOH. Patch pipette medium was (in mM): 5 NaCl, 140 CsCl, 2 $MgCl_2$, 4 Mg-ATP, 5 EGTA, 10 HEPES, adjusted to pH 7.2 with CsOH. Some cells transfected with the G1743R mutant were incubated for 24 h with 500 μM mexiletine to partially rescue the mutant-channel trafficking as in reference [14].

Ionic currents were recorded with the amplifier Axopatch 200B (Axon Instruments, CA, USA). Patch pipettes (Corning Kovar Sealing code 7052, WPI) had resistances of 1.5–2.5 MΩ, when filled with pipette medium. Currents were filtered at 10 kHz (–3 dB, 8-pole low-pass Bessel filter) and digitized at 50 kHz (NI PCI-6251, National Instruments, Austin, TX, USA). Data were acquired and analyzed with ELPHY2® software (G. Sadoc, CNRS, Gif/Yvette, France).

In order to measure peak I_{Na} amplitude and determine current-voltage relationships (I/V curves), currents were elicited from a holding potential of –120 mV by 50-ms test potentials of 0.2 Hz frequency from –100 mV to 60 mV by increments of 5 or 10 mV. For the activation-V_m protocol, currents were elicited by 100-ms depolarizing pulses applied at 0.2 Hz from a holding potential of –120 mV, in 5- or 10-mV increments between –100 and +60 mV. The steady-state inactivation-V_m protocol was established from a holding potential of –120 mV and a 2-s conditioning prepulse was applied in 5- or 10-mV increments between –140 and +30 mV, followed by a 50-ms test pulse to –20 mV at 0.2 Hz. Data for the activation-V_m and steady-state inactivation-V_m relationships of I_{Na} were fitted to the Boltzmann equation as previously reported [3]. Inactivation-kinetics time constants (τ_{fast} and τ_{slow}) were measured by fitting with a double exponential function using Clampfit (Molecular Devices, CA, USA).

2.4 Rat Neonatal Cardiomyocyte (RNC) Isolation and Transfection

Neonate 1-day-old rats were euthanized by decapitation. Their hearts were dissected, digested with collagenase A (Roche Diagnostics, Meylan, France) and incubated in culture medium at 37 °C, 5% CO_2 after 90 min preplating on 60 mm plastic dishes to remove fibroblasts. Non-adherent cells were plated at a density of 4 × 10^5 cells/well on 35 mm dishes containing glass coverslips coated with 10 mg/mL laminin (Roche Diagnostics) in cul-

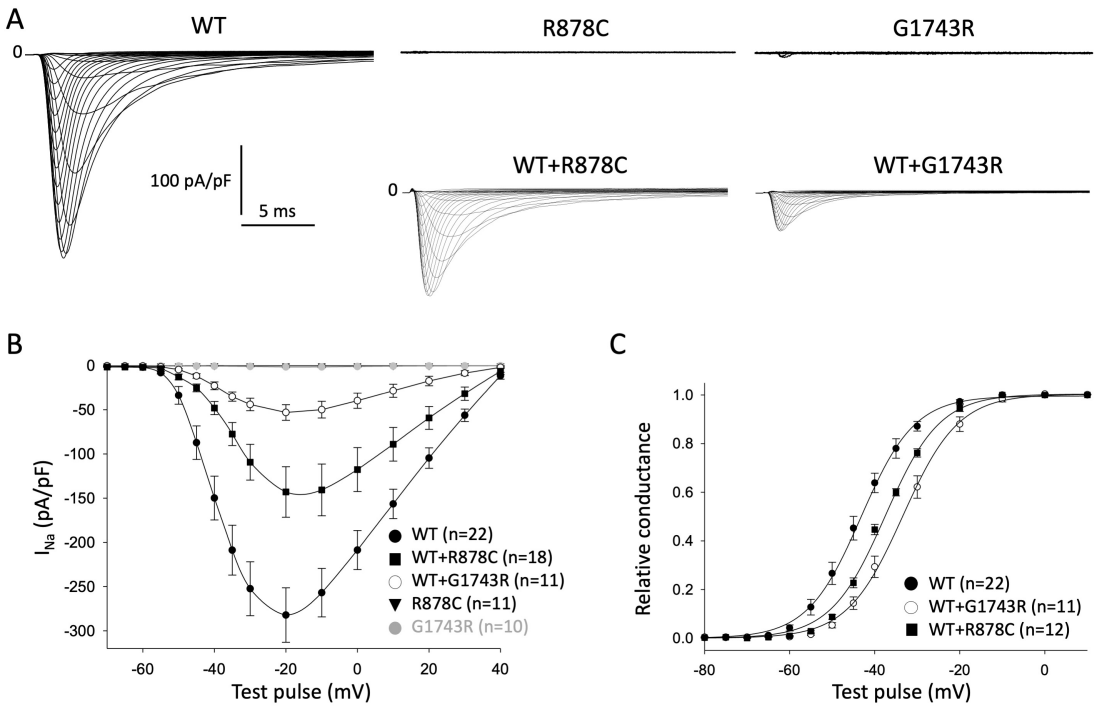

Fig. 1. Electrophysiological characterization of BrS mutants expressed alone or with WT channels to mimic patients heterozygosity. (A) I_{Na} representative Na^+-current traces measured in HEK 293 cells expressing WT, R878C, G1743R WT + R878C or WT + G1743R channels. (B) Current-voltage relationships. (C) Voltage-dependence of activation recorded from HEK 293 cells expressing WT or WT + G1743R. Note that, as opposed to cells co-expressing WT + R878C, WT + G1743R cells displayed an acute decrease in I_{Na} defining a dominant-negative effect of mutant channels on WT ones. Furthermore, we observed a significant shift of the voltage-dependent activation in presence of G1743R.

ture medium DMEM (High Glucose/L-glutamine; Gibco ref 41965 039), supplemented with 10% horse serum, 5% FBS, 1% penicillin/streptomycin, Cytosine β-D arabinofuranoside 25 mg/mL and incubated for 24 h (37 °C, 5% CO_2). Cells were transfected in a 1%-CO_2 incubator with 0.6 μg of N-terminal GFP fused constructs of WT or mutant $Na_v1.5$ using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.5 Immunocytochemistry

Indirect immunofluorescence was performed on RNC primary culture fixed with methanol for 10 min at –20 °C. Cells were then washed twice for 5 min with Phosphate Buffer Saline (PBS), blocked in PBS-5% BSA for 30 min at room temperature. Cells were incubated for one hour with primary antibodies: rabbit anti-GFP (1:300, Torrey Pines Biolabs) to detect $Na_v1.5$-GFP and mouse anti-CD4 for CD4-KKXX (1:1000, Sigma). Detection was performed after two washes with PBS and one hour of incubation with secondary antibodies: chicken anti-mouse Alexa Fluor 594, goat anti-rabbit Alexa Fluor 488 (1:1000, ThermoFisher Scientific) and the nuclear dye DAPI (1:500, Sigma) diluted in the blocking buffer. Control experiments were performed by omitting the primary antibodies.

2.6 Imaging

Labeled cardiomyocytes were observed with an Olympus epifluorescent microscope (60×). Images were acquired with a CoolSnap camera (Ropper Scientific) and analyzed with Metamorph software (Molecular devices) equipped with a 3D-deconvolution module. For each sample, series of consecutive plans were acquired (sectioning step: 0.2 μm).

2.7 Statistical Analysis

Data are presented as means ± SEM. Statistical significance was estimated with SigmaPlot® software (Systat Software Inc., San Jose, California, USA) by Student's *t*-test or ANOVA, as appropriate. $p < 0.05$ was considered significant.

3. Results

3.1 Both R878C and G1743R Mutations Abolished I_{Na}

Na^+ currents were recorded in HEK293 cells 36 h after transfection with WT or mutant channels. As previously reported, R878C abolished I_{Na} density compared to WT channels [12,13], and G1743R led to an extremely small current (–1.4 ± 0.8 pA/pF, n = 10) (Fig. 1A and Table 1) [14].

Table 1. Kinetics properties of I_{Na} recorded in HEK293 cells transfected with $Na_v1.5$ WT and mutants.

Na^+ channels	Peak current density (pA/pF)	$V_{1/2}$ activation (mV)	$V_{1/2}$ inactivation (mV)	t_{fast} (ms)
WT	–282 ± 31 (n = 22)	–44.2 ± 1.2 (n = 17)	–85 ± 8 (n = 12)	0.55 ± 0.01 (n = 13)
R878C	–0.2 ± 0.04 (n = 9) ***	na	na	na
G1743R	–1.4 ± 0.8 (n = 10) ***	na	na	na
WT + R878C	–143 ± 26 (n = 18) **	–36.6 ± 0.9 (n = 12) ***	–83.6 ± 1 (n = 13) ns	nd
WT + G1743R	–53 ± 9 (n = 11) ***	–33.2 ± 1.1 (n = 11) **	nd	nd
G1743R + mexiletine	–15.7 ± 2.3 (n = 6) ***	–27.3 ± 5.7 (n = 6) ***	nd	nd
G1743R + R878C	–25.9 ± 3.9 (n = 13) ***	–24.2 ± 3.7 (n = 11) ***	nd	nd
ΔCter	–81 ± 17 (n = 13) ***	–41.3 ± 2 (n = 12) ns	–104 ± 4 (n = 13) ***	1.13 ± 0.05 (n = 13) ***
ΔCter + R878C	nd	nd	–105 ± 6 (n = 15) ***	0.9 ± 0.02 (n = 15) ***

Data are presented as means ± SEM. Peak current density is given at –20 mV. WT indicates wild type, nd, not determined; na, not applicable; and ns, not significant. *$p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to WT channels.

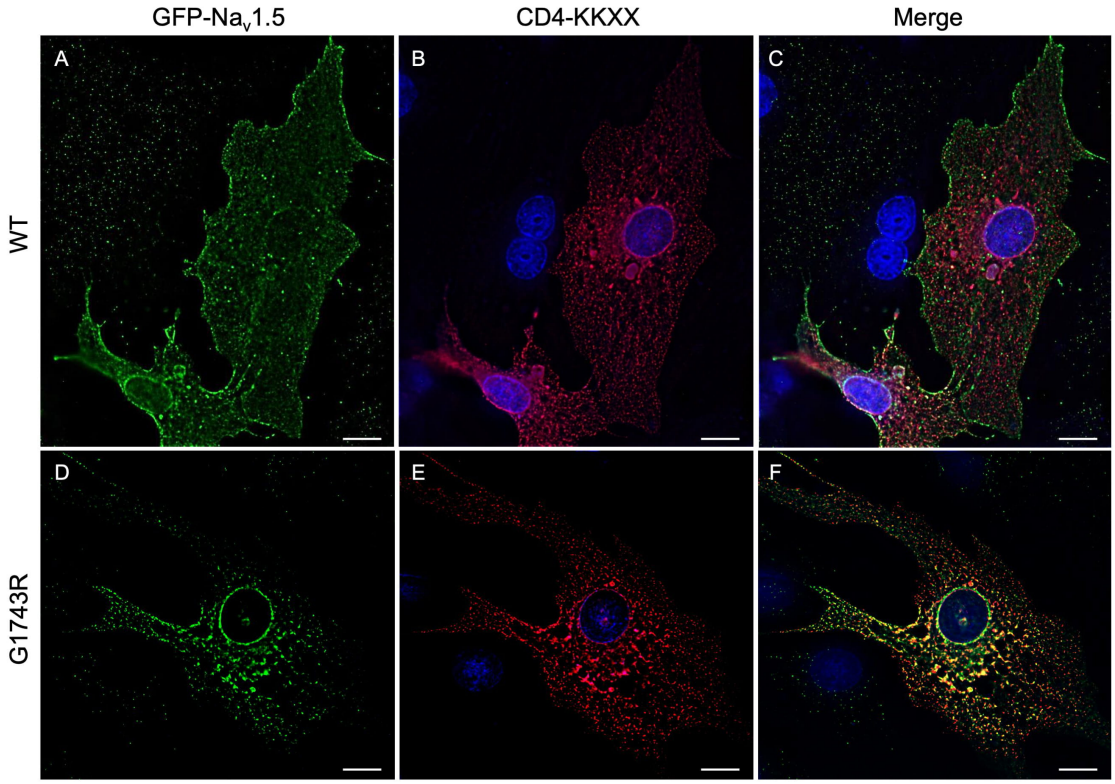

Fig. 2. Immunocytostaining of overexpressed G1743R-$Na_v1.5$ channels in RNC. Representative 3-dimensional deconvolution images (n = 5) of RNC co-transfected with GFP-$Na_v1.5$ (green) and CD4-KKXX (red). Nuclei were stained with DAPI (blue). (A, B and C) $Na_v1.5$ WT, (D, E and F) G1743R. Note that in the merged image (C) $Na_v1.5$ WT is mostly expressed at the plasma membrane, as opposed to CD4-KKXX, which is retained in the ER. In contrast, the G1743R mutant merged image (F) showed numerous internal yellow dots, indicating that $Na_v1.5$ mutant channels were mostly retained in the ER, similarly to CD4-KKXX. Scale bar: 10 μm.

3.2 G1743R Channels Led to A Dominant-Negative Impairment of I_{Na}, But R878C Channels Did Not

Earlier, we have demonstrated that mutant-$Na_v1.5$ channels could exert a dominant-negative effect on WT channels by retention of WT/mutant interacting complexes within the ER [3]. We therefore explored here whether R878C or G1743R mutants had a dominant-negative effect on WT channels. To mimic the heterozygous state of BrS patients, cells were co-transfected with WT and R878C or G1743R mutant channels in a 1:1 ratio. As previously shown [3], co-expression of R878C with WT channels led to 50% of the WT current (–143 ± 26 pA/pF, n = 18 compared to WT alone: –282 ± 31 pA/pF, n = 22) (Fig. 1A and B and Table 1). In contrast, when co-expressed with WT channels, G1743R led to an acute (≈75%) loss of I_{Na} (–53 ± 9 pA/pF, n = 11 compared to WT alone: –282 ± 31

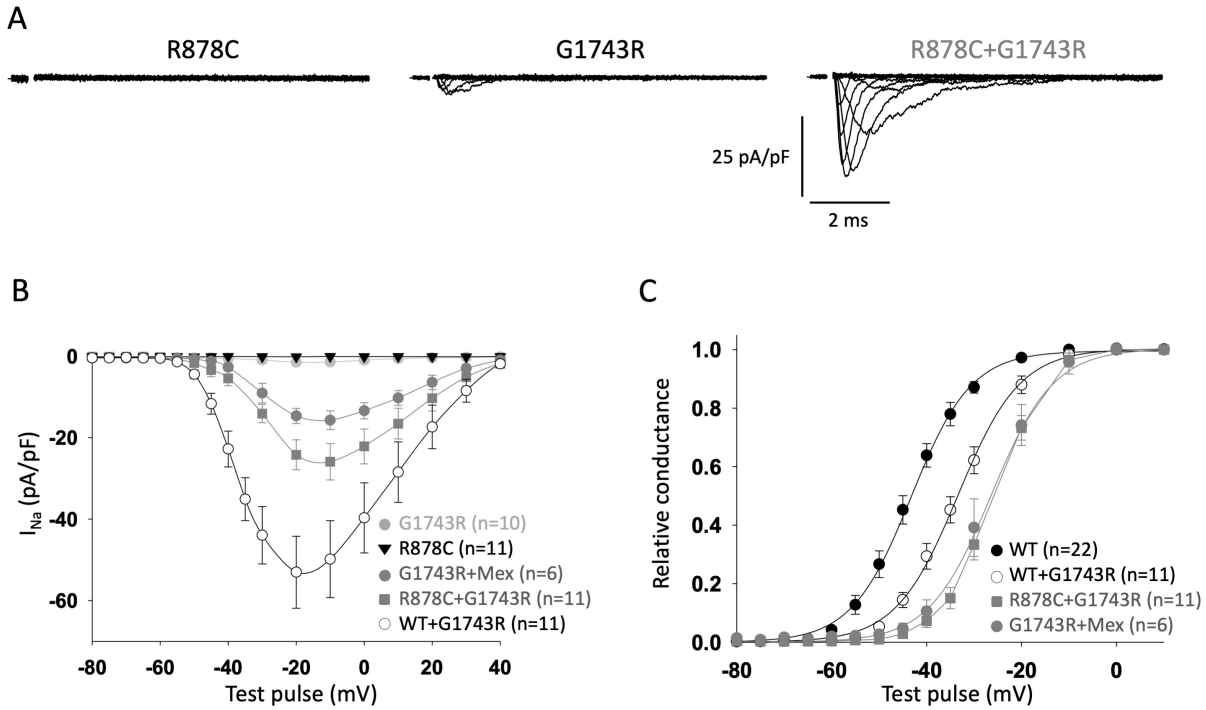

Fig. 3. Cooperation of $Na_v1.5$-mutant channels to rescue I_{Na}. (A) I_{Na} representative traces and (B) current-voltage relationships measured in HEK 293 cells expressing R878C, G1743R or both channels in a 1:1 ratio. In panel (A), 0.1 ms was omitted on current traces for the capacitance transient. (C) Voltage-dependent activation of cells expressing WT, WT + G1743R or R878C + G1743R. Mex, mexiletine. Note that the rescued current carried by R878C + G1743R mutants remained shifted positively by 19.5 mV when compared to WT channels expressed alone and by 9 mV when compared to WT + G1743R channels, to the same extent as mexiletine-rescued G1743R current.

pA/pF, n = 22), illustrating a dominant-negative effect.

Interestingly, the $V_{1/2}$ of the voltage-dependent activation was shifted towards positive values by 10.5 mV when G1743R and WT channels were co-expressed (–33.2 ± 1.1 mV, n = 11), compared to WT alone (–44.2 ± 1.2 mV, n = 22), suggesting that the presence of the G1743R mutant impairs the WT-channel's proper gating (Fig. 1C and Table 1). In contrast, the $V_{1/2}$ of steady-state inactivation remained unaffected (Table 1).

3.3 G1743R Channels Led to Dominant-Negative Impairment of I_{Na} by Retention of Mutant Proteins Within the Endoplasmic Reticulum

We assessed here whether the G1743R mutant channel's subcellular localization may contribute to its dominant-negative effect on WT channels. Rat neonatal cardiomyocytes (RNC) transfected with GFP-tagged channels were labeled with an anti-GFP antibody to assess channel localization. G1743R strongly co-localized with CD4 carrying the KKXX motif of endoplasmic reticulum (ER) retention, suggesting that the mutant is mainly retained inside ER in myocytes and very poorly expressed at the cell surface, as opposed to WT channels (Fig. 2). This result is in line with the previous study by Valdivia and coworkers who showed that the G1743R channel was mainly trapped within intracellular compartments in HEK293 cells [14]. Altogether, our results suggest that the subcellular retention of mutated G1743R channels contributed to their dominant-negative effect on WT channels. As previously reported, the R878C mutant channel was properly expressed at the plasma membrane of transfected cells [12].

3.4 R878C Channels Transcomplemented G1743R Mutant's Trafficking to Rescue I_{Na}

We have previously demonstrated a mechanism whereby Na^+ channel α-subunits were able to transcomplement each other by interacting with each other, to rescue trafficking-deficient $Na_v1.5$ mutants [3]. To assess whether the trafficking-competent R878C channels were able to rescue the trafficking-deficient G1743R ones, we co-expressed both non-functional mutant channels in HEK293 cells. Notably, cells co-expressing R878C and G1743R channels displayed current densities (–25.9 ± 3.9 pA/pF, n = 13) that were never recorded when the G1743R-mutant channels were expressed alone (–1.4 ± 0.8 pA/pF, n = 10) (Fig. 3). This rescued I_{Na} represented ≈19 times I_{Na} displayed by G1743R expressed alone, ≈ half of the current recorded in WT + G1743R transfected cells, ≈ twice the G1743R current rescued by mexiletine and ≈9% of the current recorded with WT channels alone (Fig. 3A and B

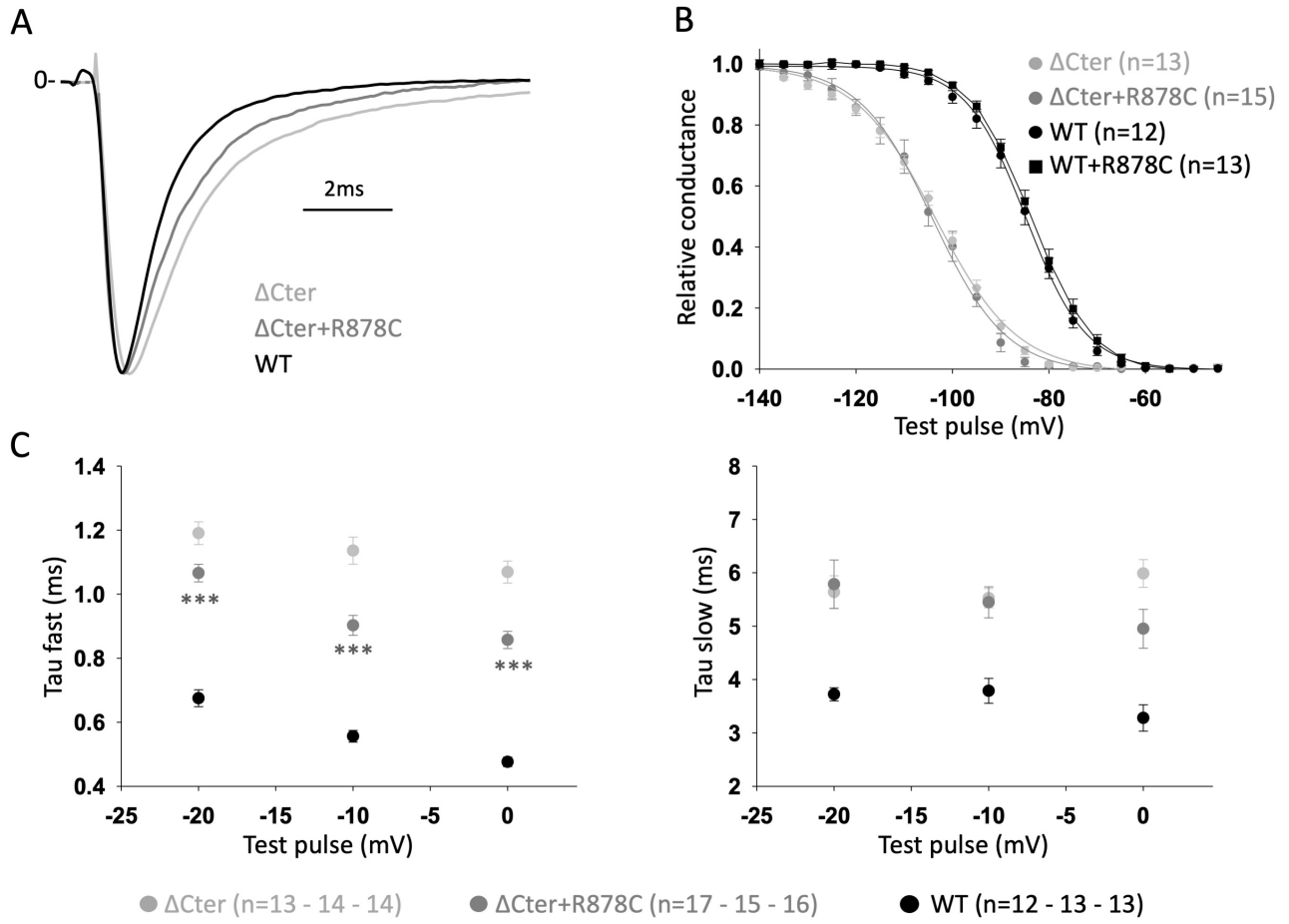

Fig. 4. Coupled-gating of $Na_v1.5$-mutant channels to rescue I_{Na}. (A) Representative I_{Na} traces at –10 mV. (B) Steady-state inactivation. (C) Fast and slow inactivation kinetics of I_{Na}. Note that the presence of R878C significantly rescued ΔCter I_{Na} fast inactivation decay (τ_{fast}). *** = $p \leq 0.001$ when compared to ΔCter expressed alone. These results were strongly supporting that R878C C-terminus played a role in the inactivation decay of ΔCter.

and Table 1), suggesting that a mechanism of transcomplementation of trafficking-deficient mutant channels by trafficking-competent ones contributed to restore I_{Na}.

3.5 G1743R Channels Transcomplemented R878C Mutant's Gating to Rescue I_{Na}

In recent studies [6,11], we reported that dominant negative effect of mutant $Na_v1.5$ channels was not only exerted through trafficking but that coupled gating could also be involved. This led us to hypothesize that such gating transcomplementation could, conversely, rescue I_{Na}. Indeed, we observed that, when R878C + G1743R were co-expressed, the voltage-dependent activation of the rescued current was drastically shifted by +19.5 mV (Fig. 3C and Table 1), suggesting that G1743R alone may not gate properly and would explain the +9 mV-shift of voltage dependence of activation in presence of WT channels (G1743R + WT) (Fig. 3C and Table 1). It is worth to note that the rescued R878C + G1743R current activation was positively shifted in the same extent as the G1743R current rescued by mexiletine (Fig. 3C and Table 1). These observations support a cooperative gating between both non-functional channels.

3.6 R878C Channels Transcomplemented ΔCter Mutant's Inactivation Kinetics

In order to confirm that a cooperative mechanism at the gating level could rescue I_{Na}, we designed another $Na_v1.5$-construct missing its C-terminus and co-expressed this trafficking-efficient ΔCter channel displaying a drastic negative shift of steady-state inactivation as well as very slow inactivation kinetics (Fig. 4A), with the trafficking-efficient but gating-deficient R878C channel. We investigated inactivation properties, i.e. steady state inactivation ($V_{1/2}$) and inactivation kinetics (τ_{fast} and τ_{slow}) of $Na_v1.5$ WT, ΔCter and ΔCter + R878C expressed in HEK293 cells. Strikingly, the fast inactivation kinetics in cells expressing ΔCter + R878C channels (τ_{fast} = 0.90 ± 0.02 ms, n = 15 vs 0.55 ± 0.01 ms, n = 13 in WT) was significantly rescued compared to cells expressing ΔCter alone (τ_{fast} =

1.13 ± 0.05 ms, n = 13) (Fig. 4C and Table 1). This result demonstrated that the presence of the full C-terminus of R878C channels was able to rescue some of the inactivation kinetics parameters of ΔCter channels, suggesting a cooperative mechanism at the gating level between these two mutant channels.

Slow inactivation kinetics (ΔCter + R878C: 5.4 ± 0.3 ms vs ΔCter: 5.5 ± 0.2 ms) and steady-state inactivation (ΔCter + R878C: $V_{1/2}$ = –105 ± 6 mV; ΔCter: $V_{1/2}$ = –104 ± 4 mV; WT: $V_{1/2}$ = –85 ± 8 mV) remained unchanged (Fig. 4B and 4C and Table 1). Also, WT + R878C fast inactivation kinetics were comparable to WT expressed alone [10].

4. Discussion

For years, the Na^+ channel α-subunits were thought to be monomers acting as independent functional entities, as opposed to K^+ channels that assemble into tetramers to be functional. However, we recently established that Na^+ channels were able to interact and form functional dimers [3,10,11]. We also demonstrated that voltage-gated Na^+ channels display coupled gating properties [6,10,11], a central mechanism for proper propagation of the action potential in cardiac cells. So far, such a gating cooperation has been described to explain a dominant-negative effect of mutant non-functional $Na_v1.5$ channels on WT channels [6,10,11]. The novelty of the present study is to delve further into the cooperative mechanisms between $Na_v1.5$ α-subunits to test whether this mechanism would be efficient to rescue I_{Na} from non-functional mutant channels. To do so, we used the ΔCter construct missing the α-subunit C-terminus and two well-documented pathogenic variants, G1743R and R878C, identified in BrS and sick sinus syndrome patients, both abolishing I_{Na} but by two different pathways: G1743R mainly by retention of the channel within the endoplasmic reticulum and R878C by gating-deficiency while normally trafficking to the membrane [12–14].

Our results showed that trafficking-competent but gating-deficient channels were able to rescue a small Na^+ current from gating-competent but trafficking-deficient channels. This breakthrough is crucial to better understand Na_v channel pathophysiology and contributes to explain the genotype-phenotype discordance often observed in BrS family members carrying Na^+-channel pathogenic variants.

4.1 G1743R and R878C Mutants Both Abolished I_{Na} Through Different Mechanisms

We and others have established that R878C mutation abolished I_{Na} by a gating deficiency, in accordance with its location in the pore of the second domain of $Na_v1.5$ [3,12,13]. It was also documented that this mutant channel normally reaches the cell membrane [12,13]. On another hand, G1743R led to the quasi-total abolition of I_{Na} because of a trafficking deficiency, since in HEK293 cells, G1743R mutant was shown to be poorly addressed to the plasma membrane even when its trafficking was partially rescued by mexiletine [14]. Our present study explored further this mechanism and showed that, in rat neonatal cardiomyocytes, G1743R mostly co-localized with CD4-KKXX, a protein carrying an ER-retention motif. Moreover, it was noteworthy that, similarly to N-terminal mutant channels we previously characterized [3], G1743R led to a dominant-negative effect on WT channels through an acute retention of the WT/mutant complexes within the ER (Fig. 2). These results strongly support our hypothesis that trafficking-deficient mutants are likely to exert a dominant-negative effect by an interaction between $Na_v1.5$ α-subunits, as early as ER compartments.

Both G1743R and R878C loss-of-function mutations have been associated with BrS and sick sinus syndrome [12–14]. However, the cellular mechanisms leading to one pathology or another remained ununderstood and even though each mutant overexpression resulted in the abolition of I_{Na}, the intrinsic mechanisms seemed radically different and may play a key role in these arrhythmia's phenotypic expression. Indeed, numerous reports have shown that a single $Na_v1.5$ mutation could induce various combinations of clinical phenotypes [15].

4.2 G1743R and R878C Mutants Cooperated in Trafficking

For the purpose of this study, we used an heterologous model of overexpression of two non-functional channels displaying none to extremely little currents in order to unequivocally demonstrate the rescue of I_{Na}. Indeed, in absence of any other Na^+ channel in HEK 293 cells, the recorded I_{Na} was necessarily the product of the non-functional mutant's expression. As shown in Fig. 2, the G1743R channels expressed alone were mostly retained in the ER, leading to an almost undetectable I_{Na}. But we demonstrated here that the trafficking-competent R878C channel, incapable of producing any current because of gating deficiency, drove the G1743R channel to the cell surface; this cooperation leading to a partial rescue of I_{Na} (Fig. 3). However, we could not exclude that R878C channels could not modify the rescued G1743R channels' kinetics. In the same way, it has to be considered that the G1743R channels likely exerts a dominant-negative effect on R878C trafficking-competent channels, as on WT channels, but the balance rescue/dominant-negative effect seemed to allow some G1743R channels to reach the cell surface.

The group of Dr. Deschenes demonstrated for the first time that co-expression of the *SCN5A* polymorphism H558R with BrS mutations could restore their trafficking defects to produce a small I_{Na} [16,17]. In the same line, we published in 2012 that $Na_v1.5$ α-subunits were capable of interacting with each other and that this interaction

was responsible for, either a dominant-negative effect of BrS mutants on WT channels, or a transcomplementation of trafficking-deficient mutants [3]. Such a cooperation between $Na_v1.5$-channel α-subunits was new in the field of Na_v channels but this concept of transcomplementation had been explored in the past in the area of CFTR [18–20] and other channels [21]. Altogether, these studies provide evidence that ion channel α-subunits are able to cooperate to rescue their trafficking defects and produce a current.

4.3 Dysfunctional Channels Cooperated With WT and With Each Other In Gating

In this work, our first argument in favor of a gating cooperation between $Na_v1.5$ α-subunits was the fact that, in cells co-expressing WT and G1743R channels, the voltage-dependence of activation was shifted towards positive voltages, amplifying the loss-of-function of I_{Na} (Fig. 1C). Secondly, the voltage-dependence of activation was even more shifted when the R878C non-conducting mutant was co-expressed with G1743R (Fig. 3C). As already observed with some N-terminal mutant channels [3], these results suggested that once the trafficking-deficient G1743R channels reached the cell surface through a cooperation between α-subunits, the voltage-dependence of activation of the channel complex is affected by the mutant channel kinetics. Indeed, if both G1743R and R878C channels were gating independently from each other, the voltage-dependent activation of cells co-expressing the mutant channels would be identical to G1743R, since R878C is not conducting.

At this point of our study, we could neither demonstrate nor exclude a cooperative gating between mutant channels occurring in addition of the observed trafficking cooperation. To this end, we co-expressed the trafficking-competent ΔCter channel displaying a drastic negative shift of steady state inactivation as well as very slow inactivation kinetics (Fig. 4A), with the trafficking-competent but gating-deficient R878C channel. Strikingly, we demonstrated that even if not conducting, the R878C channel was able to partially rescue the fast inactivation decay of the ΔCter channel (Fig. 4C). This important finding demonstrated that the C-terminus of one α-subunit can play an important role in inactivation of the other α-subunit of the dimer. This result is in accordance with the work from Gabelli *et al.* [22] presenting a crystal structure of the C-terminus of $Na_v1.5$. The authors proposed the formation of an asymmetric homodimer where both inactivation state and kinetics are regulated by a complex protein-protein interaction involving the heterotrimer calmodulin/FGF13/$Na_v1.5$-C-terminus. Our results strongly support the idea that the presence of the gating-deficient channel R878C is capable of rescuing the inactivation kinetics of ΔCter channels, but we can only speculate that this occurred by $Na_v1.5$-C-terminus domain swapping, as reported for other ion channels [23], to regulate fast inactivation of other α-subunits. Further studies will be needed to decipher these types of mechanisms, which are crucial to understand, as they most likely play a key role in the pathophysiology of Na^+ channelopathies. Another interesting point of discussion comes from the observation that R878C channels were able to partially rescue the fast inactivation decay of ΔCter channels but had no effect on slow inactivation (Fig. 4C). It is well established that the fast inactivation is regulated by the DIII-DIV linker and the C-terminus, while the slow inactivation process is mainly regulated by the C-terminus allowing to lock the channel into its closed state. According to the 3D conformation structure and the "lollipop" model from Gabelli *et al.* [22], the interaction between the two C-termini plays a crucial role to lock the channel into its closed state, which is impossible when the C-terminus is deleted as in ΔCter. We could therefore speculate that the lack of the C-terminus of ΔCter channels would not allow the dimers to enter a stable closed state allowing late re-openings.

As reported recently in the excellent review from Dixon *et al.* [24], many recent experimental findings provided evidence that several classes of ion channels appear to open and close in a coordinated, cooperative manner. Considering the cardiac Na^+ channel $Na_v1.5$, we published the first evidence that the BrS $Na_v1.5$-L325R mutant dimerized with WT channels and led to a dominant negative effect by coupled gating [11]. In the past, several studies have suggested cooperative gating of Na_v channels [25,26], e.g., Aldrich *et al.* [25] noted a tendency for even number of channels to occur within a patch during single-channel recordings. Naundorf *et al.* [27] also reported that the sharp action potential upstroke could not be explained without channels cooperation. A recent work from Zheng *et al.* [6] suggested that the heart failure-associated splice variant $Na_v1.5$-G1642X suppresses Na^+ current in heart failure patients by preventing the dimers from gating cooperatively. Finally, variants in $Na_v1.7$, which resulted in an alteration of the channel function when expressed alone, were rescued by their interaction with the WT α-subunits [28]. Very recently, the group of Dr. Kucera modeled the interactions between Na_v channels in order to examine their consequences on channel kinetics and confirmed that, if the question remains open how variants interacting with WT channels affect the Na^+ current, the condition where a variant channel potentiates the current generated by the WT leading to a gain of function, is conceivable [29].

4.4 What Consequences for the Field of Arrhythmias?

The wide variety of mutations and variants in *SCN5A* leads to a wide severity of I_{Na} dysfunction and modifications of biophysical properties. A single nucleotide variation can be associated with a decrease of trafficking, but may also affect channel biophysical properties. Thus, *in vitro* functional studies remain key to classify the pathogenicity of *SCN5A* variants leading to cardiac arrhythmias [30]. Hence, we can easily imagine some mutations

with a loss of current density due to a moderate loss of trafficking, but a gain of late I_{Na} specific to long QT syndrome. Such a mutation expressed with the WT may not lead to a significant loss of peak I_{Na} due to a transcomplementation mechanism, but may display a significant late I_{Na}, leading to a long QT syndrome phenotype. As an example, the R1860GfsX12 gain- and loss-of-function mutation in *SCN5A* underlies a complex clinical phenotype and led to a host of loss and gain of function features when expressed alone: lack of trafficking, partial degradation and drastic shift of the voltage-dependent inactivation. But, when co-expressed with WT channels, the heterozygous state displayed no major loss of peak I_{Na} and only an atrial phenotype was reported with no ventricular phenotype in affected family members [31].

As also detailed in a recent publication showing that a majority of loss-of-function variants in *SCN5A* exert a dominant-negative effect on WT $Na_v1.5$ channels [8], these findings reinforce the importance of co-expressing the two allele products of a patient in expression studies, as expression of only the mutated allele can conceal the true mutant effect leading to the patient's phenotype. Elucidating transcomplementation mechanisms could also clarify the low penetrance or the overlap syndrome often observed in related *SCN5A*-mutation carriers. We must consider the possibility that two alleles expressed together may not have the expected result of haploinsufficiency, and that mechanisms of cooperation may lead to a wide variety of phenotypes.

5. Conclusions

Collectively, our results support the notion that $Na_v1.5$ α-subunits dimerize, interact, and cooperate with each other. The study of interactions between Na_v channels is an emerging field, and understanding the underlying mechanisms will allow to develop better approaches to treat patients with congenital arrhythmia syndromes.

Abbreviations

BrS, Brugada syndrome; Na_v, Sodium voltage-gated channel; I_{Na}, Inward Sodium current; WT, Wild Type; ER, Endoplasmic Reticulum; RNC, Rat Neonatal Cardiomyocytes.

Author Contributions

JC, NN and PG designed the research study. JC performed the research. AC and ID provided help and advice in electrophysiology. JC analyzed the data. JC and NN wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All animals were cared according to the 'guide for the care and use of laboratory animals' (NIH Publication No. 85-23, revised 1996) and under supervision of authorized researchers in an approved laboratory (agreement number B75-13-08).

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Conflict of Interest

The authors declare no conflict of interest.

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